UNIVERSIDADE ESTADUAL DO CEARÁ
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
FACULDADE DE VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

JOANNA MARIA GONÇALVES DE SOUZA FABJAN

ESTUDO DE FATORES QUE AFETAM A PRODUÇÃO *IN VITRO* DE EMBRIÕES CAPRINOS E SEU USO NA PRESERVAÇÃO DA RAÇA CANINDÉ AMEAÇADA DE EXTINÇÃO

FORTALEZA
2013

Dados Internacionais de Catalogação na Publicação

Universidade Estadual do Ceará

Biblioteca Central Prof. Antônio Martins Filho

Bibliotecária Responsável – Leila Sátiro – CRB-3 / 544

F112e Fabjan, Joanna Maria Gonçalves de Souza

Estudo de fatores que afetam a produção *in vitro* de embriões caprinos e seu uso na preservação da raça Canindé ameaçada de extinção / Joanna Maria Gonçalves de Souza Fabjan. — 2013.

CD-ROM :276 f.il. (algumas color.) ; 4 ¾ pol.

"CD-ROM contendo o arquivo no formato PDF do trabalho acadêmico, acondicionado em caixa de DVD Slin (19 x 14 cm x 7 mm)".

Tese (doutorado) – Universidade Estadual do Ceará, Doutorado em Ciências Veterinárias, Fortaleza, 2013.

Área de Concentração: Reprodução e Sanidade Animal.

Orientação: Prof. Dr. Vicente José de Figueirêdo Freitas.

Coorientador: Prof. Dr. Pascal Mermillod.

1. Cabra. 2. Capra hircus. 3. Embrião. 4. FIV. 5. Risco de extinção. I. Título.

CDD: 636.089

JOANNA MARIA GONÇALVES DE SOUZA FABJAN

ESTUDO DE FATORES QUE AFETAM A PRODUÇÃO *IN VITRO* DE EMBRIÕES CAPRINOS E SEU USO NA PRESERVAÇÃO DA RAÇA CANINDÉ AMEAÇADA DE EXTINÇÃO

Tese apresentada ao Programa de Pós-Graduação em Ciências Veterinárias da Faculdade de Veterinária da Universidade Estadual do Ceará, como requisito parcial para a obtenção do título de Doutor em Ciências Veterinárias.
Área de Concentração: Reprodução e Sanidade Animal.
Linha de Pesquisa: Reprodução e sanidade de pequenos ruminantes.
Orientador: Prof. Dr. Vicente José de Figueirêdo Freitas

FORTALEZA
2013

JOANNA MARIA GONÇALVES DE SOUZA FABJAN

ESTUDO DE FATORES QUE AFETAM A PRODUÇÃO *IN VITRO* DE EMBRIÕES CAPRINOS E SEU USO NA PRESERVAÇÃO DA RAÇA CANINDÉ AMEAÇADA DE EXTINÇÃO

Tese apresentada ao Programa de Pós-Graduação em Ciências Veterinárias da Faculdade de Veterinária da Universidade Estadual do Ceará, como requisito parcial para a obtenção do título de Doutor em Ciências Veterinárias.

Aprovada em: 18/06/2013

BANCA EXAMINADORA

Prof. Dr. Vicente José de Figueirêdo Freitas
Universidade Estadual do Ceará
Orientador

Prof. Dr. Pascal Mermillod
Institut National de la Recherche Agronomique, Nouzilly, França
Coorientador

Dr. Yann Locatelli
Institut National de la Recherche Agronomique, Nouzilly, França
Examinador

Dr. Jeferson Ferreira da Fonseca
Embrapa Caprinos e Ovinos
Examinador

Prof. Dr. José Ricardo de Figueiredo
Universidade Estadual do Ceará
Examinador

Às pessoas que mais amo neste mundo,

Dedico ao meu pai, João, por ter me preparado para a vida, ser sempre o maior estimulador dos meus estudos, pelo apoio incondicional, por ser o meu exemplo de caráter e o meu melhor amigo.
Dedico à minha mãe, Joyce, por me fazer ver que nada supera amor de mãe e filha, por me amar mais do que a si própria e ser o meu ponto de equilíbrio. Agradeço por entender, mesmo com muito sofrimento, a minha ausência. Ao Gegê, meu segundo pai, por cuidar tão bem da minha mãe e ser tão especial.
Dedico à minha irmã, pelo companheirismo, amizade, admiração e por saber que ela está sempre torcendo por mim e esperando eu voltar para casa.
Dedico ao meu marido, Rok, por ser tão maravilhoso e especial para mim.

AGRADECIMENTOS

À Deus, por estar sempre ao meu lado, me orientando e protegendo em decisões difíceis e semeando a paz e o amor ao meu redor.

À Universidade Estadual do Ceará (UECE) e ao Programa de Pós-Graduação em Ciências Veterinárias (PPGCV) por permitir a realização deste curso de doutorado e pelas oportunidades recebidas. Ao ex-coordenador, prof. Marcos Fábio Gadelha Rocha, e atual coordenador, Davide Rondina, por elevar e mater o conceito 6 do nosso curso, tornando-o um dos melhores do Brasil.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela concessão de bolsa de doutorado tanto no país como no Exterior, possibilitando adquirir experiências profissionais e pessoais fantásticas.

Ao meu "doutorado", por me levar a conhecer o meu marido Rok Fabjan. Pela amizade, amor, paciência, apoio e carinho. Obrigada por entender que a nossa vida de casado deveria começar em Fortaleza, por confiar e aceitar me seguir nessa jornada, abrindo mão de absolutamente tudo. Tudo sempre dará certo para nós: *God is in our side!* Eu te amo!

A toda a minha família, por ser sempre um grande estímulo para ir para casa. Pelo total apoio e torcida. Obrigada pela alegria que sempre me esperam, como se não me vissem há uma década. Amo vocês todos!

Ao orientador prof. Dr. Vicente José de Figueirêdo Freitas, pela oportunidade recebida e confiança na execução deste projeto. Obrigada pelo incentivo, paciência, amizade e orientação. Obrigada por entender, concordar e colaborar desde o primeiro minuto com o meu grande sonho de realizar parte deste projeto no INRA/França.

Ao Laboratório de Fisiologia e Controle da Reprodução (LFCR) pelos conhecimentos adquiridos. A todos os estudantes que tive o prazer de conviver e que tornaram nossa estada tão completa e inesquecível. A doçura da Ray, a personalidade da Alê, as brincadeiras da Carlinha, a sinceridade da Maiara, a topa-tudo Iana, a alegria contagiante da Claudinha, a bondade do Agostinho, as piadas do Cacá, a ingenuidade do Talles, as discussões futebolísticas com o Vitor, a tranquilidade do Henrique, eletricidade do Dowglish, as palhaçadas do Carloide, dentre outros, fizeram com que o esforço despendido durante o doutorado tenha sido irrisório perto dos momentos de alegrias vividos. Ao meu grande companheiro, meu irmão africano, Ribrio. Morar com você foi uma experiência fantástica. Tudo foi maravilhoso pessoal.

Aos novos amigos que conheci em Fortaleza fora do LFCR, Mirley, Roberta, Elisângela, Cláudio, Maurício, Luis, Rosivaldo, Cristiano. Aos melhores vizinhos possíveis, Adriana, Vladia, Arthur e Anderson. Vocês todos me proporcionaram momentos maravilhosos.

À Profa. Dra. Luciana Magalhães Melo, sempre disposta em contribuir, apoiando em todos os momentos, um verdadeiro exemplo de pesquisadora.

Ao Prof. Dr. Dárcio Ítalo Alves Teixeira pela colaboração, convívio e amizade.

Aos funcionários do PPGCV, e, em especial, à secretária Adriana, pela assistência sempre com muito carinho. Aos funcionários Cesár e Selmar pelo auxílio nas atividades, respeito e amizade.

Ao pesquisador da Embrapa, Dr. Jeferson Ferreira da Fonseca, pela disponibilidade, experiência, incentivo contínuo e, acima de tudo, pela amizade tão especial, que significa muito para mim.

Ao meu orientador de mestrado, Prof. Ciro Alexandre Alves Torres, por entender e apoiar minha "saída". É um orgulho poder dizer que fui sua orientada.

Ao Prof. Dr. Felipe Zandonadi Brandão que mesmo longe sempre esteve muito presente. Obrigada pela confiança de sempre e amizade.

Aos Prof. Silvio Dória e Anamaria Ribeiro, dois mestres brilhantes, pelos dois anos inesquecíveis de convivência durante a especialização. Meus conhecimentos e amor pela espécie caprina hoje são diferentes depois de passar por vocês.

À Profa. Eunice Oba, pelo apoio e extrema boa-vontade nas análises de progesterona.

Ao Prof. Dr. José Ricardo de Figueiredo, pela participação na banca examinadora, que certamente contribuiu em muito para a finalização deste trabalho.

Ao INRA de Nouzilly que me aceitou como estagiária por 18 meses e me forneceu todas as melhores condições para se trabalhar. Instituição maravilhosa que terei sempre muito carinho.

Ao meu coorientador, Dr. Pascal Mermillod, que além de ser um pesquisador excepcional é iluminado. Obrigada por ter sido meu pai, amigo, orientador, e tudo mais que eu precisasse durante os 18 meses na França. Sem dúvida alguma, é uma das melhores pessoas que já conheci em minha vida. Obrigada por tudo, não existem palavras para lhe agradecer. À Severine Mermillod, pela gentileza, amizade, carinho e paciência quando eu ainda não falava francês.

Ao Dr. Yann Locateli, pelas ideias, incentivo, parceria, compania, amizade e momentos de descontração nos barzinhos de Tours à noite.

Ao Nicolas Duffard, técnico do INRA que com toda a paciência do mundo me ensinou tudo que eu precisava para seguir com o projeto. Meu grande amigo Nico, este trabalho também é seu. Parabéns futuro papai!

A toda a equipe *« interactions cellulaires et fertilité »*, em especial ao Xavier Druart, Florence Guignot, Ghylene Goudet, Cécile Douet, Guillaume Tsikis, pela recepção calorosa, almoços, brincadeiras, ensinamentos, etc. À Emilie Corbin e Christine Perreau, que nunca mediram esforços para ajudar quando necessário.

A toda a equipe genial do hospital veterinário, em especial, ao Christian, Luc, Frederic. Vocês fizeram cada momento ser verdadeiramente especial. Obrigada pela simpatia, brincadeiras, descontração total, boa vontade, etc. Vocês são formidáveis!

Ao meu *petit gateau australien*, Michael Bertoldo e às queridas amigas, Barbara Konsak, Barbara Ambruosi e Philippa Konke. Obrigada por todas as saídas, todas as cervejas, as brincadeiras, os jantares, as viagens com histórias hilariantes que nunca nos esqueceremos. Vocês tornaram esses 18 meses mágicos, indescritíveis.

Aos meus amigos brasileiros e "abrasileirados", Antoine e Vanda, Helton e Virginia, Gabi, Silvia, Ciro, vocês foram maravilhosos. Obrigada por todas as reuniões ("resenhas"), festinhas com muita música brasileira, aipim/mandioca/macaxeira, salpicão, coxinha, brigadeiro, etc.. Só quem mora fora de casa por algum tempo entende essas necessidades. À Giovanna que tive o prazer de conhecer melhor, trabalhar junto, conversar muito, desabafar, enfim, ganhei uma grande amiga.

A todos os meus amigos que conheci em Minas durante o meu mestrado, em especial à Paulinha, André, Bruninha, Gezebel, Arashiro, Pedro, Ana Maia, Giselle, Beto, Maíra, Guigui, dentre muitos outros. À minha "família mineira", Soninha, Nathália e Rafael, por todo o apoio. Vocês foram fundamentais na minha vida e nunca os esquecerei, por mais longe que a vida nos mantenha.

Aos meus inúmeros amigos do Rio de Janeiro, especialmente ao Sidney (irmão), Tat, Lét, Coxita, Leozinho, Léo, Negon, Lelê, Henrique, Marcelle, Lu, Luiza, Paulinho, por tornarem a minha vida tão especial nos momentos de lazer.

A todos que fizeram parte da minha movimentada vida acadêmica e que, direta ou indiretamente, contribuíram para a realização deste trabalho.

“Por mais títulos e diplomas que se acumulem, uma verdade permanece imutável: estamos sempre aprendendo, não importa a idade que tenhamos, e nem mesmo os títulos conquistados. O aprendizado é constante” (Selma Said)

RESUMO

Esse estudo teve por objetivos: 1) comparar os efeitos dos meios de maturação (semidefinido ou definido) em oócitos oriundos de ovários de abatedouro ou colhidos por laparoscopia (COL) sobre a cinética da maturação *in vitro* (MIV; 18 *vs*. 22 *vs*. 26 h) e competência ao desenvolvimento embrionário (submetidos à fecundação *in vitro* - FIV ou ativação partenogenética - AP); 2) avaliar o efeito das células do *cumulus* e da heparina durante a FIV e o potencial de utilização de oócitos desnudos; e 3) caracterizar a fisiologia reprodutiva de cabras nulíparas ou pluríparas da raça Canindé, além de utilizar a produção *in vitro* de embriões nesta raça ameaçada de extinção. Resultados similares entre a cinética da MIV foram obtidos para oócitos de abatedouro, contudo 22 h resultaram em maiores taxas ($P < 0,05$) que 18 h para oócitos de COL maturados em meio definido. Além disso, oócitos de abatedouro cultivados em meio definido maturaram significativamente mais rápido que os de COL (18 h e 22 h, $P < 0,05$). Oócitos de abatedouro obtiveram maior taxa de clivagem (67 *vs.* 38%; $P < 0,05$). Entretanto, após AP, os oócitos de COL apresentaram taxas semelhantes aos de abatedouro. Blastocistos vitrificados e posteriormente aquecidos apresentaram resultados similares nas taxas de sobrevivência e eclosão com relação à fonte do oócito, meios de MIV ou método de ativação. Com relação à influência da heparina e células do *cumulus* durante a FIV, a produção de blastocistos foi maior ($P < 0,05$) para os oócitos fecundados na presença de heparina (54%) do que em sua ausência (42%) ou oócitos desnudos com ou sem heparina (41 e 38%; respectivamente). Oócitos desnudos cocultivados com células do *cumulus* não diferiram significativamente dos oócitos intactos (55%). Além disso, alguns oócitos encontrados desnudos na colheita desenvolveram-se satisfatoriamente até o estádio de blastocisto, quando maturados e fecundados junto com oócitos intactos. Não houve diferença ($P > 0,05$) entre fêmeas nulíparas ou pluríparas em relação aos diversos parâmetros relativos ao estro, ovulação e progesterona plasmática. Durante a COL, em média, 12 folículos foram puncionados e nove oócitos colhidos por cabra, resultando em uma taxa de recuperação de 74%. Um total de 78 embriões foi produzido, obtendo-se uma taxa de blastocisto de 51%. Em conclusão, oócitos oriundos de abatedouro ou COL possuem diferenças na cinética da MIV e nas exigências durante a MIV e FIV, porém os oócitos de COL que seguem o seu desenvolvimento até embrião possuem qualidade similar aos de abatedouro. O uso de heparina e a associação dos oócitos com as células do *cumulus* durante a FIV

melhorou significativamente a produção *in vitro* de embriões. Este estudo demonstrou uma perspectiva interessante quanto ao uso de oócitos desnudos no momento da colheita, o que pode representar uma taxa adicional de embriões. Finalmente, pela primeira vez, foram produzidos embriões da raça Canindé pelo método *in vitro*, propiciando a formação de um banco de embriões, útil para a preservação da mesma.

Palavras-chave: Cabra. *Capra hircus.* Embrião. FIV. Risco de extinção.

ABSTRACT

This study aimed to 1) compare the effects of different maturation media (semi defined or defined) in oocytes obtained by slaughterhouse ovaries or collected by laparoscopy (LOPU) on *in vitro* maturation kinetics (IVM; 18 *vs.* 22 *vs.* 26 h) and competence to embryo development (submitted to *in vitro* fertilization, IVF or parthenogenetic activation, PA); 2) evaluate the effect of *cumulus* cells and heparin during IVF and the viability to use denude oocytes; and 3) characterize the reproductive physiology of nulliparous and pluriparous endangered Canindé goats and to produce *in vitro* embryos from this breed. Similar results between IVM kinetics were obtained for slaughterhouse oocytes, however, 22 h resulted in greater rates ($P < 0.05$) than 18 h to LOPU oocytes matured in defined medium. Furthermore, slaughterhouse oocytes cultured in defined medium matured significantly faster than those recovered by LOPU (18 h and 22 h, $P < 0.05$). Slaughterhouse oocytes reached greater cleavage rate in comparison to LOPU ones (67 *vs.* 38%; $P < 0.05$). Conversely, LOPU oocytes that were PA presented similar rates to slaughterhouse ones. Vitrified/thawed blastocysts presented similar results regarding survival and hatching rates when considering oocyte source, maturation media or activation method. Concerning heparin and *cumulus* cells effect during IVF, blastocyst yield was greater ($P < 0.05$) to oocytes that were fertilized in the presence of heparin (54%) than in its absence (42%) or to denuded oocytes with or without heparin (41% and 38%; respectively). Denuded oocytes co-cultured with *cumulus* cells did not significantly differ from intact oocytes (55%). Moreover, some oocytes that were already found denuded at collection developed well up to blastocysts when matured and fertilized together with intact oocytes. There was no difference ($P > 0.05$) between nulliparous or pluriparous Canindé females regarding the main parameters relative to estrus, ovulation and plasmatic progesterone. During LOPU, 12 follicles were puncted and nine oocytes recovered per goat in average, resulting in 74% of recovery rate. A total of 78 embryos was produced, reaching 51% of blastocyst rate. In conclusion, slaughterhouse and LOPU derived oocytes may have different IVM kinetics and require different IVF conditions. However, LOPU oocytes that are able to develop up to blastocyst have similar quality as slaughterhouse oocytes. Both the use of heparin and the association of oocytes with *cumulus* cells during IVF significantly improve goat *in vitro* embryo production. This study demonstrates an interesting perspective regarding the use of oocytes already found denuded at collection, which

may represent additional number of embryos. Finally, it was possible for the first time to produce *in vitro* embryos from Canindé goats that allowed initiating an embryo bank to be used for the breed preservation.

Keywords: Goat. Embryo. IVF. IVP. Endangered.

LISTA DE FIGURAS

REVISÃO DE LITERATURA

CAPÍTULO 1

LISTA DE TABELAS

REVISÃO DE LITERATURA

CAPÍTULO 1

CAPÍTULO 2

LISTA DE ABREVIATURAS E SIGLAS

6-DMAP : 6-Dimethylaminopyridine (6-dimetilaminopurina)

AGB : Brazilian Animal Germplasm Bank (Banco de Germoplasma Animal Brasileiro)

AI : Artificial insemination (Inseminação Artificial)

a.m. : Ante Meridium (Antes do meio-dia)

AMPc : cyclic Adenosine Monophosphate (Adenosina Monofosfato Cíclica)

ANOVA : Analysis of variance (Análise de variância)

AP : Ativação Partenogenética

ART : Assisted reproductive technologies (Tecnologias da reprodução assistida)

B2 : Menuzo's B2 medium (Meio B2)

BCB : Brilliant cresyl blue (Azul cresil brilhante)

BCS : Body condition score (Escore da condição corporal)

Bl : Blastocyst (Blastocisto)

BOEC : Bovine oviduct epithelial cells (Células epiteliais do oviduto bovino)

BSA : Bovine serum albumin (Albumina sérica bovina)

°C : Graus Celsius

cAMP : Cyclic adenosine monophosphate (Adenosina monofosfato cíclico)

CAPES : Coordenação de Aperfeiçoamento do Pessoal de Nível Superior

CE : Ceará

CENARGEN : Centro Nacional de Pesquisa de Recursos Genéticos e Biotecnologia

CEUA/UECE : Comitê de Ética Animal da Universidade Estadual do Ceará

CIDR : Controlled Internal Drug Release

CL : Corpora luteum (Corpo lúteo)

CNPq : Conselho Nacional de Desenvolvimento Científico e Tecnológico

CNRA : Centre National de la Recherche Scientifique (Centro Nacional de Pesquisa Científica)

CO_2 : Carbon dioxide (Dióxido de Carbono)

COC : *Cumulus*–oocyte complex (Complexo *cumulus*-oócito)

COFECUB : Comitê Francês de Avaliação da Cooperação Universitária com o Brasil

COL : Colheita de oócitos por laparoscopia

Cyst : Cysteamine (Cisteamina)

d : day (dia)

DNA : Deoxyribonucleic acid (Ácido desoxirribonucléico)

DOC : Denuded oocytes at collection (Oócitos desnudos na coleta)

DOCCOCF : Denuded oocytes at collection associated to COC during *in vitro* fertilization (Oócitos desnudos na coleta associados aos COC durante a fecundação *in vitro*)

DOCCOCMF : Denuded oocytes at collection associated to COC during *in vitro* maturation and fertilization (Oócitos desnudos na coleta associados aos COC durante a maturação e fecundação *in vitro*)

COCDOP : COC that were fertilized in the presence of oocytes denuded on purpose (COC que foram fecundados na presença de oócitos desnudos propositalmente)

DOP : Denuded oocytes on purpose (Oócitos desnudos propositalmente)

DOPCC : Denuded oocytes on purpose associated to *cumulus* cells (Oócitos desnudos propositalmente associados a células do *cumulus*)

DOPCOC : Denuded oocytes on purpose that were fertilized in the presence of COC (Oócitos desnudos propositalmente que foram fecundados na presença de COC)

Dr. : Doutor

E : East (Leste)

E2 : Estradiol

eCG : Equine chorionic gonadotropin (Gonadotropina coriônica equina)

EDTA : Ethylenediamine tetraacetic acid (Ácido etilenodiamino tetra-acético)

EGF : Epidermal growth factor (Fator de crescimento epidermal)

EGFR : Epidermal growth factor Receptor (Receptor do Fator de Crescimento Epidermal)

EGS : Estrus goat serum (Soro de cabra em estro)

EMBRAPA : Empresa Brasileira de Pesquisa em Agropecuária

EP : Erro Padrão

ESS : Estrus sheep serum (Soro de ovelha em estro)

FAO : Food and Agriculture Organization of the United States

FBS : Fetal Bovine Serum (Soro fetal bovino)

FCS : Fetal Calf Serum (Soro fetal de bezerro)

FF : Follicular Fluid (Fluido folicular)

FGA : Fluorogestone acetate (Acetato de Fluorogestona)

FGF : Fibroblast Growth Factor (Fator de Crescimento de Fibroblasto)

Fig. : Figure (Figura)

FIV : Fecundação *in vitro*

FSH : Hormone Stimulating Factor (Hormônio Folículo Estimulante)

FUNCAP : Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico

g : Gravidity (Gravidade)

G : Gauge (Calibre)

G1/G2 : Grade 1 and 2 oocytes (Oócitos Grau 1 e 2)

G6PD : Glucose-6-phosphate dehydrogenase (Glicose-6-fosfato desidrogenase)

GH : Growth hormone (Hormônio do crescimento)

GnRH : Gonadotropin-Releasing Hormone (Hormônio liberador de gonadotropinas)

GOEC : Goat oviduct epithelial cells (Células epiteliais do oviduto caprino)

GRB – Genetic Resource Bank (Banco de recursos genéticos)

GV : Germinal vesicle (Vesícula germinativa)

GVBD : Germinal vesicle breakdown (Quebra da vesícula germinativa)

h : Hours (Horas)

hAT III : human antithrombin III (Antitrombina humana do tipo III)

HBl : Hatched blastocyst (Blastocisto eclodido)

hCG : Human Chorionic Gonadotropin (Gonadotropina Coriônica humana)

HEPES : hydroxyethyl piperazineethanesulfonic acid (Ácido hidroxietilpiperazina etanossulfônico)

HMS : Human menopausal serum (Soro da menopausa humana)

i.e. : that is (isto é)

IGF I : Insulin-like growth factor I (Fator de crescimento semelhante à insulina I)

i.m. : Intramuscular

INRA : Institut National de la Recherche Agronomique (Instituto Nacional de Pesquisa Agronômica)

IU : International Units (Unidades internacionais)

IUCN : International Union for Conservation of Nature (União Internacional para Conservação da Natureza)

IVC : *In vitro* cultre (Cultivo *in vitro*)

IVD : *In vitro* development (Desenvolvimento *in vitro*)

IVF : *In vitro* fertilization (Fecundação *in vitro*)

IVM : *In vitro* maturation (Maturação *in vitro*)

IVEP : *In vitro* embryo production (Produção *in vitro* de embriões)

IVP : *In vitro* production (Produção *in vitro*)

kg : Quilograma

LAMOFOPA : Laboratório de Manipulação de Oócitos e Folículos Pré-Antrais

LFCR : Laboratório de Fisiologia e Controle da Reprodução

LH : Luteinizing Hormone (Hormônio Luteinizante)

LOPU : Laparoscopic ovum pick up (Aspiração ovariana por laparoscopia)

LOS : Large Offspring Syndrome (Síndrome da Cria Gigante)

MII : Metaphase II (Metáfase II)

MAP : Medroxiprogesterone Acetate (Acetato medroxiprogesterona)

mg : Miligram (Miligrama)

min : Minutes (Minutos)

min^{-1} : One per minute (Um por minuto)

MIV : Maturação *in vitro*

MIX : Sigla que designa um conjunto de substâncias, meio de maturação semidefinido

mL : Mililiter (Mililitro)

mm : Millimeter (Milímetro)

mm^3 : Cubic millimeters (Milímetro cúbico)

mmHg : Millimeters of mercury (Milímetros de mercúrio)

mM : Milimolar

MM : Maturation media (Meios de maturação)

MO : Missouri

MOET : Multiple Ovulation and Embryo Transfer (Múltipla Ovulação e Transferência de Embriões)

mOsm/L : Miliosmol/Litro

MPF : M-Phase promoting Factor (Fator Promotor de Maturação)

n : Number of oocytes (Número de oócitos)

ng : Nanogram (Nanograma)

N : North (Norte)

N_2 : Nitrogen (Nitrogênio)

NBCS : Newborn calf serum (Soro de bezerro recém-nascido)

NS : Non significant (Não significativo)

NT : Nuclear Transfer (Transferência nuclear)

O_2 : Oxygen (Oxigênio)

OEC : oviduct epithelial cells (Células epiteliais do oviduto)

oFSH : Ovine hormone stimulating fator (Hormônio Folículo Estimulante de Origem Ovina)

OPS : Open Pulled Straw

OPU : Ovum pick up (Aspiração ovariana)

P<0.05 : Probabilidade de erro menor do que 5%

P>0.05 : Probabilidade de erro maior do que 5%

p. : Página

p.m. : Post Meridium (Depois do meio-dia)

P4 : Progesterone (Progesterona)

PA : Parthenogenetic activation (Ativação Partenogenética)

PBS : Phosphate Buffered Saline (Tampão fosfato-salino)

PCR : Polymerase chain reaction (Reação em cadeia da polimerase)

pFSH : Porcine hormone stimulating fator (Hormônio Folículo Estimulante de Origem Porcina)

PFs : Preantral follicles (Folículos pré-antrais)

PGF : Prostaglandina

PGF2α : Prostaglandina F2α

pH : Potential of hydrogen (Potencial de hidrogênio)

PIV : Produção *in vitro*

PIVE : Produção *in vitro* de embriões

PIVER Program: Projet Production *In Vitro* d'Embryons de Ruminants (Projeto de produção *in vitro* de embriões em ruminantes)

PPGCV : Programa de Pós-Graduação em Ciências Veterinárias

Prof. : Professor

PVA : Polyvinyl alcohol (Álcool polivinílico)

RIA : Radioimmunoassay (Radioimunoensaio)

RNA : Ribonucleic acid (Ácido ribonucléico)

S : South (Sul)

SEM : Standard error of means (Erro padrão da média)

SNCT : Somatic Cell Nuclear Transfer (Transferência Nuclear de Células Somáticas)

SCNTi : interspecies Somatic Cell Nuclear Transfer (Transferência Nuclear de Células Somáticas interespecífica)

SOF : Synthetic Oviductal Fluid (Fluido sintético do oviduto)

SP : São Paulo

TCM 199 : Tissue culture medium 199 (Meio de cultivo tecidual 199)

TotBl : Total Blastocysts (Blastocistos totais)

TUGA : Transvaginal ultrasound-guided aspiration (Aspiração guiada por ultrassonografia transvaginal)

UECE : Universidade Estadual do Ceará

UEPAO : Unité expérimentale de Physiologie Animale de l'Orfrasière (Unidade Experimental de Fisiologia Animal de Orfrasière)

UMR: Unité mixte de Recherche (Unidade Mista de Pesquisa)

USA : United States of America (Estados Unidos da América)

UV : Ultraviolet Light (Luz ultravioleta)

v : Volume

vs. : Versus

W : West (Oeste)

WTA : Watanabe

x : times (vezes)

μg : Microgram (Micrograma)

μL : Microliter (Microlitro)

μM : Micromolar

% : Percentage (Porcentagem)

~ : Approximately (Aproximadamente)

± : more or less (mais ou menos)

° : Degrees (Graus)

- : Absence (Ausência)

+ : Presence (Presença)

SUMÁRIO

1 INTRODUÇÃO

A caprinovinocultura desenvolvida no Nordeste brasileiro teve sua origem com a chegada de animais de raças européias trazidas pelos jesuítas e colonizadores portugueses por volta de 1535. Durante quase quatrocentos anos, a caprinocultura evoluiu numericamente, quase sem interferência direta do homem. De modo geral, os animais foram criados de forma extensiva e para se adaptarem às condições edafoclimáticas predominantes na região, desenvolveram mecanismos biológicos apropriados, resultando em vários grupos e/ou raças naturalizadas da região. Esta adaptação ao sistema de produção promoveu uma redução da capacidade produtiva dos rebanhos em termos de carne, leite e tamanho corporal. Apesar da seleção natural ter ocorrido no sentido negativo da produção, o Nordeste possui hoje, para suas condições de semiárido, material genético de excelente qualidade para produção de pele, carne de baixo teor de gordura e uma adequada produção de leite, desde que seja adotado um nível mínimo de tecnologia (Revisado por FONSECA; SOUZA, 2011).

Entretanto, desde o início do século XX, algumas raças comerciais, selecionadas em regiões temperadas, têm sido importadas pelo Brasil. Embora mais produtivas, a maior parte destas não possui características adaptativas, encontradas em naturalizadas. Ainda assim, elas gradualmente vieram substituindo-as de tal forma que as raças naturalizadas, em sua maior parte, encontram-se em risco de extinção (MARIANTE et al., 2009). A conservação de raças ameaçadas de extinção, como a Canindé, é fundamental para a preservação da biodiversidade. Ações que objetivem a preservação destes animais são importantes para que este material genético não seja perdido de forma definitiva. Estas ações podem estar baseadas, sobretudo, no uso de modernas biotécnicas reprodutivas.

Diferentes biotecnologias reprodutivas têm sido descritas como sendo capazes de contribuir de forma importante para a conservação de diferentes espécies, dando suporte à sobrevivência das populações existentes. Dentre as biotécnicas já estabelecidas ou em atual desenvolvimento, destacam-se a inseminação artificial (IA), a múltipla ovulação e transferência de embrião (MOTE), a colheita oocitária por laparoscopia (COL) seguida pela produção *in vitro* de embriões (PIVE), dentre outras. Devido à repetibilidade da PIVE (BALDASSARRE; KARATZAS, 2004), sua aplicação tem sido proposta como uma estratégia valiosa para a preservação de espécies ameaçadas (LOCATELLI et al., 2006). Além do uso potencial da técnica para a

conservação de espécies ou ainda raças caprinas ameaçadas, a PIVE é uma biotécnica reprodutiva sofisticada que permite aumentar a eficiência reprodutiva e o melhoramento genético dos animais envolvidos. Para Comizzoli et al. (2000) a PIVE seguida de transferência embrionária é a melhor maneira para alcançar o melhoramento genético em ambos os gêneros, masculino e feminino. Vale salientar que a técnica é ainda essencial para o estabelecimento de novas biotécnicas, tais como a clonagem e transgênese. O processo da PIVE engloba a colheita dos oócitos a partir de folículos antrais, a maturação *in vitro* destes oócitos, a fecundação dos oócitos maturados e, por fim, o desenvolvimento dos embriões resultantes (FREITAS; MELO, 2010). O sucesso da PIVE depende da eficiência de todas as etapas do processo. No Brasil, a PIVE ainda é incipiente em caprinos, porém atraente, e responderá, sem dúvida, por ampla parcela futura das atividades em tecnologia de embriões em caprinos, a exemplo do que ocorre em bovinos (FONSECA; SOUZA; CAMARGO, 2010).

Para uma melhor compreensão da relevância deste estudo, a revisão de literatura a ser apresentada nos tópicos seguintes, contemplará três artigos de revisão. O primeiro artigo (em processo de submissão) relata a situação atual da PIVE em pequenos ruminantes. O segundo artigo (em processo de submissão) enfatiza os principais fatores que afetam e as etapas da técnica de COL, especificamente em caprinos. Já o terceiro artigo (publicado) abrange as diferentes biotécnicas reprodutivas aplicadas à conservação de ruminantes ameaçados de extinção. Os demais capítulos que compõem a tese são artigos técnico-científicos englobando os diferentes fatores afetando a técnica de PIVE em caprinos, especialmente durante a etapa de maturação (Capítulo 1 – artigo em processo de submissão) e de fecundação *in vitro* (Capítulo 2 – artigo publicado). O terceiro (Capítulo 3 – artigo aceito para publicação) avalia os parâmetros reprodutivos fisiológicos e o perfil plasmático de progesterona durante o ciclo estral da cabra Canindé e reporta os primeiros embriões produzido *in vitro* nesta raça.

2 REVISÃO DE LITERATURA

PARTE I: PRODUÇÃO *IN VITRO* DE EMBRIÕES EM PEQUENOS RUMINANTES: MELHORIAS RECENTES E PESQUISAS FUTURAS

Resumo

Além do uso potencial da técnica de produção *in vitro* de embriões (PIV) para o melhoramento animal, esta é também essencial para o estabelecimento de novas biotecnologias como a clonagem e transgênese. Adicionalmente, o conhecimento da fisiologia do oócito e do embrião adquirida durante a PIV pode estimular o desenvolvimento de outras técnicas como, por exemplo, a escolha de marcadores moleculares para seleção genômica de embriões pré-implantacionais, além de beneficiar a reprodução assistida em seres humanos. A PIV consiste atualmente em um dos objetivos principais da agropecuária, incluindo pequenos ruminantes. A heterogeneidade dos oócitos coletados de folículos antrais via laparoscopia (COL) ou a partir de ovários de fêmeas abatidas, permanece um desafio enorme para o sucesso da MIV e ainda limita as taxas de desenvolvimento embrionário. Além disso, a menor qualidade dos embriões de PIV em comparação aos produzidos *in vivo*, restringe a utilização mais ampla desta técnica promissora. Desta forma, muitos estudos têm sido relatados buscando a determinação de condições mais adequadas para a maturação, fecundação e desenvolvimento *in vitro* objetivando maximizar a produção e a qualidade dos embriões obtidos. Esta revisão tem como objetivo apresentar o panorama atual da produção *in vitro* de embriões em pequenos ruminantes, descrevendo importantes etapas para o seu sucesso, relatando os avanços recentes e ainda os principais obstáculos identificados para sua propagação.

Palavras-chave: blastocisto, embrião, caprino, PIV, oócito, ovino

Esta parte da revisão de literatura corresponde ao artigo que será submetido ao periódico **Theriogenology** (Qualis: A2, Fator de Impacto: 2,082).

In vitro production of small ruminant embryos: Late improvements and further research

Joanna Maria Gonçalves de Souza-Fabjan[1,2], Barbara Panneau[1], Nicolas Duffard[1,3], Yann Locatelli[1,3], Vicente José de Figueiredo Freitas[3], José Ricardo de Figueiredo[4], Pascal Mermillod[1*]

1 INRA, UMR7247, Physiologie de la Reproduction et des Comportements, INRA, CNRS, Université de Tours, Haras Nationaux. 37380 Nouzilly, France
2 Laboratory of Physiology and Control of Reproduction, Veterinary School, State University of Ceará, Av. Dedé Brasil, 1700, 60740-90, Fortaleza, CE, Brazil
3 Museum National d'Histoire Naturelle, Réserve de la Haute Touche, 36290, Obterre, France
4 Faculty of Veterinary, Laboratory of Manipulation of Oocyte and Preantral Follicles (LAMOFOPA), State University of Ceará, Fortaleza, Ceará, Brazil

* Corresponding author.
Tel.: +33 247 42 79 20
E-mail address: Pascal.Mermillod@tours.inra.fr

Running title: *In vitro* production of small ruminant embryos

Abstract

Beyond the potential use of *in vitro* embryo production (IVP) in breeding schemes, this technique is also required for the establishment of new biotechnologies such as cloning and transgenesis. Additionally, the knowledge of oocyte and embryo physiology acquired through IVP techniques may stimulate the further development of other techniques such as marker assisted and genomic selection of preimplantation embryos and also benefit to assisted procreation in human being. *In vitro* embryo production is currently a major objective for livestock industries, including small ruminants. The

heterogeneity of oocytes collected from growing follicles by laparoscopic ovum pick up (LOPU) or in ovaries of slaughtered females, remains an enormous challenge for IVM success, and still limits the rate of embryo development. In addition, the lower quality of the IVP embryos, compared to *in vivo* derived counterparts, translates into a poor cryosurvival which restricts the wider use of this promising technology. Therefore, many studies have been reported in an attempt to determine the most suitable protocol for *in vitro* maturation, fertilization and development in order to maximize embryo production rate and quality. This review aims to present the current panorama of *in vitro* embryo production in small ruminants, describing important steps for its success, reporting the recent advances and also the main obstacles identified for its improvement and dissemination.

Key words: blastocyst, embryo, goat, IVP, oocyte, sheep

1. Introduction

Throughout the world, the importance of small ruminants as providers of essential food – meat and dairy products – has been well reported. Beyond genetic selection for productive traits, reproductive efficiency is one of the most important factors to improve goat and sheep production. After artificial insemination and multiple ovulation - embryo transfer (MOET) schemes, *in vitro* production of embryos (IVP) represents the third generation of techniques aimed at a better control of animal reproduction [1, 2]. This technique involves four major steps: oocyte collection, oocyte *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* development of the resulting embryos up to the blastocyst stage (IVD), at which these embryos can be efficiently cryopreserved or transferred into the uterus of synchronized recipient females. These different steps are now well established in domestic ruminant species

(cattle, sheep and goat). IVP is also under progress in non ruminant species like horse [3] and pig [4], although far less efficient than in ruminants. Despite heavy research efforts during the past 30 years, the variability of the number and quality of the oocytes collected and the low viability of frozen – thawed *in vitro* produced embryos still limit the large-scale use of this technology [5-7].

In small ruminants, *in vivo* embryo recovery requires surgical procedures that impair repeated embryo production from individual donors. Therefore, *in vitro* embryo production has some advantages such as reliability [8], reproducibility [9], the possibility to collect oocytes from females hormonally stimulated or not [10], the use of pre-pubertal donors [11, 12], senile [13], pregnant females or even in *post-mortem* cases. Moreover, oocyte and embryo techniques are necessary to develop other biotechnologies, such as cloning and transgenesis [14] and it has been proposed as a valuable strategy for the conservation of endangered species [15, 16]. Furthermore, gametes and embryo technologies give access to basic research on cell cycle and gene expression regulations, for instance, and could help the identification of new targets for marker assisted genomic selection of precise reproductive characters.

Studies concerning IVF in mammals have been initiated as early as 1878 and the first domestic animal born after IVF was a rabbit, in 1959 [17]. Some years later, Hanada [18] reported the first goat birth after IVF using *in vivo* maturated oocytes and only in 1993, a study was published reporting for the first time a development to term after transfer of an embryo produced totally *in vitro* in this species [19]. The technique is extremely versatile and thus has been intensely studied in the last years. However, despite considerable efforts aiming to improve IVP steps, success rates are still far from *in vivo* derived embryos [20, 21]. This review aims to present the current situation of *in vitro* embryo production in small ruminants, describing important steps for its success,

reporting the recent advances and also the main obstacles identified for its improvement and dissemination, as well as clues for further progress of this technology.

2. Oocytes: source and recovery

The first step of IVP is the recovery of oocytes showing developmental competence. Oocyte developmental competence may be defined as its ability to resume and achieve meiosis, be fertilized, to develop into embryo and give rise to normal and fertile offspring after normal gestation. Oocyte developmental competence thus reflects oocyte's intrinsic quality. In mammals, developmental competence is acquired progressively by the oocyte during folliculogenesis process and increases with the size of follicle, to reach its maximum at time of ovulation [22, 23].

Immature oocytes may be recovered from slaughterhouse ovaries or from live animals. Slaughterhouse ovaries provide a cheap and abundant source of oocytes that can be recovered by follicle aspiration, slicing or follicle dissection. These large numbers of oocytes from unknown females are helpful for research and improvement of IVP conditions. This strategy may be of interest for females of high genetic value that had to be culled [24], which obviously consists in a single use of this animal but can provide a last offspring from theses valuable females. However, the use of IVP for genetic improvement or diffusion requires repeated oocytes recovery from live females with high genetic value in order to maximize the number of embryos that can be produced and optimize genetic gain. It is possible to obtain immature oocytes by follicular aspiration performed by abdominal laparotomy [25], but this method presents the disadvantage of generating adherences, avoiding repetitions of the procedure. In cattle, the transvaginal ultrasound-guided aspiration technique based on ovarian

mobilization through rectum wall is currently the most used technique to successfully obtain oocytes [26, 27]. However, in small ruminants this method is not adapted as ovaries are difficult to grasp through the rectum. Graff et al. [28] obtained lower numbers of oocytes from goats submitted to transvaginal ultrasound-guided aspiration as compared to those submitted to laparoscopy (4.3 *vs.* 11.5 oocytes collected per female). LOPU procedure appeared less stressful, less invasive, lasts less (each session takes between 10 and 20 min in does and ewes) and can be repeated at short intervals without affecting oocyte developmental competence [8-10, 29, 30].

In goat, meiotic, cleavage and development rates were positively correlated with the size of follicles after IVM, IVF and IVD [31, 32]. In consequence, antral follicles from 3-5 mm may be preferentially aspirated for subsequent IVP. At the step of recovery of immature oocytes, the integrity of COC defined by density of *cumulus* cells and homogeneity of oocyte cytoplasm are the main criteria to look for, as *cumulus* cells play important role during IVM process [33]. To respect the integrity of COC structure and optimize their recovery rate, the material and aspiration conditions used during LOPU are of importance [34]. Depending on laboratories, the needle diameter used varies from 16 to 21 G and the vacuum connected to the needle is regulated from 25 to 70 mmHg for both goats and sheep. Under these conditions, oocyte recovery rates range from 40 to 90%, and the number of harvested structures may reach frequently around 12-13 oocytes per female in different laboratories [1, 8, 10, 35].

In sheep, Rodriguez and al. [36] compared different aspiration devices and flow rates for aspiration. With the increase of aspiration flow rate, the proportion of good quality oocytes decreased dramatically (69.5% to 28.3% with aspiration flows of 10 and 50 mL/min respectively, $P < 0.05$). Tubulure employed may also affect oocyte recovery and its quality. Thin and intermediate tubings were more effective as laminar flow

during aspiration of COC within tubulure to prevent damages on COC. In experiments performed on slaughterhouse ovaries, follicle size did not affect recovery rate, but proportion of good quality oocytes was higher for large (78%) and medium (64%) follicles ($P < 0.05$). They observed that 18 G promoted a significant better oocyte recovery rate than the 20 G needle, whereas no influence was noted in oocyte quality after aspiration using both diameters.

The aspiration device is also important to respect female tract integrity. It has been shown that repeated ovum pick-up doesn't affect fertility of donor sheep, even when repeated up to 20 times [9]. In the same way, after repeated LOPU in sheep no complication such as adherences and fibroses and normal histology of ovaries were observed [30]. This may confirm that laparoscopy is a minimally invasive procedure. Besides that, the repetition of ovum collections did not cause painful discomfort to the animals, detrimental to animal welfare [30].

2.1. Influence of ovarian status, stimulation, age and season

As mentioned earlier, oocyte developmental competence is acquired progressively during mammalian folliculogenesis to reach a maximum at time of ovulation. In small ruminants, emergence of large antral follicle occurs in different waves during reproductive cycle, 2-4 in goats [37] and 2-3 waves in sheep [38]. Selection of ovulatory follicle(s) may occur during the last wave whereas others follicles become atretic. Competent oocytes may be recovered from theses follicles for IVP before atresia resumption. As oocyte developmental competence increases with follicular size, a strategy for improving number of embryos per females relies on

decreasing selection pressure during folliculogenesis for maximizing number of large antral follicles.

Therefore, smaller follicles present on the ovary at the end of progestagen treatment may be stimulated by administration of purified exogenous gonadotropin such as FSH or eCG, increasing follicle survival [30, 39, 40]. This strategy must be employed at precise moment of emergence of follicular waves, when follicle survival depends on FSH. Two main strategies were assessed in small ruminants. The first one concerned the administration of gonadotropin before follicular aspiration by LOPU at random time of the reproductive cycle [9]. However, this appeared unsatisfying regarding prevention of atresia [40], as this event may have already occurred for a follicular population. The second and most used nowadays represents the association of progestagen treatment to gonadotropin (Fig. 1). Its steady and continuous release is ensured by the administration of intravaginal sponges impregnated with progestagen (fluorogestone acetate, FGA or medroxiprogesterone acetate, MAP) or progesterone (Controlled Internal Drug Release, CIDR) which inhibit the endogenous secretion of luteinizing hormone (LH), allowing regression of dominant follicle(s) by atresia.

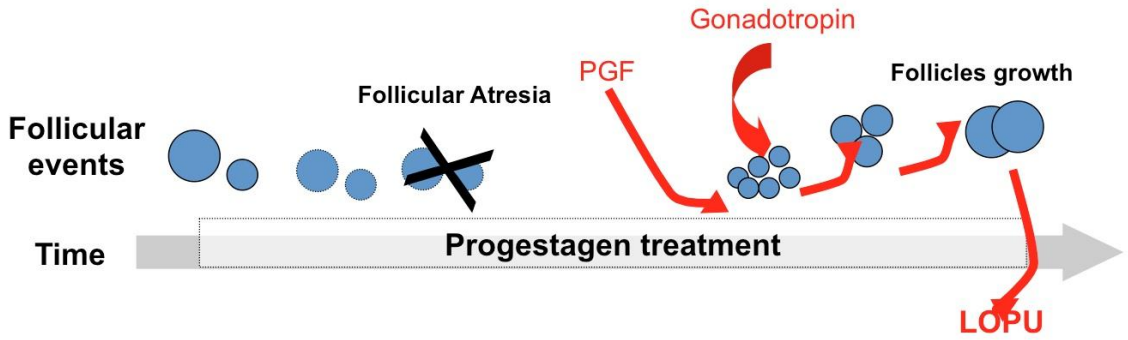

Fig. 1. Diagrammatic representation of protocols used to synchronize estrus and to stimulate healthy estrogenic follicle growth. Prostaglandin (PGF) is administered normally just before the end of progestagen treatment. At progestagen removal, laparoscopic ovum pick up (LOPU) is performed in goats or sheep. Adapted from Reby et al. [42]

Gonzalez-Bulnes et al. [43] suggested that progestagen may adversely affect general aspects and the alterations caused are even greater in superovulatory protocols, when combined with high FSH doses. In comparison to ewes that received just prostaglandins for estrus synchronization, ovulatory follicles from progestagen-treated ewes showed deficiencies in different phases: first, in the secretion of estradiol during the preovulatory phase; second, in the ability to ovulate an oocyte able to be fertilized and develop into a viable embryo; and third, in the secretion of progesterone by the subsequent corpora lutea [44]. Likewise, Berlinguer et al. [45] demonstrated that ewes treated with FSH after estrus synchronization without progestagens (just prostaglandins) produced a higher oocyte recovery rate and oocytes with higher competence to cleave and to develop up to the blastocyst stage. Although it is important to associate estrus synchronization to stimulatory treatments, more studies should be performed in order to verify the real benefits of progestagen in these procedures.

It is well described that FSH treatment prior to LOPU increases the number of ovarian follicles and oocytes collected from each female [9, 10, 46]. Morton et al. [10]

reported similar IVD rates in ewes submitted to treatment with or without FSH prior to LOPU; although, these results were obtained in relation to the cleaved blastocysts. In fact, although not statistically different, when we take into account ewes that received FSH (~59%) they had a lower cleavage rate than those not treated (~79%). Similarly, in another study, a lower cleavage rate was obtained in FSH-stimulated does rather than in the control group, but this difference was balanced by the numerically higher developmental rates to the blastocyst stage in the FSH-treated group [46]. In our laboratory, we observed that does receiving high dose of ovine FSH (1 IU) had greater proportion of zygotes reaching the blastocyst stage than goats treated with half dose [47]. In sheep, Baldassarre et al. [29] observed no difference in the IVD of oocytes collected from females treated with different FSH administration regimes. Conversely, the use of a stimulation protocol for goats with a higher number of FSH injections (5 *vs* 3) resulted in G1/G2 COC's with higher Epidermal Growth Factor Receptor (EGFR) expression in *cumulus* cells which is correlated with an elevated meiotic competence following IVM [48]. Abdullah et al. [49] reported an increase in IVP efficiency when ovarian stimulation started 72 h before LOPU when compared with 36 or 60 h. Furthermore, an increase in blastocyst rate after IVP and cryotolerance to vitrification were reported when FSH was administered in constant *vs.* decreasing doses before LOPU [50]. Therefore, both purity and dose of gonadotropin, stimulation regimen employed and timing of administration are of particular importance, affecting not only follicles/oocytes yield but also oocyte intrinsic quality.

The use of GnRH antagonist prior to the stimulatory treatment has been proposed to synchronize emergence of follicular waves regarding ovarian stimulation and thus maximizing responses to exogenous FSH by reducing the effect of dominant follicles in MOET programs [51]. When applied to small ruminants, the antagonist

treatment overcomes the problem of non-responding females. However, terminal follicular growth was impaired in goats and the beneficial effect of this treatment on the ovulation rate was negated by an increase in the proportion of unfertilized ova and degenerated embryos. Using such treatment, it is likely that high response observed (more than 28 corpora lutea per goat) may also have impaired female's physiology, especially regarding maternal environment or oocyte developmental competence. When this strategy was applied for LOPU/IVP, it enhanced follicular stimulation but also affected the oocyte competence to develop after IVF and IVD [52] indicating that oocyte itself was affected by treatment.

Some studies focused on effect of age of donor on oocyte yield and developmental competence in small ruminants. The use of prepubertal animals is a real possibility for embryo IVP, allowing to reduce the interval between generations and to accelerate the processes of genetic improvement in selection schemes [53]. The effect of the goat donors age on oocyte quality, as well as the effect of follicle and oocyte size were both reviewed [21]. Generally, prepubertal animals are good responders to hormonal stimulation with exogenous gonadotropin. It has been shown in various ruminant species that oocytes collected from prepubertal animals are less competent for development than those collected from adult females (cattle: [54]; sheep: [25]; goat: [25, 55]. Moreover, ultrastructural and functional deficiencies are reported in prepubertal goat oocytes [21]. In goats, the high ovarian response of prepubertal animal was associated with lower developmental competence and lower embryo cryotolerance [56]. On the other hand, it has been recently shown that the developmental competence of oocytes obtained from large follicles ($\geq$ 3 mm) were equally competent between non treated prepubertal and adult goat [57]. These results may suggest that oocytes of good quality may be recovered from prepubertal animals in physiological hormonal

environment and that ovarian stimulation in prepubertal animal may require further adjustment. With decrease of ovarian reserve and impaired hormonal environment, aged females may be associated with poor reproductive performance. In goat, LOPU/IVP has shown to be an efficient strategy for the reproductive rescue of aged goats of high genetic value [13]. Berlinguer et al. [58] reported that reproductive aging in sheep is associated with impaired follicle functionality and an increase in the proportion of oocytes showing morphological abnormalities. Total oocyte yield was not affected by age but amount of oocyte suitable for IVP was decreased (7%). Despite these observations, the developmental competence of oocytes after IVP and embryo cryotolerance was not affected by aging process.

In addition to the age of the females, their physiological status (season, feeding,…) could also influence the quality of LOPU oocytes. Regarding the evaluated breeding season, the number of aspirated follicles was similar, whereas the proportion of good quality COC (Grade I/II) was enhanced in the breeding season. However, interestingly, the cleavage rate of LOPU oocytes was higher in the anestrous season and blastocyst development rate was also higher in the same period when using slaughterhouse oocytes (unpublished data). Similarly, season was shown to influence the number and competence of recovered oocytes in sheep, but cleavage rate tended to be higher in the anestrous season [59]. Recently, oocyte developmental competence in buffalo was also shown to be affected by different seasons since a higher cleavage and embryo yields were recorded in autumn compared to spring, with intermediate results in summer and winter [60]. A subcutaneous implant of melatonin improved sheep oocyte developmental competence during the anestrous season [59]. It was earlier demonstrated that LOPU could be performed at different seasons with little or no important change in overall response [61]. It is likely that the progestagen exposure and

ovarian stimulation may be sufficient to overcome a possible seasonal effect in small ruminants.

3. *In vitro* maturation (IVM)

During folliculogenesis, the oocyte undergoes a progressive differentiation (ultrastructure, organites, RNA and protein storage,…) leading to the acquisition of meiotic competence and finally to developmental competence [22, 62]. During this differentiation, the oocyte is maintained at meiotic prophase stage (germinal vesicle) by follicular environment, impairing chromosomes condensation and thus allowing the continuation of transcription activities, important for its final developmental competence [63]. As the follicular inhibition is stopped by LH surge or by removing oocyte from its follicle, the maturation occurs. Oocyte maturation includes meiotic resumption and progression to the fertilizable stage of metaphase II after emission of the first polar body (transition from oocyte I to oocyte II), as well as related events in oocyte cytoplasm and surrounding *cumulus* cells [63]. Therefore, the result of IVM depends on the intrinsic quality of immature oocytes but also the maturation conditions can widely modulate the final competence of IVM [64]. It is clear that the whole process of oocyte differentiation and maturation is coordinated by surrounding somatic cells (granulosa, *cumulus*) through a constant dialog mediated by follicular fluid and through the network of gap junctions maintained between these compartments [23,65, 66]. It was well demonstrated that *in vitro* matured oocytes are compromised in their developmental capacity compared with those matured *in vivo* [5, 67-69]. The lower potential of IVM oocytes is probably related to the heterogeneity of the oocytes obtained, in terms of differentiation status, and to inappropriate conditions used in IVM. This results in a relatively low rate of oocytes finally reaching the blastocyst stage,

which is one of the main limitations of IVP from immature oocytes in mammals. While many studies aim at searching for molecular biomarkers of oocyte quality in follicular cells [70] or in oocyte itself [71], the morphological evaluation of COC (*cumulus* cells number and appearance, homogeneity of oocyte cytoplasm) remains the only non-invasive way to select homogeneous COC population after collection [72, 73]. Nonetheless, the assessment of the COC morphology prior to IVM is proved to be a poorly accurate predictor of oocyte intrinsic meiotic competence [48], resulting in a great variability in the production of embryos. Brilliant cresyl blue (BCB) staining may represent a good complement to simple morphological evaluation of COC. BCB staining reflects activity of glucose-6-phosphate dehydrogenase (G6PD), an enzyme synthesized in growing oocytes but with decreased activity in oocytes that have finished their growth phase. This staining allows the selection of two populations of COC with different competences, BCB- oocytes being more competent than BCB+ [74]. It has been shown that BCB staining also reflects mitochondrial activity [71] and apoptosis regulator genes expression [75] in oocytes.

In vitro maturation is commonly performed using tissue culture media enriched with amino acids and glucose (usually TCM 199), supplemented with hormones and heat inactivated serum. In general, the most commonly used system for IVM in small ruminants is TCM 199 medium supplemented with FSH (porcine or ovine origin), LH, estradiol (E2) and 10% fetal calf serum (FCS) [1, 76]. Our group used follicular fluid (FF) from non-atretic and large follicles as IVM medium supplement (10%) for some years with good results [1, 5]. However, all complex supplements such as FCS, estrus goat or sheep serum (EGS or ESS) or FF lead to a lack of reproducibility, since they present high chemical variations among sources or among batches from the same source [21, 77] and expose to sanitary risks. For these reasons, there has been a trend to use

more defined maturation media. Our laboratory, in order to make IVM simpler, more safe and repeatable, proposed a maturation medium using just defined compounds – TCM 199 supplemented with 10 ng/mL EGF and 100 µM cysteamine – and obtained good results in embryo development of adult goat oocytes [20]. Indeed, EGF family of growth factors has been pointed out as a possible intrafollicular mediator of the LH preovulatory surge in rodent species [78] and the addition of EGF to the IVM medium has been shown to improve matured oocyte quality in several species [72, 79, 80]. Cysteamine is a stable permeating precursor of glutathione metabolism which increases the reduced glutathione content of matured oocytes, improving their post fertilization viability by facilitating sperm nucleus decondensation and pronuclei formation [81, 82]. These simplified maturation conditions (TCM 199, EGF, Cysteamine) provide good and reliable results in terms of cleavage and development rate [1] for slaughterhouse derived COC. However, recent experiments performed in our lab observed that LOPU derived COC may have different requirements during IVM and more complexes media would be necessary in order to achieve high IVP rates (article in preparation).

The alteration of basic maturation conditions can significantly affect oocyte competence as reflected by the morula and blastocyst yield after IVF [83]. The use of vitamins in IVM also promoted beneficial effects in overall blastocyst development, cleavage rate and in the mean number of blastocyst cells [84]. It was shown [83] that cleavage rate and developmental capacity of cleaved ovine oocytes matured in undefined medium (FBS) were higher than those matured in semidefined (BSA) or defined medium (PVA). This result indicates that serum may have an additional effect beyond EGF, IGF-I and cysteamine (possibly an effect of serum components) leading to a better completion of cytoplasmic maturation in oocytes. Serum is a highly complex combination of components, including proteins, fatty acids, vitamins, trace elements,

hormones and growth factors [85]. The same group demonstrated later that the maturation media containing FCS supplemented with human menopausal serum (HMS), ESS, or EGS supported better rates of IVM, IVF and embryo development than bovine or ovine follicular fluid [86]. Also in the presence of FCS, the supplementation with GH or FSH reached similar blastocyst rates, but a lower rate was obtained when both hormones were concomitantly used [87]. Different interactions may exist among the substances and more studies are needed to establish which molecules or combinations to optimize this step [88]. Regarding IVM physical conditions, the literature does not vary considerably and IVM is usually performed incubating COC in large groups (50-100) in 4-well plates with 500 µL of medium, under 5% CO_2 in air at 38 to 39 °C with maximum humidity for 22-27 h [20, 35, 89].

Another way to improve the whole success of IVP would be to increase the intrinsic quality of the oocytes before IVM by allowing them to complete *in vitro* the late differentiation process that occurs into the growing follicle *in vivo* [64]. This prematuration step would require mimicking *in vitro* the meiotic inhibition signal provided by the follicle, to allow the oocyte to continue his transcription and translation activities at the germinal vesicle stage. The key element of meiotic resumption control cascade in the mammalian oocyte is the M-Phase promoting Factor (MPF) kinase which can induce chromosome condensation, germinal vesicle breakdown and spindle formation by phosphorylating specific target proteins [63]. Several chemical methods aimed at inhibiting MPF activation [90] or MPF kinase activity [91, 92] have been proposed. These methods provided efficient meiotic block *in vitro* but did not allow positive progression of treated oocytes toward improved competence, since several MPF independent pathways related to maturation were still activated [93]. More recently, some interesting results have been obtained in cattle by acting upstream MPF

activation on phosphodiesterase 3, the enzyme which regulates cAMP level in *cumulus* cells, and in turn MPF activation in the oocyte [94]. This more physiological inhibition process is able to increase final oocyte intrinsic quality before gonadotropin addition allowing meiotic resumption. In addition, the same group showed that oocyte secreted factors could be involved in an autocrine-paracrine manner in the regulation of oocyte quality [95]. Such oocyte secreted factors have been shown to increase the developmental competence of prepubertal goat oocytes [96]. These interesting findings may open the way for new improvement of IVM success in small ruminants.

4. *In vitro* fertilization (IVF)

The IVF technique should be able to produce embryos using both female and male gametes. Studies of sperm capacitation for IVF purposes have provided evidence that male variability, the origin of sperm (ejaculated or cauda-epididymal, fresh or frozen-thawed) and the presence of seminal plasma play crucial roles in *in vitro* embryo production in cattle [68]. Either fresh or frozen-thawed semen can be used to fertilize matured oocytes. In any case, it is essential to assess live or dead sperm cells. IVF rates following Percoll or swim-up separation of motile spermatozoa did not differ significantly, regarding the rates of single pronucleus formation, normal fertilization with two pronuclei and polyspermic fertilization in goats [69].

It has been suggested that procedures used in bovine IVP can be applied in small ruminants after minimal modifications, mainly during sperm capacitation and IVF, such as the reduction in the centrifugation speed and the addition of sheep serum in IVF medium for sperm capacitation [35]. Capacitation is a crucial process that mammalian sperms require to achieve fertilizing ability and the importance of capacitating agents for the success of IVF has been already described in many species [69]. Heparin has

been shown to increase fertilization rate in cattle [97] and is thus widely used for spermatozoa capacitation in this species. Similarly, there are some reports confirming the role of heparin in goats and sheep [35, 46]. However, some reports indicate an adverse effect on fertilization [98] or lesser embryo development and quality [99]. Interestingly, a different pattern of heparin effect when using goat fresh or frozen–thawed sperm was reported, probably due to a capacitating action of sperm freezing [46]. We have recently demonstrated that greater numbers of blastocysts were obtained from COC fertilized in the presence of 5 µg/mL heparin than those without heparin, suggesting that the addition of heparin to the fertilization medium improves sperm capacitation of frozen-thawed goat sperm [89]. The treatment of sperm with ionomycin has also been shown to increase the fertilization rate in goats [100].

Regarding IVF conditions for small ruminants, sperm concentrations vary from 1 to $3.5x10^6$ cells/mL with spermatozoa and oocytes co-incubated for 16-20 h at 38-39 °C in humidified atmosphere of 5% CO_2 in air [8, 35, 46]. In our laboratory we routinely use a pool of two or three straws from different males [20] since this may increase the repeatability of IVF results (unpublished observations). In pre-pubertal goat oocytes, it was possible to achieve elevated sperm penetration with $4x10^6$ cells/mL without increasing polyspermy [101], which is a major concern when utilizing a high sperm concentration. In sheep and goats, polyspermy is the main abnormality detected after IVF, affecting almost 20% of the inseminated oocytes, much higher than what is observed with the *in vivo* situation [102]. Some recent sheep IVF data show that sex-sorted spermatozoa elicit equal or greater cleavage and blastocyst rates than non-sorted spermatozoa [103]. Despite its interesting applicability, in the consulted literature there are no reports related to the use of sexed spermatozoa in goat IVF.

A high variation is observed between bucks or rams and also between ejaculates from the same animal in terms of fertilizing ability *in vitro* and embryo development, as it happens in bulls. Currently, in most IVP laboratories, male selection is based on their capacity to fertilize oocytes in previously established IVF conditions. This practice could make it easier to solve the problem and maintain IVP. However, genetic IVP requires the use of a specific male, which is generally of unknown *in vitro* fertility. In order to make this technique more applicable in the field, these variations should be better understood and IVF conditions optimized for each specific male of interest. This optimization should be based on the number of spermatozoa used and/or heparin concentration. A capacitation treatment before insemination could be tried for lower fertility males.

The appropriate time of *cumulus* cells removal from oocytes during IVP also remained controversial. Evaluating recent studies, some reports describe that oocytes are denuded immediately after IVM [10, 20, 59, 73], just after IVF [35, 57] or even partially denuded after IVM with *cumulus*-free presumptive zygotes denuded after IVF [104]. Without direct comparison of these two procedures, it is difficult to precisely analyze their respective interests. In our laboratory *cumulus* oophorus was usually removed following maturation, before IVF [5]. Nonetheless, we recently evidenced the importance of *cumulus* cells during IVF step [89] and currently goat COC are denuded just after IVF. This parameter may also be important while setting up conditions adapted to specific males, with the presence of *cumulus* cells being able to regulate the sperm capacitation process.

5. *In vitro* development (IVD) and embryo quality

After IVF, the presumptive zygotes are removed from the fertilization medium and placed in an embryo culture medium that allows the development up to a stage that is compatible with its transfer to the recipient uterus. It has been clearly demonstrated in cattle that while the rate of success of IVP in terms of blastocysts yield rely on oocyte intrinsic quality and maturation conditions, the quality of the resulting blastocysts (cryosurvival, viability) relies on the conditions encountered during earlier steps of development [105]. It was demonstrated that bovine *in vitro*-derived embryos show certain differences when compared to embryos produced *in vivo* in relation to their morphology, timing of development, resistance to low temperature [106], embryo metabolism [107] and especially gene expression [108, 109]. One of the most dramatic effect of *in vitro* environment is the modification of embryo lipid metabolism leading to increased storage of triglycerides and decreased phospholipids production [110], translating into altered membrane fluidity and, in turn, lower cryoresistance. *In vitro* conditions may also induce sex ratio deviation, due to metabolic advantage of male embryos in *in vitro* environment [111].

Although different culture media have been successfully used for small ruminant embryo development (TCM 199, B2,…) the most widely used medium is the synthetic oviduct fluid (SOF). First developed for sheep embryos [112], the SOF medium has then been used and adapted for other species like cattle [113], pig [114] and goat [1]. Sequential media more adapted to changing embryo requirements have also been successfully used for small ruminants [115, 116]. To ensure embryo confinement, which facilitates the action of autocrine factors known to stimulate early development, embryos are usually cultured in mineral oil overlaid droplets of medium (1 µL/embryo). This confinement improves the development to the blastocyst stage [117]. This is

particularly important when working with LOPU, since a high variation in female response can be observed and, in any case, the number of embryos per female remains low.

Embryo development media are usually supplemented with various protein sources (BSA, FCS, growth factors), although these supplements may be associated to "large offspring syndrome" (LOS) in several species, including small ruminants [118]. Indeed, serum can induce morphological and physiological differences in embryos [104]. Therefore, a chemically defined medium for culturing IVP embryos may allow to overcome some inconvenient of the use of complex biological additives such as serum (sanitary risks, batch effect, LOS and embryo metabolic defects), but results still consist in lower development. Consequently, while setting up IVP conditions, it is important to evaluate the quality of the embryos produced in addition to the final rate of development. It is also important to keep in mind that early embryos, as any eukaryotic cells, have active securities to recognize genome abnormalities and consequently block the cell cycle progression and drive the affected cell to apoptosis [119]. Therefore, selecting fast growing embryos or accelerating development kinetics by modifying culture conditions may favor embryos which lost their protections against genetic abnormalities and finally lead to the transfer of genetically defected embryos.

The best way to assess embryo quality or viability is to check their capacity of establishing pregnancy after transfer to recipients and consequently give birth to normal offspring. However, embryo transfers being heavy and costly in domestic species, various methods have been proposed to approximate embryo quality in laboratory experiments. Some reliable indicators of embryo viability are: the evaluation of the level of expression of specific gene sets, embryo metabolism, kinetic of development and the resistance to cryopreservation [23]. The lower quality of IVP embryos is

probably due to inappropriate culture conditions compared to natural maternal environment. Early development taking place in the oviduct, many studies have been aimed at understanding the physiology of this organ [120] and to mimic *in vitro* its beneficial effect on embryo quality. Indeed, developing IVP embryos in oviducts of a transient recipient allows to reach the same level of quality than *in vivo* derived embryos [53]. This recipient female could be from another species, ewes or rabbit does being the most frequently used, although mouse oviducts could also provide valuable results [121]. However, since this methodology is heavy and requires the presence of transient recipients and two surgeries, efforts have been done to reproduce this maternal environment in culture.

The first successes of ruminant embryo development up to the blastocyst stage *in vitro* have been obtained by using co-culture systems involving oviduct epithelial cells [122, 123]. Further evolution of this technique involved use of new media especially designed to support early embryo development based on the composition of oviduct fluid, such as SOF medium [124]. Rodríguez-Dorta et al. [20] used the SOF medium on goat oviduct epithelial cells (GOEC) monolayers for co-culture of goat IVP embryos to evaluate the effect of cells on embryo survival after vitrification. Although the rate of zygotes reaching the blastocyst stage was significantly higher in SOF (28%) as compared with GOEC (20%), after direct transfer of vitrified-thawed embryos, kidding rate and embryo survival rate were significantly higher in GOEC co-culture when compared to the SOF medium (Table 1).

Table 1. Success rate after direct transfer of goat IVP embryos, adapted from [2].

Treatment	Recip. n	Emb. n	Pregnant Day 34 n (%)	Pregnant Day 90 n (%)	Kidding n (%)	Kids born n (%)
SOF Fresh	13	26	12[a] (92)	12[a] (92)	12[a] (92)	16[a] (62)
SOF vit.	29	58	6[b] (21)	4[b] (14)	4[b] (14)	5[b] (9)
GOEC vit.	18	36	13[a] (72)	10[c] (56)	10[c] (56)	12[c] (33)

a,b,c Values with different superscripts in the same column are significantly different (Chi Square, $P<0.05$).

The oviducts used to generate GOEC primocultures come usually from slaughtered animals, and due to heterogeneous cell origin, they can introduce some variability. Therefore, further research using more reliable GOEC co-culture or purified GOEC factors is still required in view to enhance the success rate of IVP in goats. Goat IVM/IVF oocytes were successfully co-cultured with GOEC that were simultaneously collected at the same moment as immature oocytes [46]. However, in cases when LOPU is applied, there is no opportunity to collect oviduct epithelial cells. An interesting possibility would be to use oviducts from other species (cattle), easier to obtain and providing high number of cells. Indeed, the positive effect of OEC is not species specific [125] and we obtained similar results with BOEC (bovine cells) than those described for GOEC (goat cells), in terms of development rate and quality (article in preparation). Surprisingly, porcine cells were even more effective in supporting bovine embryos development than with bovine derived cells [126]. The mechanisms of the beneficial effects of co-culture are not well understood. Oviduct hosts the first events of reproduction, such as oocyte maturation, selection, transport and storage of sperm, fertilization of the ovum, and early embryonic development [127]. These activities may

rely on the production of embryotrophic factors by oviduct cells, the depletion of potentially harmful substances, the modification of the medium components to better fit embryo requirements or even the modification of physico-chemical environment parameters such as pH or oxygen concentration [128]. In addition, the oviduct may act on early reproductive processes through a combination of these mechanisms.

Regarding embryo quality, as described above, COC morphology evaluation prior to IVM is not sufficient as a good predictor of oocyte intrinsic meiotic competence [129]. Han et al. [32] showed that developmental competence of goat oocytes with the same COC morphology, follicle size and grade of *cumulus* expansion may be different. This strongly indicates that the developmental potential of an oocyte is determined by multifactorial interactions. Researchers have begun to report studies aiming to determine the relationship between oocyte quality and their competence to develop up to blastocyst following IVF. EGFR may indeed be a good candidate marker for indirect prediction of goat oocyte quality [129]. Nonetheless, fewer and less conclusive results have been obtained about the relationship between oocyte and blastocyst quality. It was suggested that the conditions of embryo culture have a crucial role in determining blastocyst quality [57, 130].

The investigation of molecular markers using highly sensitive techniques, such as microarray and real-time PCR, is a promising approach for studies in COC gene expression for a better inference of oocyte competence to development [65, 131]. Successful embryonic development is dependent on a rigorous, time- and site-specific gene expression program of appropriate genes. Identification of these differentially expressed genes and the analysis of their pattern of expression are powerful tools to gain information about functions relevant to processes such as the oocyte competence. These biochemical markers for embryo development would be able to improve

pregnancy by optimizing oocyte and embryo selection. The differentially expressed genes may be important markers of the oocyte's ability to reach the blastocyst stage and allow direct assessment of the fertility potential of an individual oocyte without compromising its integrity [65]. A first class of genes is required for normal folliculogenesis and fertilization. Other genes are maternal effect genes, i.e. they are dispensable until fertilization but essential for proper embryo development. Besides those, there is some functional importance of other oocyte-enriched genes that remains to be assessed [132]. Moreover, some important research could be done in order to evaluate the genic expression profile of oocytes obtained after LOPU or slaughterhouse ovaries.

6. Related technologies

6.1. Cloning and transgenesis

Some biotechnologies associated with genetic engineering, such as transgenesis and cloning are strongly related to the IVP methods. Additionally, small ruminants are presented as an excellent model for these techniques.

Tremendous interest in cloning has been generated in the recent years. It is the asexual production of genetically identical animals that can be obtained by nuclear transfer (NT). The NT involves the transfer of nuclei from serum-starved fetal or adult cells into enucleated oocytes matured *in vivo* or *in vitro*. Fetal fibroblast cell or a variety of adult cells can be used. Since the birth of "Dolly" [133], an ewe derived from the transfer of an adult somatic cell to an enucleated oocyte, research on cloning of somatic cells has gained force. In goats, several reports have been published about the birth of

clones, most of these reports pertaining to the production of genetically modified animals [134, 135].

A transgenic animal may be defined as one containing recombinant DNA molecules in its genome that were introduced by genetic engineering manipulation. Transgenic small ruminants are mainly produced by two techniques: NT [134, 135] and pronuclear microinjection of DNA [136, 137]. Recently, however, Pereyra-Bonnet et al. [138] obtained transgenic sheep embryos using the sperm-mediated gene transfer technique. After the birth of first transgenic mammal [139], transgenic sheep were obtained few years after that [140]. On the other hand, goat is particularly an efficient system of producing recombinant proteins as they produce considerable amounts of milk and incur lower investment and maintenance costs than cows. Currently, the human antithrombin III (hAT-III) produced by transgenic goats is the unique recombinant protein from animal bioreactor that was approved for clinical use in Europe [141], and in the USA [142]. Other examples of human recombinant proteins expressed in the milk of transgenic goats are alpha-1-antitrypsin, blood clotting factor IX, granulocyte colony-stimulating factor, growth hormone, prolactin and tissue plasminogen activator [143]. Thus, the transgenic technology was validated using a goat model as a viable alternative method for the production of recombinant pharmaceutical proteins.

6.2. Manipulation of preantral follicle-enclosed oocytes in goats and sheep

Preantral follicles (PFs) form a far larger oocyte reservoir (90% of all ovarian follicles) compared with the number of antral follicles. However, the majority (99.9%) of preantral and antral follicles become atretic during their growth and maturation.

Therefore, recovery of PFs before atresia followed by *in vitro* culture (IVC) might increase the availability of fertilizable oocytes, enhance the knowledge of the mechanisms involved in ovarian folliculogenesis, complement other reproductive technologies such as IVP, nucleus transfer, transgenic animal production, embryonic stem cell development and aid in studies on reproductive toxicology.

Taking into consideration that farm or endangered animals are normally found far away from the laboratory, one step extremely important is the preservation of ovarian tissue during transportation. This step aims to ensure good quality oocytes enclosed in preantral follicle for further cryopreservation and or IVC. Experiments performed by our group demonstrated that the best temperature for the transportation of caprine or ovine preantral follicles enclosed in ovarian tissue is 4 °C independently of media used. Methods for the isolation of caprine PFs include mechanical and enzymatic procedures. While mechanical isolation of PFs is mainly performed using 25 G needles [144] or a tissue chopper [145], enzymatic isolation is performed by proteolytic digestion, incubating the ovarian tissue with non-specific enzymes such as collagenase [144] or trypsin [146].

PFs, isolated or enclosed in the ovarian tissue, can be cryopreserved by applying conventional slow freezing or vitrification methods. Both methods are efficient and possess advantages and disadvantages. Recently, we have shown that ovarian tissue-enclosed PFs from sheep and goats respond differently to the same cryopreservation protocol [147]. Furthermore, caprine PFs present some peculiarities that might influence cryopreservation, such as the absence of concentration of cytoplasmic components in the juxta-nuclear region as observed in other species [148]. Recovery of gonadal function and harvesting of mature viable oocytes after auto-transplantation of frozen–

thawed caprine ovarian tissue are the best results reported so far [149]. In addition, using this procedure live offsprings were produced in sheep [150].

Caprine PFs are usually cultured either in ovarian cortical slices or after isolation. Although IVC of PFs enclosed in cortical slices is practical, non-time-consuming, maintains threedimensional follicle architecture and preserves interactions between follicles and surrounding stroma cells, the cortical tissue may act as a barrier to IVC medium perfusion, resulting in the growth of primordial follicles until the secondary stage only [151]. Conversely, IVC of isolated PFs allows monitoring of individual follicles throughout the growing period, but is time-consuming, may be affected by the isolation procedure, demands more sophisticated IVC systems and is often applied to secondary and not to primordial and primary follicles. A major achievement was the *in vitro* development of isolated secondary PFs up to antral stages and subsequent production of mature oocytes and embryos in goats [152, 153] and sheep [154].

7. Conclusions

In vitro embryo production has a great potential for efficient propagation of valuable females after obtaining oocytes generated by LOPU or slaughterhouse ovaries. However, oocyte and embryo physiology need to be better understood in order to improve the technique and to produce a large number of good quality embryos of high genetic merit for production traits or for biodiversity preservation.

Three different research lines appear promising in this view:

- Based on the comparison of requirements between LOPU and slaughterhouse oocytes and on new meiotic inhibition strategies, it would

be possible to set up innovative maturation treatments beneficial to oocyte intrinsic quality.

- The fertilization step of IVP should be adapted to be able to enter any male for genetic IVP. A systematic check of the most appropriate IVF parameters with slaughterhouse oocytes should be designed and used for any new male.
- A deeper study of oviduct embryo interaction and dialog will allow identifying the embryotrophic factors produced by the oviduct epithelium. These factors could then be used as supplement of semi defined culture media.

Finally, *in vitro* technologies bring up new hopes for an increased accuracy of selection scheme and improved efficiency of genetic gain.

Acknowledgments

J.M.G. Souza was supported by CAPES and Ceará State University. The authors wish to thank CAPES-COFECUB bilateral framework for financial support of collaboration between the Ceará State University and INRA on goat IVP (Grant 728/11, 2011-2013). This work was supported by a grant from Région Centre, France (PIVER program, #200800030493, 2008-2011).

References

[1] Cognié Y, Poulin N, Locatelli Y, Mermillod P. State-of-the-art production, conservation and transfer of *in-vitro*-produced embryos in small ruminants. Reprod Fertil Dev 2004;16(4):437-45.

[2] Basrur PK, King WA. Genetics then and now: breeding the best and biotechnology. Rev Sci Tech 2005;24(1):31-49.

[3] Hinrichs K. *In vitro* production of equine embryos: state of the art. Reprod Domest Anim 2010;45(2):3-8.

[4] Gil MA, Cuello C, Parrilla I, Vazquez JM, Roca J, Martinez EA. Advances in swine *in vitro* embryo production technologies. Reprod Domest Anim 2010;45(2):40-8.

[5] Cognié Y, Baril G, Poulin N, Mermillod P. Current status of embryo technologies in sheep and goat. Theriogenology 2003;59(1):171-88.

[6] Lonergan P, Fair T. *In vitro*-produced bovine embryos: dealing with the warts. Theriogenology 2008;69(1):17-22.

[7] Hansen PJ, Block J, Loureiro B, Bonilla L, Hendricks KE. Effects of gamete source and culture conditions on the competence of *in vitro*-produced embryos for post-transfer survival in cattle. Reprod Fertil Dev 2010;22(1):59-66.

[8] Baldassarre H, Karatzas CN. Advanced assisted reproduction technologies (ART) in goats. Anim Reprod Sci 2004;82-83:255-66.

[9] Stangl M, Kühholzer B, Besenfelder U, Brem G. Repeated endoscopic ovum pick-up in sheep. Theriogenology 1999;52(4):709-16.

[10] Morton KM, de Graaf SP, Campbell A, Tomkins LM, Maxwell WM, Evans G. Repeat ovum pick-up and *in vitro* embryo production from adult ewes with and without FSH treatment. Reprod Domest Anim 2005;40(5):422-8.

[11] Baldassarre H, Wang B, Pierson J, Neveu N, Sneek L, Lapointe J, Cote F, Kafidi N, Keefer CL, Lazaris A, Karatzas CN. Prepubertal propagation of transgenic

cloned goats by laparoscopic ovum pick-up and in vitro embryo production. Cloning Stem Cells 2004;6(1):25-9.

[12] Morton KM. Developmental capabilities of embryos produced in vitro from prepubertal lamb oocytes. Reprod Domest Anim 2008;43(2):137-43.

[13] Baldassarre H, Rao KM, Neveu N, Brochu E, Begin I, Behboodi E, Hockley DK. Laparoscopic ovum pick-up followed by *in vitro* embryo production for the reproductive rescue of aged goats of high genetic value. Reprod Fertil Dev 2007;19(5):612-6.

[14] Baldassarre H, Wang B, Keefer CL, Lazaris A, Karatzas CN. State of the art in the production of transgenic goats. Reprod Fertil Dev 2004;16(4):465-70.

[15] Comizzoli P, Mermillod P, Mauget R. Reproductive biotechnologies for endangered mammalian species. Reprod Nutr Dev 2000; 40(5):493-504.

[16] Locatelli Y, Vallet JC, Huyghe FP, Cognié Y, Legendre X, Mermillod P. Laparoscopic ovum pick-up and in vitro production of sika deer embryos: effect of season and culture conditions. Theriogenology 2006;66(5):1334-42.

[17] Chang, M.C. Fertilization of rabbit ova *in vitro*. Nature 1959;184(7):466-7.

[18] Hanada, A. *In vitro* fertilization in goats. Jpn. J Anim Reprod 1985,31:21-26.

[19] Crozet N, De Smedt V, Ahmed-Ali M, Sévellec C. Normal development following *in vitro* oocyte maturation and fertilization in the goat. Theriogenology 1993;39:206.

[20] Rodríguez-Dorta N, Cognié Y, González F, Poulin N, Guignot F, Touzé JL, Baril G, Cabrera F, Alamo D, Batista M, Gracia A, Mermillod P. Effect of coculture

with oviduct epithelial cells on viability after transfer of vitrified in vitro produced goat embryos. Theriogenology 2007;68(6):908-13.

[21] Paramio, M.T. *In vivo* and *in vitro* embryo production in goats. Small Rumin Res 2010;89:144-14.

[22] Mermillod, P, Oussaid, B, Cognié, Y. Aspects of follicular and oocyte maturation that affect the developmental potential of embryos. J Reprod Fertil 1999;449-460.

[23] Mermillod P, Dalbiès-Tran R, Uzbekova S, Thélie A, Traverso JM, Perreau C, Papillier P, Monget P. Factors affecting oocyte quality: who is driving the follicle? Reprod Domest Anim 2008;43(2):393-400.

[24] Tibary A, Anouassi A, Khatir, H. Update on reproductive biotechnologies in small ruminants and camelids. Theriogenology 2005;64(3):618-38.

[25] Ptak G, Loi P, Dattena M, Tischner M, Cappai P. Offspring from one-month-old lambs: studies on the developmental capability of prepubertal oocytes. Biol Reprod 1999;61(6):1568-74.

[26] Viana JH, De Almeida Camargo LS, De Moraes Ferreira A, De Sa WF, De Carvalho Fernandes CA, De Pinho Marques Junior A. Short intervals between ultrasonographically guided follicle aspiration improve oocyte quality but do not prevent establishment of dominant follicles in the Gir breed (Bos indicus) of cattle. Anim Reprod Sci 2004;84(1-2):1-12.

[27] Bols PE. Puncture of immature ovarian follicles in bovine assisted reproduction. Verh K Acad Geneeskd Belg, 2005;67(3):177-202.

[28] Graff KJ, Meintjes M, Dyer VW, Paul JB, Denniston RS, Ziomek C, Godke RA. Transvaginal ultrasound-guided oocyte retrieval following FSH stimulation of domestic goats. Theriogenology 1999;51(6):1099-119.

[29] Baldassarre H, Furnus CC, De Matos DG, Pessi H. *In vitro* production of sheep embryos using laparoscopic folliculocentesis: alternative gonadotrophin treatments for stimulation of oocyte donors. Theriogenology 1996;45(3):707-17.

[30] Teixeira PP, Padilha LC, Oliveira ME, Motheo TF, da Silva AS, Barros FF, Coutinho LN, Flôres FN, Lopes MC, Bandarra MB, Silva MA, Vasconcelos RO, Rodrigues LF, Vicente WR. Laparoscopic ovum collection in sheep: Gross and microscopic evaluation of the ovary and influence on ooctye production. Anim Reprod Sci 2011;127(3-4):169-175.

[31] Crozet N, Ahmed-Ali M, Dubos MP. Developmental competence of goat oocytes from follicles of different size categories following maturation, fertilization and culture *in vitro*. J Reprod Fertil 1995;103(2):293-8.

[32] Han ZB, Lan GC, Wu YG, Han D, Feng WG, Wang JZ, Tan JH. Interactive effects of granulosa cell apoptosis, follicle size, *cumulus*-oocyte complex morphology, and *cumulus* expansion on the developmental competence of goat oocytes: a study using the well-in-drop culture system. Reproduction 2006; 132(5):749-58.

[33] Gandolfi F, Brevini TAL. *In vitro* maturation of farm animal oocytes: a useful tool for investigating the mechanisms leading to full-term development. Reprod Fertil Dev 2010;22:495-507.

[34] Bols PE, Ysebaert MT, Van Soom A, de Kruif A. Effects of needle tip bevel and aspiration procedure on the morphology and developmental capacity of bovine compact *cumulus* oocyte complexes. Theriogenology 1997;47(6):1221-36.

[35] Cox JF, Alfaro V. *In vitro* fertilization and development of OPU derived goat and sheep oocytes. Reprod Domest Anim 2007;42(1):83-7.

[36] Rodríguez C, Anel L, Alvarez M, Anel E, Boixo JC, Chamorro CA, de Paz P. Ovum Pick-up in Sheep: a Comparison between Different Aspiration Devices for Optimal Oocyte Retrieval. Reprod Domest Anim 2006;41:106-113.

[37] de Castro T, Rubianes E, Menchaca A, Rivero A. Ovarian dynamics, serum estradiol and progesterone concentrations during the interovulatory interval in goats. Theriogenology 1999;52:399-411.

[38] Evans AC, Duffy P, Hynes N, Boland MP. Waves of follicle development during the estrous cycle in sheep. Theriogenology 2000;53(3):699-715.

[39] Avelar SRG, Moura RR, Sousa FC, Pereira AF, Almeida KC, Melo CHS, Teles-Filho ACA, Baril G, Melo LM, Teixeira DIA, Freitas VJF. Oocyte production and in vitro maturation in Canindé goats following hormonal ovarian stimulation. Anim Reprod 2012;9(1):1-7.

[40] Gibbons A, Pereyra Bonnet F, Cueto MI, Catala M, Salamone DF, Gonzalez-Bulnes A. Procedure for maximizing oocyte harvest for *in vitro* embryo production in small ruminants. Reprod Domest Anim 2007;42:423-426.

[41] Jablonka-Shariff A, Reynolds LP, Redmer DA. Effects of gonadotropin treatment and withdrawal on follicular growth, cell proliferation, and atresia in ewes. Biol Reprod 1996;55(3):693-702.

[42] Reby D, Charlton BD, Locatelli Y, McComb K. Oestrous red deer hinds prefer male roars with higher fundamental frequencies. Proc Biol Sci 2010;277:2747-2753.

[43] Gonźalez-Bulnes A, Baird DT, Campbell BK, Cocero MJ, García-García RM, Inskeep EK, López-Sebastián A, McNeilly AS, Santiago-Moreno J, Souza CJ,

Veiga-López A. Multiple factors affecting the efficiency of MOET in sheep and goats. Reprod Fertil Dev 2004;16:421-435.

[44] Gonzalez-Bulnes A, Veiga-Lopez A, Garcia P, Garcia-Garcia RM, Ariznavarreta C, Sanchez MA, Tresguerres JA, Cocero MJ, Flores JM. Effects of progestagens and prostaglandin analogues on ovarian function and embryo viability in sheep. Theriogenology 2005;63:2523-2534.

[45] Berlinguer F, Gonzalez-Bulnes A, Succu S, Leoni G, Mossa F, Bebbere D, Ariznavarreta C, Tresguerres JA, Veiga-Lopez A, Naitana S. Effects of progestagens on follicular growth and oocyte developmental competence in FSH-treated ewes. Domest Anim Endocrinol 2007;32:303-314.

[46] Katska-Ksiazkiewicz L, Ryńska B, Gajda B, Smorag Z. Effect of donor stimulation, frozen semen and heparin treatment on the efficiency of *in vitro* embryo production in goats. Theriogenology 2004;62(3-4):576-86.

[47] Locatelli Y, Poulin N, Baril G, Touzé JL, Bouttier A, Cognié Y, Mermillod P. FSH treatment before laparoscopic ovum pick-up in goat influences quantity and quality of recovered oocytes. Reprod Fertil Dev 2004;16(4):513.

[48] Almeida KC, Pereira AF, Alcântara Neto AS, Avelar SR, Bertolini LR, Bertolini M, Freitas VJ, Melo LM. Real-time qRT-PCR analysis of EGF receptor in *cumulus*-oocyte complexes recovered by laparoscopy in hormonally treated goats. Zygote 2011;19(2):127-36.

[49] Abdullah RB, Liow SL, Rahman AN, Chan WK, Wan-Khadijah WE, Ng SC. Prolonging the interval from ovarian hyperstimulation to laparoscopic ovum pick-up improves oocyte yield, quality, and developmental competence in goats. Theriogenology 2008;70:765-771.

[50] Berlinguer F, Leoni G, Bogliolo L, Pintus PP, Rosati I, Ledda S, Naitana S. FSH different regimes affect the developmental capacity and cryotolerance of embryos derived from oocytes collected by ovum pick-up in donor sheep. Theriogenology 2004;61(7-8):1477-86.

[51] Cognié Y. State of the art in sheep-goat embryo transfer. Theriogenology 1999; 51(1):105-16.

[52] Berlinguer F, Gonzalez-Bulnes A, Succu S, Leoni GG, Veiga-Lopez A, Mossa F, Garcia-Garcia RM, Bebbere D, Galioto M, Cocero MJ, Naitana S. GnRH antagonist enhance follicular growth in FSH-treated sheep but affect developmental competence of oocytes collected by ovum pick-up. Theriogenology 2006;65(6):1099-109.

[53] Galli C, Crotti G, Notari C, Turini P, Duchi R, Lazzari G. Embryo production by ovum pick up from live donors. Theriogenology 2001;55(6):1341-57.

[54] Revel F, Mermillod P, Peynot N, Renard JP, Heyman Y. Low developmental capacity of in vitro matured and fertilized oocytes from calves compared with that of cows. J Reprod Fertil 1995;103(1):115-20.

[55] Mogas T, Palomo MJ, Izquierdo MD, Paramio MT. Developmental capacity of in vitro matured and fertilized oocytes from prepubertal and adult goats. Theriogenology 1997;47(6):1189-203.

[56] Leoni GG, Succu S, Satta V, Paolo M, Bogliolo L, Bebbere D, Spezzigu A, Madeddu M, Berlinguer F, Ledda S, Naitana S. *In vitro* production and cryotolerance of prepubertal and adult goat blastocysts obtained from oocytes collected by laparoscopic oocyte-pick-up (LOPU) after FSH treatment. Reprod Fertil Dev 2009;21:901-908.

[57] Romaguera R, Moll X, Morató R, Roura M, Palomo MJ, Catalá MG, Jiménez-Macedo AR, Hammami S, Izquierdo D, Mogas T, Paramio MT. Prepubertal goat oocytes from large follicles result in similar blastocyst production and embryo ploidy than those from adult goats. Theriogenology 2011;76(1):1-11.

[58] Berlinguer F, Gonzalez-Bulnes A, Spezzigu A, Contreras-Solis I, Succu S, McNeilly AS, Naitana S, Leoni GG. Effect of aging on follicular function may be relieved by exogenous gonadotropin treatment in a sheep model. Reproduction 2012;144(2):245-255.

[59] Vázquez MI, Abecia JA, Forcada F, Casao A. Effects of exogenous melatonin on in vivo embryo viability and oocyte competence of undernourished ewes after weaning during the seasonal anestrus. Theriogenology 2010;74(4):618-26.

[60] Di Francesco S, Boccia L, Campanile G, Di Palo R, Vecchio D, Neglia G, Zicarelli L, Gasparrini B. The effect of season on oocyte quality and developmental competence in Italian Mediterranean buffaloes (Bubalus bubalis). Anim Reprod Sci 2011;123(1-2):48-53.

[61] Pierson J, Wang B, Neveu N, Sneek L, Côté F, Karatzas CN, Baldassarre H. Effects of repetition, interval between treatments and season on the results from laparoscopic ovum pick-up in goats. Reprod Fertil Dev 2004;16(8):795-9.

[62] Sirard MA, Richard F, Blondin P, Robert C. Contribution of the oocyte to embryo quality. Theriogenology 2006;65(1):126-36.

[63] Mermillod P, Marchal, R. Mammalian oocyte maturation. M S-Medecine Sciences 1999;15(2):148-156.

[64] Mermillod P, Marchal R. *In-vitro* maturation of oocytes from domestic species. Hum Reprod 1999;14:384-385.

[65] Assidi M, Dufort I, Ali A, Hamel M, Algriany O, Dielemann S, Sirard MA. Identification of potential markers of oocyte competence expressed in bovine *cumulus* cells matured with follicle-stimulating hormone and/or phorbol myristate acetate *in vitro*. Biol Reprod 2008;79(2):209-22.

[66] Salhab M, Tosca L, Cabau C, Papillier P, Perreau C, Dupont J, Mermillod P, Uzbekova S. Kinetics of gene expression and signaling in bovine *cumulus* cells throughout IVM in different mediums in relation to oocyte developmental competence, *cumulus* apoptosis and progesterone secretion. Theriogenology 2011;75(1):90-104.

[67] Sutton ML, Gilchrist RB, Thompson JG. Effects of *in-vivo* and *in-vitro* environments on the metabolism of the *cumulus*-oocyte complex and its influence on oocyte developmental capacity. Hum Reprod Update 2003;9(1):35-48.

[68] Smorag Z, Katska-Ksiazkiewicz L, Skrzyszowska M, Jura J, Gajda B, Bochenek M. Animal reproduction biotechnology in Poland. Int J Dev Biol 2008;52(2-3):151-5.

[69] Khatun M, Bhuiyan MM, Ahmed JU, Haque A, Rahman MB, Shamsuddin M. *In vitro* maturation and fertilization of prepubertal and pubertal black Bengal goat oocytes. J Vet Sci 2011;12(1):75-82.

[70] Salhab M, Papillier P, Perreau C, Guyader-Joly C, Dupont J, Mermillod P, Uzbekova S. Thymosins beta-4 and beta-10 are expressed in bovine ovarian follicles and upregulated in *cumulus* cells during meiotic maturation. Reprod Fertil Dev 2010;22(8):1206-21.

[71] Catalá MG, Izquierdo D, Uzbekova S, Morató R, Roura M, Romaguera R, Papillier P, Paramio MT. Brilliant Cresyl Blue stain selects largest oocytes with

highest mitochondrial activity, maturation-promoting factor activity and embryo developmental competence in prepubertal sheep. Reproduction 2011;142(4):517-27.

[72] Guler A, Poulin N, Mermillod P, Terqui M, Cognié Y. Effect of growth factors, EGF and IGF-I, and estradiol on *in vitro* maturation of sheep oocytes. Theriogenology 2000;54(2):209-18.

[73] Katska-Ksiazkiewicz L, Opiela J, Rynska B. Effects of oocyte quality, semen donor and embryo co-culture system on the efficiency of blastocyst production in goats. Theriogenology 2007;68(5):736-44.

[74] Rodríguez-González E, López-Béjar M, Velilla E, Paramio MT. Selection of prepubertal goat oocytes using the brilliant cresyl blue test. Theriogenology 2002; 57(5):1397-409.

[75] Opiela J, Katska-Ksiazkiewicz L, Lipiński D, Słomski R, Bzowska M, Ryńska B. Interactions among activity of glucose-6-phosphate dehydrogenase in immature oocytes, expression of apoptosis-related genes Bcl-2 and Bax, and developmental competence following IVP in cattle. Theriogenology 2008;69(5):546-55.

[76] Cocero MJ, Alabart JL, Hammami S, Martí JI, Lahoz B, Sánchez P, Echegoyen E, Beckers JF, Folch J. The Efficiency of In vitro Ovine Embryo Production Using an Undefined or a Defined Maturation Medium is Determined by the Source of the Oocyte. Reprod Domest Anim 2011;46(3):463-70.

[77] Kishida R, Lee ES, Fukui Y. *In vitro* maturation of porcine oocytes using a defined medium and developmental capacity after intracytoplasmic sperm injection. Theriogenology 2004;62(9):1663-76.

[78] Park JY, Su YQ, Ariga M, Law E, Jin SL, Conti M. EGF-like growth factors as mediators of LH action in the ovulatory follicle. Science 2004;303(5658):682-4.

[79] Lonergan P, Carolan C, Van Langendonckt A, Donnay I, Khatir H, Mermillod P. Role of epidermal growth factor in bovine oocyte maturation and preimplantation embryo development *in vitro*. Biol Reprod 1996;54(6):1420-9.

[80] Marques MG, Nicacio AC, de Oliveira VP, Nascimento AB, Caetano HV, Mendes CM, Mello MR, Milazzotto MP, Assumpção ME, Visintin JA. *In vitro* maturation of pig oocytes with different media, hormone and meiosis inhibitors. Anim Reprod Sci 2007;97(3-4)375-81.

[81] de Matos DG, Furnus CC, Moses DF, Baldassarre H. Effect of cysteamine on glutathione level and developmental capacity of bovine oocyte matured *in vitro*. Mol Reprod Dev, 1995;42(4):432-6.

[82] de Matos DG, Gasparrini B, Pasqualini SR, Thompson JG. Effect of glutathione synthesis stimulation during *in vitro* maturation of ovine oocytes on embryo development and intracellular peroxide content. Theriogenology 2002;57(5):1443-51.

[83] Shabankareh HK, Zandi M. Developmental potential of sheep oocytes cultured in different maturation media: effects of epidermal growth factor, insulin-like growth factor I, and cysteamine. Fertil Steril 2010;94(1): 335-340.

[84] Bormann CL, Moige Ongeri E, Krisher RL. The effect of vitamins during maturation of caprine oocytes on subsequent developmental potential *in vitro*. Theriogenology 2003;59:1373-1380.

[85] Harper KM, Brackett BG. Bovine blastocyst development after follicle-stimulating hormone and platelet-derived growth factor treatment for oocyte maturation *in vitro.* Zygote 1993;1(1):27-34.

[86] Shabankareh HK, Sarsaifi K, Mehrannia T. *In vitro* maturation of ovine oocytes using different maturation media: effect of human menopausal serum. J Assist Reprod Genet 2011;28(6):531-537.

[87] Shirazi A, Shams-Esfandabadi N, Ahmadi E, Heidari B. Effects of Growth Hormone on Nuclear Maturation of Ovine Oocytes and Subsequent Embryo Development. Reprod Domest Anim 2010;45:530-536.

[88] Fonseca JF, Souza JMG, Camargo LSA. Produção de Oócitos e Embriões de Pequenos Ruminantes: Passado, Presente e Futuro. Acta Sci Vet 2010;38(2):337-369.

[89] Souza JM, Duffard N, Bertoldo MJ, Locatelli Y, Corbin E, Fatet A, Freitas VJ, Mermillod P. Influence of heparin or the presence of *cumulus* cells during fertilization on the *in vitro* production of goat embryos. Anim Reprod Sci 2013;138:82-89.

[90] Sirard MA, First NL. *In vitro* inhibition of oocyte nuclear maturation in the bovine. Biol Reprod 1988;39(2):229-34.

[91] Lonergan P, Khatir H, Carolan C, Mermillod P. Bovine blastocyst production in vitro after inhibition of oocyte meiotic resumption for 24 h. J Reprod Fertil 1997;109(2):355-365.

[92] Mermillod P, Tomanek M, Marchal R, Meijer L. High developmental competence of cattle oocytes maintained at the germinal vesicle stage for 24 hours in culture by specific inhibition of MPF kinase activity. Mol Reprod Dev 2000;55(1):89-95.

[93] Vigneron C, Perreau C, Dupont J, Uzbekova S, Prigent C, Mermillod P. Several signaling pathways are involved in the control of cattle oocyte maturation. Mol Reprod Dev 2004:69(4):466-74.

[94] Gilchrist RB, Thompson JG. Oocyte maturation: emerging concepts and technologies to improve developmental potential *in vitro*. Theriogenology 2007; 67(1):6-15.

[95] Gilchrist RB, Lane M, Thompson JG. Oocyte-secreted factors: regulators of *cumulus* cell function and oocyte quality. Hum Reprod Update 2008;14(2):159-77.

[96] Romaguera R, Morató R, Jiménez-Macedo AR, Catalá M, Roura M, Paramio MT, Palomo MJ, Mogas T, Izquierdo D. Oocyte secreted factors improve embryo developmental competence of COCs from small follicles in prepubertal goats. Theriogenology 2010;74(6):1050-9.

[97] Parrish JJ, Susko-Parrish J, Winer MA, First NL. Capacitation of bovine sperm by heparin. Biol Reprod 1988;38(5):1171-80.

[98] Malik RK, Lohan IS, Dhanda OP. Tuli RK. Test for the acrosomal reaction of goat spermatozoa treated with heparin. Small Rumin Res 1997;26:163-166.

[99] Cognié Y, Poulin N, Pignon P, Sulon J, Beckers JF, Guerin Y. Does heparin affect developmental ability of IVP goat oocytes? in *11th AETE*. 1995. Hannover, Germany.

[100] Wang B, Baldassarre H, Tao T, Gauthier M, Neveu N, Zhou JF, Leduc M, Duguay F, Bilodeau AS, Lazaris A, Keefer C, Karatzas CN. Transgenic goats produced by DNA pronuclear microinjection of *in vitro* derived zygotes. Mol Reprod Dev 2002;63(4):437-43.

[101] Palomo MJ, Mogas T, Izquierdo D, Paramio MT. The influence of sperm concentration, length of the gamete co-culture and the evolution of different sperm parameters on the *in vitro* fertilization of prepubertal goat oocytes. Zygote 2010;18(4):345-55.

[102] Coy P, Aviles M. What controls polyspermy in mammals, the oviduct or the oocyte? Biological reviews of the Cambridge Philosophical Society, 2010. 85(3):593-605.

[103] de Graaf SP, Beilby KH, Underwood SL, Evans G, Maxwell WM. Sperm sexing in sheep and cattle: the exception and the rule. Theriogenology 2009;71(1): 89-97.

[104] Karami Shabankareh H, Sarsaifi K, Mehrannia T. *In vitro* maturation of ovine oocytes using different maturation media: effect of human menopausal serum. J Assisted Reprod Gen 2011;28(6):531-7.

[105] Rizos D, Ward F, Duffy P, Boland MP, Lonergan P. Consequences of bovine oocyte maturation, fertilization or early embryo development *in vitro* versus *in vivo*: implications for blastocyst yield and blastocyst quality. Mol Reprod Dev, 2002;61(2):234-48.

[106] Massip A, Mermillod P, Dinnyes A. Morphology and biochemistry of *in-vitro* produced bovine embryos: Implications for their cryopreservation. Hum Reprod 1995;10(11):3004-3011.

[107] Donnay I, Partridge RJ, Leese HJ. Can embryo metabolism be used for selecting bovine embryos before transfer? Reprod Nutr Dev 1999;39(5-6):523-33.

[108] Rizos D, Clemente M, Bermejo-Alvarez P, de La Fuente J, Lonergan P, Gutiérrez-Adán A. Consequences of *in vitro* culture conditions on embryo development and quality. Reprod Domest Anim 2008;43(4):44-50.

[109] Lonergan P, Fair T, Corcoran D, Evans AC. Effect of culture environment on gene expression and developmental characteristics in IVF-derived embryos. Theriogenology 2006;65(1):137-52.

[110] Abd El Razek IM, Charpigny G, Delalleau N, Marquant-Leguienne B, Mermillod P, Guyader-Joly C, Humblot P. Lipid composition of *in vivo* and *in vitro* produced bovine embryos and of *in vitro* embryos showing different osmotic behavior. Theriogenology 2000;53:46.

[111] Gutiérrez-Adán A, Lonergan P, Rizos D, Ward FA, Boland MP, Pintado B, de la Fuente J. Effect of the *in vitro* culture system on the kinetics of blastocyst development and sex ratio of bovine embryos. Theriogenology 2001;55(5):1117-26.

[112] Tervit HR, Whittingham DG, Rowson LE. Successful culture *in vitro* of sheep and cattle ova. J Reprod Fertil 1972;30(3):493-7.

[113] Takahashi Y, First NL. *In vitro* development of bovine one-cell embryos: Influence of glucose, lactate, pyruvate, amino acids and vitamins. Theriogenology 1992;37(5):963-78.

[114] Marchal R, Feugang JM, Perreau C, Venturi E, Terqui M, Mermillod P. Meiotic and developmental competence of prepubertal and adult swine oocytes. Theriogenology 2001;56(1):17-29.

[115] Wang B, Baldassarre H, Pierson J, Cote F, Rao KM, Karatzas CN. The *in vitro* and *in vivo* development of goat embryos produced by intracytoplasmic sperm injection using tail-cut spermatozoa. Zygote 2003;11(3):219-27.

[116] Garcia-Garcia RM, Ward F, Fair S, O'Meara CM, Wade M, Duffy P, Lonergan P. Development and quality of sheep embryos cultured in commercial G1.3/G2.3 sequential media. Anim Reprod Sci 2007;98(3-4):233-40.

[117] Ferry L, Mermillod P, Massip A, Dessy F. Bovine embryos cultured in serum-poor oviduct-conditioned medium need cooperation to reach the blastocyst stage. Theriogenology 1994;42(3):445-53.

[118] Sinclair KD, McEvoy TG, Maxfield EK, Maltin CA, Young LE, Wilmut I, Broadbent PJ, Robinson JJ. Aberrant fetal growth and development after in vitro culture of sheep zygotes. J Reprod Fertil 1999;116(1):177-86.

[119] Mu XF, Jin XL, Farnham MM, Li Y, O'Neill C. DNA damage-sensing kinases mediate the mouse 2-cell embryo's response to genotoxic stress. Biol Reprod 2011;85(3):524-35.

[120] Leese HJ, Hugentobler SA, Gray SM, Morris DG, Sturmey RG, Whitear SL, Sreenan JM. Female reproductive tract fluids: composition, mechanism of formation and potential role in the developmental origins of health and disease. Reprod Fertil Dev 2008;20(1):1-8.

[121] Rizos D, Ramirez MA, Pintado B, Lonergan P, Gutierrez-Adan A. Culture of bovine embryos in intermediate host oviducts with emphasis on the isolated mouse oviduct. Theriogenology 2010;73(6):777-85.

[122] Eyestone WH, First NL. Co-culture of early cattle embryos to the blastocyst stage with oviducal tissue or in conditioned medium. J Reprod Fertil 1989;85(2):715-20.

[123] Gandolfi F, Moor RM. Stimulation of early embryonic development in the sheep by co-culture with oviduct epithelial cells. J Reprod Fertil 1987;81(1):23-8.

[124] Fukui Y, McGowan LT, James RW, Pugh PA, Tervit HR. Factors affecting the *in-vitro* development to blastocysts of bovine oocytes matured and fertilized *in vitro*. J Reprod Fertil 1991;92(1):125-31.

[125] Yadav PS, Saini A, Kumar A, Jain GC. Effect of oviductal cell co-culture on cleavage and development of goat IVF embryos. Anim Reprod Sci 1998;51(4): 301-6.

[126] Pavasuthipaisit K, Lhuangmahamongkol S, Tocharus C, Kitiyanant Y, Prempree P. Porcine oviductal cells support *in vitro* bovine embryo development. Theriogenology 1994;41(5):1127-1138.

[127] Buhi WC, Alvarez IM, Kouba AJ. Oviductal regulation of fertilization and early embryonic development. J Reprod Fertil 1997;52:285-300.

[128] Goncalves RF, Staros AL, Killian GJ. Oviductal fluid proteins associated with the bovine zona pellucida and the effect on *in vitro* sperm-egg binding, fertilization and embryo development. Reprod Domest Anim 2008;43(6):720-9.

[129] Almeida KC, Pereira AF, Alcântara Neto AS, Avelar SR, Bertolini LR, Bertolini M, Freitas VJ, Melo LM. Real-time qRT-PCR analysis of EGF receptor in *cumulus*-oocyte complexes recovered by laparoscopy in hormonally treated goats. Zygote 2011;19(2):127-36.

[130] Rizos D, Ward F, Boland MP, Lonergan P. Effect of culture system on the yield and quality of bovine blastocysts as assessed by survival after vitrification. Theriogenology 2001;56(1):1-16.

[131] Wrenzycki C, Herrmann D, Niemann H. Messenger RNA in oocytes and embryos in relation to embryo viability. Theriogenology 2007;68(1):77-83.

[132] Thélie A, Papillier P, Pennetier S, Perreau C, Traverso JM, Uzbekova S, Mermillod P, Joly C, Humblot P, Dalbiès-Tran R. Differential regulation of abundance and deadenylation of maternal transcripts during bovine oocyte maturation *in vitro* and *in vivo*. BMC Dev Biol 2007;7:125.

[133] Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. Viable offspring derived from fetal and adult mammalian cells. Nature 1997;385:810-813.

[134] Baguisi A, Behboodi E, Melican DT, Pollock JS, Destrempes MM, Cammuso C, Williams JL, Nims SD, Porter CA, Midura P, Palacios MJ, Ayres SL, Denniston RS, Hayes ML, Ziomek CA, Meade HM, Godke RA, Gavin WG, Overström EW, Echelard Y. Production of goats by somatic cell nuclear transfer. Nat Biotechnol, 1999;17:456-461.

[135] Keefer CL, Keyston R, Lazaris A, Bhatia B, Begin I, Bilodeau AS, Zhou FJ, Kafidi N, Wang B, Baldassarre H, Karatzas CN. Production of cloned goats after nuclear transfer using adult somatic cells. Biol Reprod 2002;66(1):199-203.

[136] Freitas VJ, Serova IA, Andreeva LE, Dvoryanchikov GA, Lopes ES Jr, Teixeira DI, Dias LP, Avelar SR, Moura RR, Melo LM, Pereira AF, Cajazeiras JB, Andrade ML, Almeida KC, Sousa FC, Carvalho AC, Serov OL. Production of transgenic goat (*Capra hircus*) with human Granulocyte Colony Stimulating Factor (hG-CSF) gene in Brazil. An Acad Bras Cienc 2007;79:585-592.

[137] Ko JH, Lee CS, Kim KH, Pang MG, Koo JS, Fang N, Koo DB, Oh KB, Youn WS, Zheng GD, Park JS, Kim SJ, Han YM, Choi IY, Lim J, Shin ST, Jin SW, Lee KK, Yoo OJ. Production of biologically active human granulocyte colony stimulating factor in the milk of transgenic goat. Transgenic Res 2000;9:215-222.

[138] Pereyra-Bonnet F, Gibbons A, Cueto M, Sipowicz P, Fernández-Martín R, Salamone D. Efficiency of sperm-mediated gene transfer in the ovine by laparoscopic insemination, *in vitro* fertilization and ICSI. J Reprod Dev 2011; 57:188-196.

[139] Gordon JW, Scangos GA, Plotkin DJ, Barbosa JA, Ruddle FH. Genetic transformation of mouse embryos by microinjection of purified DNA. Proc Nat Acad Sci 1980;77:7380-7384.

[140] Hammer RE, Pursel VG, Rexroad CE Jr, Wall RJ, Bolt DJ, Ebert KM, Palmiter RD, Brinster RL. Production of transgenic rabbits, sheep and pigs by microinjection. Nature 1985;315:680-683.

[141] Schmidt C. Belated approval of first recombinant protein from animal. Nat. Biotechnol 2006;24:877.

[142] Kling J. First US approval for a transgenic animal drug. Nat. Biotechnol 2009;27.

[143] Moura RR, Melo LM, Freitas VJF. Production of recombinant proteins in milk of transgenic and non-transgenic goats. Braz Arch Biol Technol 2011;54:927-938.

[144] Demeestere I, Delbaere A, Gervy C, Van Den Bergh M, Devreker F, Englert Y. Effect of preantral follicle isolation technique on in vitro follicular growth, oocyte maturation and embryo development in mice. Hum Reprod 2002;17: 2152-2159.

[145] Lucci CM, Amorim CA, Báo SN, Figueiredo JR, Rodrigues AP, Silva JR, Gonçalves PB. Effect of the interval of serial sections of ovarian in the tissue chopper on the number of isolated caprine preantral follicles. Anim Reprod Sci 1999;56:39-49.

[146] Itoh T, Hoshi H. Efficient isolation and long-term viability of bovine small preantral follicles *in vitro.* In Vitro Cell Dev Biol Anim 2000;36:235-240.

[147] Faustino LR, Santos RR, Silva CM, Pinto LC, Celestino JJ, Campello CC, Figueiredo JR, Rodrigues AP. Goat and sheep ovarian tissue cryopreservation: effects on the morphology and development of primordial follicles and density of stromal cell. Anim Reprod Sci 2010;122:90-97.

[148] Lucci CM, Silva RV, Carvalho CA, Figueiredo R, Báo N. Light microscopical and ultrastructural characterization of goat preantral follicles. Small Rumin Res 2001;41:61-69.

[149] Santos RR, Knijn HM, Vos PL, Oei CH, van Loon T, Colenbrander B, Gadella BM, van den Hurk R, Roelen BA. Complete follicular development and recovery of ovarian function of frozen–thawed, autotransplanted caprine ovarian cortex. Fertil Steril 2009;91:1455-1458.

[150] Salle B, Demirci B, Franck M, Rudigoz RC, Guerin JF, Lornage J. Normal pregnancies and live births after autograft of frozen-thawed hemi-ovaries into ewes. Fertil Steril 2002;77:403-408.

[151] Martins FS, Celestino JJ, Saraiva MV, Matos MH, Bruno JB, Rocha-Junior CM, Lima-Verde IB, Lucci CM, Báo SN, Figueiredo JR. Growth and differentiation factor-9 stimulates activation of goat primordial follicles *in vitro* and their progression to secondary follicles. Reprod Fertil Dev 2008;20:916-924.

[152] Magalhães DM, Duarte AB, Araújo VR, Brito IR, Soares TG, Lima IM, Lopes CA, Campello CC, Rodrigues AP, Figueiredo JR. *In vitro* production of a caprine embryo from a preantral follicle cultured in media supplemented with growth hormone. Theriogenology 2011;75:182-188.

[153] Saraiva MV, Rossetto R, Brito IR, Celestino JJ, Silva CM, Faustino LR, Almeida AP, Bruno JB, Magalhães DM, Matos MH, Campello CC, Figueiredo JR.

Dynamic medium produces caprine embryo from preantral follicles grown *in vitro.* Reprod Sci 2010;17(12):1135-1143.

[154] Arunakumari G, Shanmugasundaram N, Rao VH. Development of morulae from the oocytes of cultured sheep preantral follicles. Theriogenology 2010;74:884-894.

PARTE II: COLHEITA DE OÓCITOS POR LAPAROSCOPIA EM CAPRINOS: DO TRATAMENTO HORMONAL AOS POSSÍVEIS DESTINOS DOS OÓCITOS

Resumo

Independentemente se a coleta de oócitos é parte de um programa de produção *in vitro* de embriões (PIVE) e/ou promoverá avanços biotecnológicos, os aspectos metodológicos nas técnicas de colheita de oócitos são imprenscídiveis. Para alcançar sucesso de forma ótima, número suficiente de oócitos de boa qualidade é prequisito para diversas técnicas reprodutivas e a colheita de oócitos por laparoscopia (COL) é a mais recomendada técnica. Entretanto, a variabilidade na quantidade e qualidade de oócitos colhidos ainda limita o uso desta tecnologia. Atualmente, uma grande variação é relatada na literatura com taxas de colheita de oócitos variando de 40 a 90% e o número de estruturas colhidas por fêmea entre quatro e 14. Esta variabilidade pode ocorrer tanto em função de variáveis não controláveis, como raça, idade e características intrínsecas da cabra, como devido a aspectos controláveis, como o tratamento superestimulatório, tipo da agulha, pressão de aspiração, dentre outros. Acredita-se que novas pesquisas devam contribuir significativamente para a melhoria da técnica de COL. Esta revisão objetiva relatar os diferentes fatores que influenciam a resposta de cabras doadoras após COL, apresentando as principais etapas para recuperação oocitária assim como modificações técnicas propostas para melhoria da eficiência da COL. Além disso, discutir sobre potencias aplicações de oócitos caprinos depois de sua recuperação por COL e resultados gerais sobre a técnica no mundo.

Palavras-chave: biotecnologia, caprino, FIV, LOPU, oócito.

A parte II dessa revisão de literatura corresponde ao artigo que será submetido à **Revista Brasileira de Ciência Veterinária** (Qualis: B3).

Laparoscopic ovum pick up (LOPU) in goats: from hormonal treatment to oocytes possible destinations

Colheita de oócitos por laparoscopia em caprinos: do tratamento hormonal aos possíveis destinos dos oócitos

Joanna Maria Gonçalves de Souza-Fabjan[1,2], Yann Locatelli[1,3], Vicente José de Figueiredo Freitas[2], Pascal Mermillod[1*]

1 INRA, UMR7247, Physiologie de la Reproduction et des Comportements, INRA, CNRS, Université de Tours, Haras Nationaux. 37380 Nouzilly, France

2 Laboratory of Physiology and Control of Reproduction, Veterinary School, State University of Ceará, Av. Dedé Brasil, 1700, 60740-90, Fortaleza, CE, Brazil

3 Museum National d'Histoire Naturelle, Réserve de la Haute Touche, 36290, Obterre, France

Running title: LOPU in goats

* Corresponding author.
Tel.: +33 247 42 79 20
E-mail address: Pascal.Mermillod@tours.inra.fr

Summary

Whether we are collecting oocytes from valuable donors as part of an *in vitro* embryo production (IVEP) program and/or to promote advances in biotechnology as cloning and transgenesis, all depends on the development of methodological aspects aimed at the techniques of recovering oocytes. To achieve success optimally, sufficient number of good quality oocytes is the prerequisite for various reproductive techniques and laparoscopic ovum pick up (LOPU) is the recommended technique for obtaining them from live goats. However, the variability of the quantity and quality of the oocytes collected still limits the large-scale use of this technology. Under the current conditions, too large variety is reported with oocyte recovery rates ranging from 40 to 90%, and the number of harvested structures per female between four and 14 oocytes in different laboratories. This variability can occur either for non-controlled variables in some cases such as breed, age and intrinsic characteristics of the doe or due to aspects that we are able to control, as stimulation treatment, type of needle, aspiration pressure, among others. We believe that new investigations should contribute to significant improvement of LOPU methods. This review aims to report different factors influencing goat donor response for LOPU, presenting main steps for oocytes recovery as well as technical modifications proposed for improving LOPU efficiency. Furthermore, discuss about the potential applications of goat oocytes after their recovery by LOPU and overall results in goats worldwide.

Keywords: biotechnology, goat, IVF, LOPU, oocyte

1. Introduction

The potential application of IVEP, cloning or transgenesis depends on the development of reliable, repeatable and efficient techniques for recovery of good quality oocytes, among other factors (Gibbons et al., 2008). In order to recover oocytes, earlier reports used ovariectomy via laparotomy (Younis et al., 1991; Keskintepe et al., 1994). Another possibility was the use of laparotomy, where the oocytes are aspirated from intact ovaries (Ptak et al., 1999a), but it is well known that it could lead to adherences or infections. Studies involving slaughtered healthy goats were also published (Crozet et al., 1995). However, unless any organizational reason aiming to reduce the herd is involved (Katska- Ksiazkiewicz et al., 2007) or if it is part of the experimental design (Vazquez et al., 2010b) this method is unacceptable nowadays in healthy females. In cattle, the transvaginal ultrasound-guided aspiration (TUGA) technique is currently the most used technique to successfully obtain oocytes (Viana et al., 2004; Bols 2005). However, Graff et al. (1999) obtained lower numbers of oocytes from goats submitted to TUGA as compared to those submitted to laparoscopy (4.3 *vs.* 11.5 oocytes collected per doe).

Currently, goat oocytes are mostly collected from slaughterhouse ovaries or, less often, by laparoscopic ovum pick up (LOPU) from live animals. Slaughterhouse ovaries may provide a cheap and abundant source of large number of oocytes by follicles aspiration, slicing or follicle dissection (Martino et al., 1994). These oocytes from unknown females are helpful for research. However, in diverse countries, the number of goats slaughtered is reduced and consequently it is difficult to carry out proper experiments using their ovaries (Tan et al., 2011). Therefore, LOPU may be an alternative and important source of oocytes. Moreover, the use of IVEP for genetic improvement or diffusion requires collecting oocytes from given females with high

economic or genetic merit. LOPU procedure is less stressful, less invasive, lasts less (each session takes between 10 and 20 min in does) than laparotomy and can be repeated at short intervals without reducing oocyte developmental competence. Therefore, LOPU is the recommended technique for obtaining good-quality oocytes from live donors (Baldassarre and Karatzas, 2004; Pierson et al., 2004; Baldassarre et al., 2007).

Whether we are collecting oocytes from valuable donors as part of an IVEP program, or we are collecting oocytes from standard goats to be used as recipient cytoplasts in a SCNT program, it all starts here (Baldassarre, 2012). The development of LOPU began in the 1990s and there is still little information available from immature oocytes collected from live goats. Despite heavy research efforts, the variability of the number and quality of the oocytes collected still limits the large-scale use of this technology. Under the current conditions, oocyte recovery rates range from 40 to 90%, and the number of harvested structures per female between 4 and 14 oocytes in different laboratories. This variability can occur either for non-controlled variables in some cases such as breed, age and intrinsic characteristics of the doe or due to aspects that we are able to control, as stimulation treatment, type of needle, aspiration pressure, among others. This review will focus on: i, different factors influencing goat donor response for LOPU; ii, presenting the main steps for oocytes recovery as well as technical modifications proposed for improving LOPU efficiency; iii, the potential applications of goat oocytes after their recovery by LOPU; and iv, overall results in goats worldwide.

2. Before LOPU – Hormonal treatment and other factors affecting ovarian response

2.1. Hormonal treatment – different protocols for ovarian stimulation

Different attempts of hormonal stimulation have been evaluated in order to improve the quantity, quality and development competence of oocytes from does submitted to LOPU (Gibbons et al., 2008). The goats should be heat synchronized and stimulated with gonadotropin. These protocols enable synchronous recruitment of a larger population of follicles. Progestagen or progesterone is used for estrus synchronization during 10 to 14 d and usually remains until the moment of donor aspiration in order to block ovulations. Indeed, a dose of prostaglandin F2α analogue is also used to promote luteolysis. Hormonal treatments proposed by different research groups worldwide in goats are represented in Fig. 1. The use of GnRH analogue for one week prior to the stimulatory regime in goats adversely affected the number of follicles available and of oocytes recovered by LOPU (Baldassarre et al., 2001).

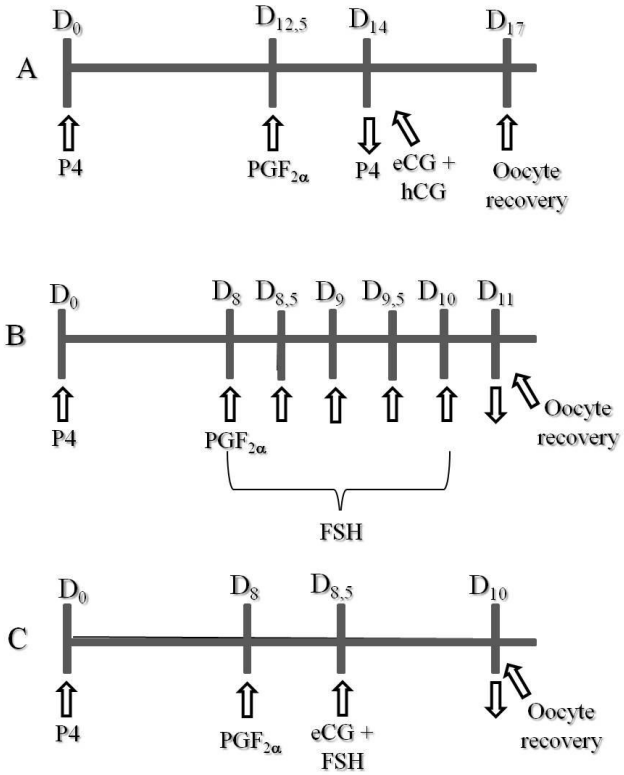

Fig 1. Stimulation treatments proposed by different research groups for LOPU in goats using A: no FSH, B: five doses of FSH or C: eCG associated to FSH, called oneshot. A. P4 = CIDR, $PGF_{2\alpha}$ = 125 µg cloprostenol, eCG = 1500 IU, hCG = 250 IU, (Tan et al.,

2011); B. P4 = 60 mg MAP, $PGF_{2\alpha}$ = 50 μg cloprostenol, pFSH = 30/30/20/20/20 mg (Avelar et al., 2012); C. P4 = 60 mg MAP, $PGF_{2\alpha}$ = 125 μg cloprostenol, eCG = 300 IU, pFSH = 80 mg (Baldassarre and Karatzas, 2004).

It is well described that FSH stimulation prior to oocyte recovery increases production efficiency with significantly more follicles punctured and oocytes retrieved from each female (Katska-Ksiazkiewicz et al., 2004). Porcine derived-FSH (pFSH; 20 *vs* 40 mg) and FSH of equine origin (eFSH; 20 *vs* 40 mg) were compared. Goats that received 40 mg pFSH allowed the recovery of 14.8 oocytes per doe, greater ($P<0.05$) than 10.5 when they received 40 mg eFSH or 6-7 ooytes when only 20 mg p/eFSH was administered (Graff et al., 1999). Generally, a higher FSH stimulation dose for donor females produces more large follicles which are easier to visualize and aspirate (Graaf et al., 1999). We observed that does receiving high dose of ovine FSH (1 IU) had greater proportion of zygotes reaching the blastocyst stage than goats treated with half dose (Locatelli et al., 2004). However, interestingly, Katska-Ksiazkiewicz et al. (2004) reported a lower cleavage rate in FSH-stimulated goats rather than in the control group, but this difference was balanced by the numerically higher developmental rates to the blastocyst stage in the FSH-treated group. Morton et al. (2005) reported similar IVD rates in ewes submitted to treatment with or without FSH prior to LOPU, although, these results were obtained in relation to the cleaved blastocysts. In fact, although not statistically different, when we take into account ewes that received FSH (~59%) they had a lower cleavage rate than those not treated (~79%). Shortly, these data suggest that FSH may enhance the amount of oocytes recovered per doe, however, some reports indicate an adversely effect in further cleavage rate although it does not affect development rates.

Perhaps either the positive or negative effect of FSH may be in relation to its plan of administration. Protocols using decreasing doses of FSH or a constant dose every 12 or 24 h were reported (Gibbons et al., 2008). Ewes treated with decreasing doses of FSH administered in 12 h interval, presented greater number of big follicles (>5 mm) than those submitted to constant doses (4 doses of 24 IU every 12 h). However, blastocyst rates were similar for untreated ewes (13.7%) or those treated with decreasing FSH doses (11.8%), but were higher from ewes treated with a constant FSH dose (20.4%). A possible explanation for the lower embryo development capacity in the treatment which used FSH in decreasing doses is that the first two very high doses could have induced to a rapid and abnormal follicular development (Berlinguer et al., 2004). With regard to the number of doses, the higher number of FSH injections (5 *vs* 3) resulted in Grade1/2 COC's with higher Epidermal Growth Factor Receptor expression in *cumulus* cells that was correlated with an elevated meiotic competence following IVM in goats (Almeida et al., 2011). Current treatments mostly consist in multi-injection FSH regimes, but the protocols are very labor intensive and rather stressful to the animals because of excessive handling. Therefore, the "Oneshot" regime, in which only a combination of FSH and eCG is given as a single treatment (Fig. 1) is a good option, which is less expensive and requires less labor input LOPU (Baldassarre and Karatzas, 2004). LOPU is performed at 10, 24, 36 or 48 h after the end of gonadotrophic administration. Stimulation with a single dose has been carried out at 24, 36 or 48 h before LOPU (Gibbons et al., 2008). It was recently demonstrated that a prolonged interval from FSH/hCG to LOPU improved oocyte retrieval rate and oocyte quality in goats. LOPU at 60 or 72 h after FSH/hCG optimized yields of good quality oocytes for IVM and embryo production in comparison to 36 h interval (Abdullah et al., 2008).

2.2. Individual characteristics

Besides stimulation treatment, it is also important to consider intrinsic factors of goat donors, in particular the body condition score, breed, age and genetic merit, since they may influence the quality and number of oocytes obtained (Cognié et al., 2004). Surely, the individual response of the goats would be one of the most important factors in the variability of the final effectiveness of the technique. A very large variability was observed among individual cows within a range from 0 to 128 oocytes (Pontes et al., 2011) and in goats, ranging from 4 to 33 follicles and 2 to 12 oocytes recovered (Gibbons et al., 2007). Such variability shows that the individual response constitutes an important role which is difficult to overcome (Pontes et al., 2011).

2.3. Age

The use of prepubertal animals is a real possibility for IVEP, allowing to reduce the interval between generations and to accelerate the processes of genetic improvement in selection schemes (Galli et al., 2001). The effect of goat donors age on oocyte quality, as well as the effect of follicle and oocyte size were reviewed (Paramio, 2010). Oocytes collected from prepubertal animals are less competent for development than those collected from adult goats (Mogas et al., 1997; Ptak et al., 1999b). Moreover, ultrastructural and functional deficiencies are reported in prepubertal goat oocytes (Paramio, 2010). However, it has been recently shown that the real problem of prepubertal goats is that they have more small follicles than adults, but the developmental competence of oocytes obtained from large follicles (≥ 3 mm) were equally competent between prepubertal and adult goats (Romanguera et al., 2011). On the other hand, Baldassarre et al., (2007) demonstrated that LOPU-IVEP could be

successfully used to extend the reproductive life of valuable aged goats that have acquired difficulties becoming pregnant by artificial insemination after multiple kiddings.

2.4. Season

The season could also influence the quality of LOPU oocytes. Regarding the season studied, the number of aspirated follicles was similar, whereas the proportion of good quality COC (Grade I/II) was enhanced in the breeding season in goats. However, interestingly, the cleavage rate of LOPU oocytes and blastocyst development rate of slaughterhouse oocytes were both greater in the anestrous season (unpublished data). Similarly, season was shown to influence the number and competence of recovered oocytes in sheep and cleavage rate tended to be higher in the anestrous season (Vazquez et al., 2010a). Recently, oocyte developmental competence in buffalo was also shown to be affected by different seasons since a higher cleavage and embryo yields were recorded in autumn compared to spring, with intermediate results in summer and winter (Di Francesco et al., 2011). In cattle, the number of follicles and collected oocytes significant decreased during the hot season, limitedly in empty Japanese black cows. It is noteworthy that pregnant cows showed a significant higher proportion of cleavage and blastocyst rates and freezable embryos than empty cows (Takuma et al., 2010). A subcutaneous implant of melatonin improved oocyte developmental competence during the anestrous season (Vazquez et al., 2010). Therefore, there are some approaches that could be used to overcome the season effect.

2.5. Breed

In cattle, Pontes et al. (2010) reported a large-scale commercial program for IVEP from dairy donors. The number of viable oocytes per OPU session was 12.1 (Gir), 8.0 (Holstein), 16.8 (1/4 Holstein x 3/4 Gir), and 24.3 (1/2 Holstein-Gir crossbred cows), significantly different among breeds. An indirect influence of the breed is the pattern of ovaries concerning their size. It was observed in sheep that ovaries greater than 5×7×9 mm^3 in size yielded a greater number of oocytes, compared to ovaries less than that, by the aspiration method (Wani et al., 1999). However, apparently the follicular size in goats does not affect the efficiency of oocyte recovery and their morphologic quality (Gibbons et al., 2007).

2.6. Interval of LOPU

The time between LOPU in successive treatments varies between 4 and 16 d. With respect to carrying out successive procedures in the same doe, various authors have pointed out that it affects neither the quantity of ovarian follicles nor the quality of the oocytes obtained (Gibbons et al., 2008). In cattle, there is a general agreement that twice-weekly aspiration yields a higher number of viable oocytes and transferable embryos than once-weekly aspiration, since it doubles the frequency of follicular waves, which becomes uncoupled from the estrous cycle because of inhibition of ovulation (Boni, 2012). However, there is no study in goats using this system and the most similar work was reported by Gibbons et al. (2007) when they performed LOPU every 4 d, with three sessions within 8 d. The authors found no significant differences in follicular development and oocyte quality among the three LOPU sessions.

3. During LOPU – The procedure and latest improvements

3.1. The procedure

The females should be deprived of feed and water for 36 h and 24 h, respectively, prior to laparoscopy (Avelar et al., 2012). The surgical field, cranial to the udder, should be shaved and disinfected (Gibbons et al., 2007). In goats, good sedation and anesthesia for adequate immobilization are essential for efficient aspiration. Commonly, the animal is placed in an inverted position on a cradle at a 45° angle, in order to prevent accidents when the trocar is inserted into the abdomen (Silva et al., 2012) (Fig. 2). Local administration of lidocain should be applied to the puncture sites of the trocars and three small incisions (3-5 mm) should be made. An endoscope may be inserted into the abdominal cavity through a trocar, cranial to the udder and to the left of the midline. This trocar is connected to a CO_2 tank via the CO_2 insufflator inserted to insufflate the abdominal cavity with CO_2. Once the peritoneum cavity was expanded, a second trocar is inserted into the right side (opposite from the first one) of the abdomen for passing the grasping forceps. The uterine horns are gently manipulated to allow visualization of each stimulated ovary. The aspiration is more efficient if atraumatic grasping forceps is used to stabilize the mesovarium, making it possible for the technician to turn the ovary in different directions for better positioning, visualization, and follicle aspiration. The last trocar is inserted in the midline for passing oocyte retrieval needle (Fig. 2). The objective is to enter the follicle from the side, with the needle in a direction parallel to the base of the follicle, or if not possible, puncture should be perpendicular to the follicle wall. Once the needle is inside the follicle, it must be gently rotated to ensure that as much of the follicle contents as possible is aspirated (Baldassarre et al., 1994) (Fig. 3). In cattle, this technique showed a significant improvement of approximately 30% of the recovery rate (Sasamoto et al.,

2003) due to a better detachment of the COC by curettage of the follicular wall during the follicle aspiration. Observation of the vessels on the follicle wall during laparoscopy make it possible to choose a less vascular site to introduce the needle, minimizing the loss of follicular fluid and oocytes, and aspirating follicular fluid containing little or no blood. All follicles visible on the surface of the ovaries more than 2 mm are aspirated using a needle connected to an aspiration and flushing system (Fig. 3). It is noteworthy that if the follicle is too big, comparable to the size of a cyst (varies according to breed) the aspiration should not be performed to the collection tube. The use of heparin is essential both in the aspiration medium and in the periodic washing of the circuit for flow of aspiration fluid to prevent coagulation and blockage of the system. Finally, the trocar orifices are treated with a local antibiotic-cicatrizing solution. The collection tube containing aspirated fluid (3-5 mL) with COCs is dispensed into a sterile Petri dish (90 mm) for COCs searching under a stereomicroscope (Tan et al., 2011; Avelar et al., 2012; Silva et al., 2012). Some small differences are reported such as the use of a discard cannula for intrauterine artificial insemination called “Aspic for pellet insemination in sheep” for follicle puncture in goat donors (Gibbons et al., 2007).

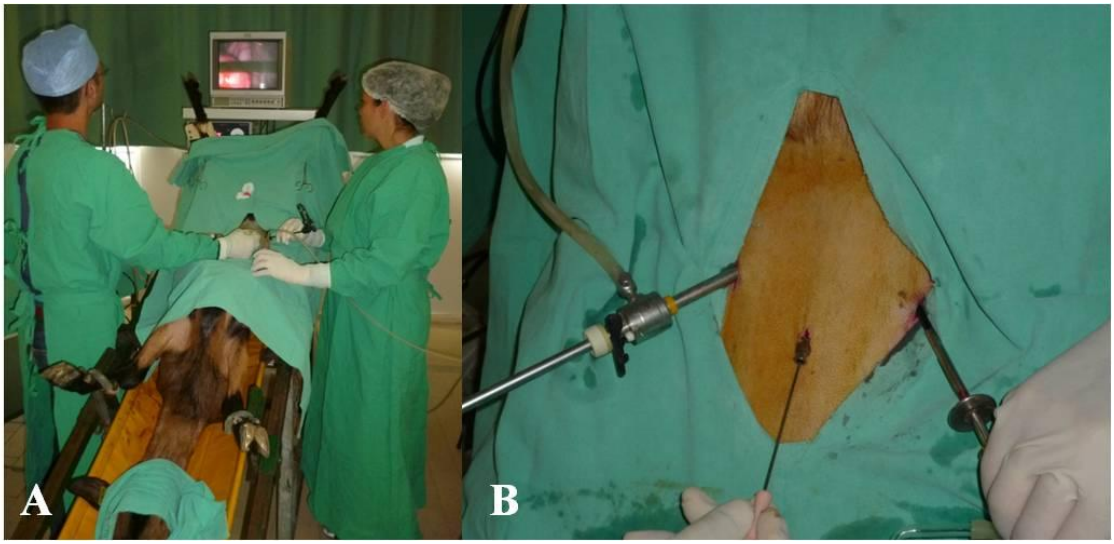

Fig. 2.A. General view of LOPU system in goats. B. View of three trocarters position during LOPU.

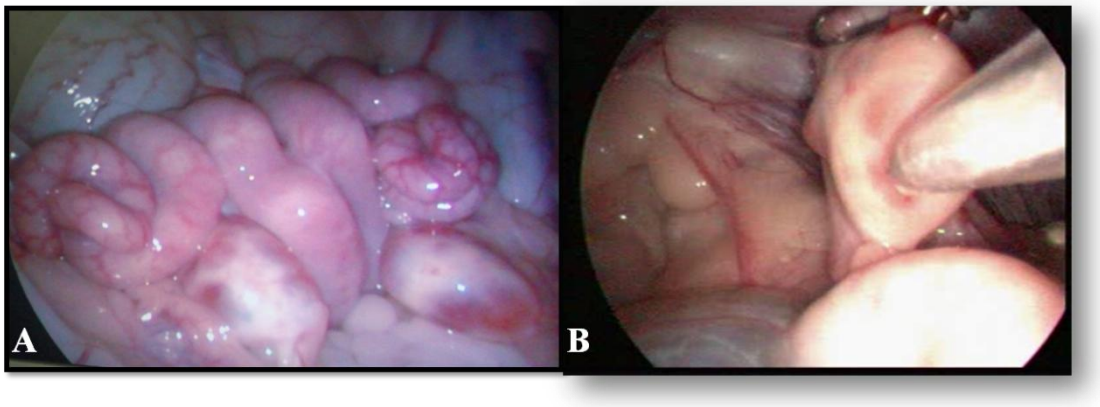

Fig. 3. A. Reproductive tract of a goat showing both ovaries with good response, i.e., follicles available for puncture. B. Follicle aspiration through needle specific for LOPU.

3.2 Important aspects for achieving high recovery rate

The effectiveness of this technique is based on the quantity and quality of the oocytes recovered. Besides the stimulation treatment, high variability in the results may happen due to type of needle, aspiration pressure and flow rate (Gibbons et al., 2008). The aspiration conditions used during LOPU are extremely important to respect the COC structure while optimizing their recovery rate (oocytes recovered/follicle punctioned) (Bols et al., 1997). Briefly, these are some of the considerations with respect to the variables enunciated.

3.2.1. Size of follicle

Follicle size does not affect recovery rate, but proportion of good quality oocytes was higher for large (77.9%) and medium (64.4%) follicles in sheep (Rodriguez et al., 2006). These authors stated that large follicles are aspirated more easily, but due to their dense content, the number of oocytes recovered is low. Commonly, a direct relationship between follicle size, oocyte diameter and developmental capacity is reported.

Similarly, Crozet et al. (1995) observed in goats that COC obtained from big follicles have better *in vitro* maturation than those from small follicles.

3.2.2. Needle and tubing

Regarding the type of needle, the variables are the diameter of the needle and the length of the bevel. The diameter could modify the quality of the oocytes, since a smaller needle diameter would submit the oocyte to shearing forces which could remove some of the covering of the *cumulus* cells. The bevel determines the portion of the needle that is introduced into the follicle to detach and aspirate the oocyte, directly affecting the percentage of retrieval (Bols et al., 1997).

In bovine, higher recovery rates with wide diameter needles (18 G) were obtained in comparison with 19 and 21 G needles (Bols et al., 1997). However, Rodriguez et al. (2006) reported that needle gauge did not affect aspiration device efficiency in sheep. Various authors used different sizes of needles varying from 16 to 23 G, obtaining good results for recovery and oocyte quality (Gibbons et al., 2008), but no studies were found in the literature comparing different needle diameters for LOPU in goats. Our group observed that 18 G needle promoted a significant better oocyte recovery rate in goats than the 20 G needle, whereas no influence was noted in oocyte quality (unpublished data).

Moreover, short needles produced the best results for aspiration, because of the short passage from the follicle to the silicon tubing, which is less traumatic for the oocyte than the steel needle (Rodriguez et al., 2006). Interestingly, thin and intermediate tubings were more effective in terms of proportion of good quality oocytes, probably due to less turbulence when the oocyte passed from the needle to the tubing (overall

efficiency rates: 34.9%, 32.3% and 28.1% for 1, 2 and 3 mm respectively) (Rodriguez et al., 2006).

3.2.3. Aspiration pressure

Aspiration pressure is another factor to define. The vacuum connected to the needle is regulated from 25 to 70 mmHg for goats. It was earlier reported that the optimal aspiration pressures using a vacuum pump for goats are between 50 and 70 drops min $^{-1}$ (Baldassarre et al., 2003a; Koeman et al., 2003) while lower pressures such as 25 mmHg show lower rates of recovery in sheep (Alberio et al., 2002) and as 100 mmHg result in smaller proportion of good quality COC (Morton et al., 2008). However, our group demonstrated that pressure at 30 mmHg in goats was efficient and reached high recovery rate regarding to treatment (84%; Avelar et al., 2012). It should be noted that it is possible to reduce and simplify the cost of LOPU, by using conventional syringes adapted for this purpose. It is also possible to use a cannula for intrauterine artificial insemination in sheep with or without vacuum control (Gibbons et al., 2008).

In cattle, for all needle types, more oocytes were recovered at the highest aspiration. On the other hand, the proportion of oocytes surrounded by compact *cumulus* and *in vitro* produced blastocysts decreased progressively as the vacuum increased (Bols et al., 1997). It is noteworthy that high vacuums, although resulting in high recovery rates, can affect the quality of the oocytes recovered (Cognié et al., 2004). Most part of the studies evaluating aspiration pressures have focused on the collection of oocytes, and have defined the optimal oocyte aspiration pressure as based on the number/quality of oocytes recovered. However, the aspiration pressure during oocyte recovery may affect subsequent embryonic development *in vitro* (Tervit et al., 1996).

The optimal aspiration pressure for oocyte recovery may not be the optimal pressure for embryo development.

3.2.4. Aspiration flow rate

Variable flow rates may be obtained when authors measure flow rate as mmHg rather than mL water/min. In these circumstances, a change in needle gauge affects aspiration flow rate (narrow needles have lower flow rates than wide ones, for the same aspiration pressure). The aspiration flow rate measured in mL water/min allows achieving uniformity when using any combination of the elements for LOPU. Aspiration flow rate significantly affects the proportion of good quality oocytes in sheep (69.5%, 50.5%, 44.8%, 36.5% and 28.3% for flows of 10, 20, 30, 40 and 50 mL/min respectively) (Rodriguez et al., 2006). Moreover, the best LOPU devices differ for each follicle size. Small (< 3 mm) and medium follicles (3-5 mm) need low-aspiration flow rates and thin tubing for optimal results. On the contrary, pre-ovulatory large follicles (> 5 mm) showed the best rates when aspiration flow rates were high and tubing was wide (Crozet et al., 1995). Fluid flow of 7 to 7.5 mL/min was used in goats and the percentage of good quality oocytes was approximately 70% (Avelar et al., 2012).

Depending of all these factors already mentioned the success of this technique will be greater or smaller. It is estimated that each LOPU session supplies from 4 to 14 oocytes per goat donor, with a rate of oocyte recovery of 40 to 90% (Baldassarre and Karatzas, 2004; Pierson et al., 2004; Gibbons et al., 2007; Avelar et al., 2012).

4. After LOPU – Applications and potential

It was earlier demonstrated that LOPU is a reliable and effective technique for the recovery of goat oocytes for production of zygotes that were DNA microinjected in

order to obtain efficient transgenesis rates (Baldassarre et al., 2003a). The same group also produced cloned live kids in a nuclear transfer program by the use of LOPU oocytes (Baldassarre et al., 2003b). Intracytoplasmic sperm injection (ICSI) was also performed in LOPU derived oocytes in goats (Abdullah et al., 2008). Another perspective for the their use was reported by Cox et al. (2002) when they transferred oocytes to inseminated does generating a model to study sperm function *in vivo.* LOPU efficiently resets the > 2 mm diameter follicular population to zero which in effect guarantees that the new population will be uniformly renewed with less atresia. By performing OPU in cattle 24 h prior to superovulatory treatment, an improved efficiency of superovulatory response was found (Boni, 2012). This association could be also important in goats.

Currently, LOPU is implicitly associated to IVEP in goats. Most part of research papers published in two different worldwide databasis (pubmed and scopus) refers to the use of LOPU oocytes for IVEP. Through consulting journals indexed by both databasis, it is possible to estimate that approximately only 10 countries work with LOPU in goats in the world: United States of America (Graaf et al., 1999), Chile (Cox et al., 2002; Cox and Alfaro, 2007), Canada (Baldassarre et al., 2003, Pierson et al., 2004; Baldassarre et al., 2007) Argentina (Gibbons et al., 2007), Bangladesh (Rahman et al., 2007), Malaysia (Abdullah et al., 2008; Tan et al., 2011), France (Locatelli et al., 2004), Italy (Leoni et al., 2009), Brazil (Almeida et al., 2011; Avelar et al., 2012) and Spain (Morato et al., 2011). IVEP involves four major steps: oocyte collection, oocyte *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* development (IVD) of the resulting embryos up to the blastocyst stage, at which these embryos can be efficiently cryopreserved or transferred into the uterus of synchronized recipient females. These steps will be briefly detailed.

After successful oocytes recovery, COC are classified according to the number of *cumulus* cells layers and good quality COC are submitted to IVM. Our group observed that slaughterhouse oocytes had greater cleavage rate than LOPU oocytes, respectively, after IVM in more complex medium: TCM 199 supplemented with follicular fluid (FF) and oFSH (89.5% *vs.* 64.5%); or under control medium, EGF and cysteamine (87.5% *vs.* 37.3%). For LOPU oocytes, both cleavage and blastocyst development rates were increased significantly when FF-FSH was used (Locatelli et al., 2008). These data clearly indicate that oocytes requirements during IVM may differ according to their origin. It remains unclear if their difference in terms of response to IVM treatments may be related to FSH stimulation prior to LOPU session or to *post mortem* changes in oocyte responsiveness in slaughterhouse group. Regarding IVM physical conditions, the literature does not vary considerably and it is usually performed incubating COC in large groups under 5% CO_2 in air at 38 to 39 °C with maximum humidity for 22-27 h.

After the step of IVM, the matured oocytes should undergo IVF. Either fresh or frozen-thawed semen can be used to fertilize matured oocytes. In any case, it is essential to assess live or dead sperm cells, usually by the use of Percoll or swim-up separation (Khatun et al., 2011). The most widely used medium is the modified synthetic oviduct fluid (SOF), added by serum, antibiotics and other substances, varying according to the laboratories. The inclusion of heparin as capacitant agent in the fertilization medium significantly improves IVEP in goats (Souza et al., 2013). Regarding IVF conditions for goats, sperm concentrations vary from 1 to $3.5x10^6$ cells/mL with spermatozoa and oocytes co-incubated for 16-20 h at 38 to 39 °C in humidified atmosphere of 5% CO_2 in air (Cognié et al., 2004). Some recent sheep IVF data show that sex-sorted spermatozoa elicit equal or greater cleavage and blastocyst rates than non-sorted spermatozoa (de

Graaf et al., 2009) and a large-scale commercial program for IVEP in cattle using sexed sperm has been performed (Pontes et al., 2010). Despite its interesting applicability, up to now there are no reports related to the use of sexed spermatozoa in goat IVF.

The last step in order to produce *in vitro* the embryo is the IVD, which occurs immediately after the end of IVF. We recently showed that the oocytes should be denuded, i.e., free of the attached *cumulus* cells after IVF (Souza et al., 2013), washed and placed in an embryo culture medium that allows the development up to a stage that is compatible with its transfer to the recipient uterus. The SOF medium supplemented with BSA and fetal calf serum has been successfully used for goats and embryos are usually cultured in mineral oil overlaid droplets of medium (1 µL/embryo). This is particularly important when working with LOPU, since a high variation in female response can be observed and often the number of embryos per female remains low. The presumptive zygotes are incubated at 38 to 39 °C in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2. After 6 to 8 days, the developed embryos can be transferred to recipients or cryopreserved (Cognié et al., 2004).

5. Conclusions

Developments in laparoscopes have led to increased use of laparoscopy and LOPU has been greatly spread over the recent years. Successive collections by laparoscopy have demonstrated the feasibility of obtaining high quantity and quality of oocytes for IVEP, cloning or transgenesis. It is noteworthy that laparoscopy is a minimally invasive procedure, not perforating other organs of the reproductive system (Teixeira et al., 2011), as it happens when using ultrasound-guided OPU in cattle. However, it is still necessary to evaluate the best combination of variables considering the diameter of needle, aspiration pressure and flow rate, recovery rate, oocyte quality

and its competence to development for the use of LOPU in goats more efficient. Under the current conditions, too large variety is reported with oocyte recovery rates ranging from 40 to 90%, and the number of harvested structures per female between 4 and 14 oocytes in different laboratories. Depending of all these factors already mentioned the success of this technique will be greater or smaller. Limits to LOPU application are still represented by lower pregnancy rate of *in vitro vs. in vivo* produced embryos. In addition, commercially, a big challenge is to convince the breeder to use this tool to add value to the herds or flocks, given the relative high cost of the service. We believe that new investigations should contribute to significant improvement of LOPU methods.

Acknowledgments

J.M.G. Souza was supported by CAPES and Ceará State University. The authors wish to thank CAPES-COFECUB bilateral framework for financial support of collaboration between the Ceará State University and INRA on goat IVEP (Grant 728/11, 2011-2013). This work was supported by a grant from Région Centre, France (PIVER program, #200800030493, 2008-2011).

References

Abdullah, R.B., Liow, S.L., Rahman, A.N.M.A., Chan, W.K., Wan-Khadijah, W.E., Ng, S.C., 2008. Prolonging the interval from ovarian hyperstimulation to laparoscopic ovum pick-up improves oocyte yield, quality, and developmental competence in goats. Theriogenology 70, 765-771.

Alberio, R., Olivera, J., Roche, A., Alabart, J., Folch, J., 2002. Performance of a modified ovum pick-up system using three different FSH stimulation protocols in ewes. Small Ruminant Research 46, 81-87.

Almeida, K.C., Pereira, A.F., Alcantara Neto, A.S., Avelar, S.R., Bertolini, L.R., Bertolini, M., Freitas, V.J., Melo, L.M., 2011. Real-time qRT-PCR analysis of EGF receptor in *cumulus*-oocyte complexes recovered by laparoscopy in hormonally treated goats. Zygote 19, 127-136.

Avelar, S.R.G., Moura, R.R., Sousa, F.C., Pereira, A.F., Almeida, K.C., Melo, C.H.S., Teles-Filho, A.C.A., Baril, G., Melo, L.M., Teixeira, D.I.A., Freitas, V.J.F., 2012. Oocyte production and in vitro maturation in Canindé goats following hormonal ovarian stimulation. Animal Reproduction 9, 1-7.

Baldassarre, H., 2012. Practical aspects for implementing in vitro embryo production and cloning programs in sheep and goats. Animal Reproduction 9, 188-194.

Baldassarre, H., de Matos, D.G., Furnus, C.C., Castro, T.E., Cabrera Fischer, E.I., 1994. Technique for efficient recovery of sheep oocytes by laparoscopic folliculocentesis. Animal Reproduction Science 35, 145-150.

Baldassarre, H., Keefer, C., Gauthier, M., Bhatia, B., Begin, I., Pierson, J., Laurin, D., Trigg, T., Downey, B.R., Karatzas, C.N., 2001. Laparoscopic ovum pick up and zygote recovery in goats treated with deslorelin implants before superovulation. Theriogenology 55, 510.

Baldassarre, H., Keefer, C., Wang, B., Lazaris, A., Karatzas, C.N., 2003a. Nuclear transfer in goats using in vitro matured oocytes recovered by laparoscopic ovum pick-up. Cloning and stem cells 5, 279-285.

Baldassarre, H., Rao, K.M., Neveu, N., Brochu, E., Begin, I., Behboodi, E., Hockley, D.K., 2007. Laparoscopic ovum pick-up followed by in vitro embryo production

for the reproductive rescue of aged goats of high genetic value. Reproduction, fertility, and development 19, 612-616.

Baldassarre, H., Wang, B., Kafidi, N., Gauthier, M., Neveu, N., Lapointe, J., Sneek, L., Leduc, M., Duguay, F., Zhou, J.F., Lazaris, A., Karatzas, C.N., 2003b. Production of transgenic goats by pronuclear microinjection of in vitro produced zygotes derived from oocytes recovered by laparoscopy. Theriogenology 59, 831-839.

Berlinguer, F., Leoni, G., Bogliolo, L., Pintus, P.P., Rosati, I., Ledda, S., Naitana, S., 2004. FSH different regimes affect the developmental capacity and cryotolerance of embryos derived from oocytes collected by ovum pick-up in donor sheep. Theriogenology 61, 1477-1486.

Bols, P.E., 2005. Puncture of immature ovarian follicles in bovine assisted reproduction. Verh K Acad Geneeskd Belg 67, 177-202.

Bols, P.E., Ysebaert, M.T., Van Soom, A., de Kruif, A., 1997. Effects of needle tip bevel and aspiration procedure on the morphology and developmental capacity of bovine compact *cumulus* oocyte complexes. Theriogenology 47, 1221-1236.

Boni, R., 2012. Ovum pick-up in cattle: a 25 yr retrospective analysis. Animal Reproduction 9, 362-369.

Cognie, Y., Poulin, N., Locatelli, Y., Mermillod, P., 2004. State-of-the-art production, conservation and transfer of in-vitro-produced embryos in small ruminants. Reproduction, fertility, and development 16, 437-445.

Cox, J.F., Alfaro, V., 2007. In vitro fertilization and development of OPU derived goat and sheep oocytes. Reproduction in domestic animals = Zuchthygiene 42, 83-87.

Cox, J.F., Zavala, A., Saravia, F., Rivas, C., Alfaro, V., 2002. Fertilization efficiency of in vitro matured oocytes transferred to oviducts of inseminated goats: a model to

assess in vivo fertilization performance of goat spermatozoa. Theriogenology 58, 1-8.

Crozet, N., Ahmed-Ali, M., Dubos, M.P., 1995. Developmental competence of goat oocytes from follicles of different size categories following maturation, fertilization and culture in vitro. Journal of reproduction and fertility 103, 293-298.

de Graaf, S.P., Beilby, K.H., Underwood, S.L., Evans, G., Maxwell, W.M., 2009. Sperm sexing in sheep and cattle: the exception and the rule. Theriogenology 71, 89-97.

Di Francesco, S., Boccia, L., Campanile, G., Di Palo, R., Vecchio, D., Neglia, G., Zicarelli, L., Gasparrini, B., 2011. The effect of season on oocyte quality and developmental competence in Italian Mediterranean buffaloes (Bubalus bubalis). Animal reproduction science 123, 48-53.

Galli, C., Crotti, G., Notari, C., Turini, P., Duchi, R., Lazzari, G., 2001. Embryo production by ovum pick up from live donors. Theriogenology 55, 1341-1357.

Gibbons, A., Pereyra Bonnet, F., Cueto, M., Catala, M., Salamone, D., González-Bulnes, A., 2007. Procedure for maximizing oocyte harvest for in vitro embryo production in small ruminants. Reproduction in Domestic Animals 42, 423-426.

Gibbons, A., Pereyra Bonnet, F., Cueto, M.I., Salamone, D., Catala, M., 2008. Recovery of sheep an goat oocytes by laparoscopy. Acta Scientiae Veterinariae 36, 223-230.

Graff, K.J., Meintjes, M., Dyer, V.W., Paul, J.B., Denniston, R.S., Ziomek, C., Godke, R.A., 1999. Transvaginal ultrasound-guided oocyte retrieval following FSH stimulation of domestic goats. Theriogenology 51, 1099-1119.

Katska-Ksiazkiewicz, L., Opiela, J., Rynska, B., 2007. Effects of oocyte quality, semen donor and embryo co-culture system on the efficiency of blastocyst production in goats. Theriogenology 68, 736-744.

Katska-Ksiazkiewicz, L., Rynska, B., Gajda, B., Smorag, Z., 2004. Effect of donor stimulation, frozen semen and heparin treatment on the efficiency of in vitro embryo production in goats. Theriogenology 62, 576-586.

Keskintepe, L., Darwish, G.M., Younis, A.I., Brackett, B.G., 1994. In vitro development of morulae from immature caprine oocytes. Zygote 2, 97-102.

Khatun, M., Bhuiyan, M.M., Ahmed, J.U., Haque, A., Rahman, M.B., Shamsuddin, M., 2011. In vitro maturation and fertilization of prepubertal and pubertal black Bengal goat oocytes. Journal of veterinary science 12, 75-82.

Koeman, J., Keefer, C.L., Baldassarre, H., Downey, B.R., 2003. Developmental competence of prepubertal and adult goat oocytes cultured in semi-defined media following laparoscopic recovery. Theriogenology 60, 879-889.

Leoni, G.G., Succu, S., Satta, V., Paolo, M., Bogliolo, L., Bebbere, D., Spezzigu, A., Mardeddu, M., Berlinguer, F., Ledda, S., Naitana, S., 2009. In vitro production and cryotolerance of prepubertal and adult goat blastocysts obtained from oocytes collected by laparoscopic oocyte-pick-up (LOPU) after FSH treatment. Reproduction, Fertility and Development 21, 901-908.

Locatelli, Y., Poulin, N., Baril, G., Touze, J.L., Bouttier, A., Cognie, Y., Mermillod, P., 2004. FSH treatment before laparoscopic ovum pick-up in goat influences quantity and quality of recovered oocytes. Reprod Fertil Dev 16, 513.

Locatelli, Y., Poulin, N., Baril, G., Touze, J.L., Fatet, A., Beckers, J.F., Mermillod, P., 2008. In vitro maturation treatment affects developmental competence of laparoscopic ovum pickup-derived oocytes in follicle-stimulating hormone-stimulated goats. Reproduction Fertility And Development 20, 182-183.

Martino, A., Palomo, M.J., Mogas, T., Pammio, M.T., 1994. Influence of the collection technique of prepubertal goat oocytes on in vitro maturation and fertilization. Theriogenology 42, 859-873.

Mogas, T., Palomo, M.J., Izquierdo, M.D., Paramio, M.T., 1997. Developmental capacity of in vitro matured and fertilized oocytes from prepubertal and adult goats. Theriogenology 47, 1189-1203.

Morato, R., Romanguera, R., Izquierdo, D., Paramio, M.T., Mogas, T., 2011. Vitrification of in vitro produced goat blastocysts: Effects of oocyte donor age and development stage. Cryobiology 63, 240-244.

Morton, K.M., de Graaf, S.P., Campbell, A., Tomkins, L.M., Maxwell, W.M., Evans, G., 2005. Repeat ovum pick-up and in vitro embryo production from adult ewes with and without FSH treatment. Reprod Domest Anim 40, 422-428.

Morton, K.M., Maxwell, W.M.C., Evans, G., 2008. Effect of Aspiration Pressure during Oocyte Harvesting on Oocyte Recovery and in vitro Development of Ovine Oocytes. Reproduction in Domestic Animals 43, 106-110.

Paramio, M.T., 2010. In vivo and in vitro embryo production in goats. Small Ruminant Research 89, 144-114!

Pierson, J., Wang, B., Neveu, N., Sneek, L., Cote, F., Karatzas, C.N., Baldassarre, H., 2004. Effects of repetition, interval between treatments and season on the results from laparoscopic ovum pick-up in goats. Reproduction, fertility, and development 16, 795-799.

Pontes, J.H.F., Melo Sterza, F.A., Basso, A.C., Ferreira, C.R., Sanches, B.V., Rubin, K.C.P., Seneda, M.M., 2011. Ovum pick up, in vitro embryo production, and pregnancy rates from a large-scale commercial program using Nelore cattle (Bos indicus) donors. Theriogenology 75, 1640-1646.

Pontes, J.H.F., Silva, K.C.F., Basso, A.C., Rigo, A.G., Ferreira, C.R., G.M.G., S., Sanches, B.V., Porcionato, J.P.F., Vieira, P.H.S., Faifer, F.S., Sterza, F.A.M., Schenk, J.L., Seneda, M.M., 2010. Large-scale in vitro embryo production and pregnancy rates from Bos taurus, Bos indicus, and indicus-taurus dairy cows using sexed sperm. Theriogenology 74, 1349-1355.

Ptak, G., Dattena, M., Loi, P., Tischner, M., Cappai, P., 1999a. Ovum pick-up in sheep: efficiency of in vitro embryo production, vitrification and birth of offspring. Theriogenology 52, 1105-1114.

Ptak, G., Loi, P., Dattena, M., Tischner, M., Cappai, P., 1999b. Offspring from one-month-old lambs: studies on the developmental capability of prepubertal oocytes. Biology of reproduction 61, 1568-1574.

Rahman, A.N.M.A., Abdullah, R.B., Embong, W.K.W., 2007. Goat embryo development from in vitro matured oocytes of heterogeneous quality through intracytoplasmic sperm injection technique. Biotechnology 6, 373-382.

Rodriguez, C., Anel, L., Alvarez, M., Anel, E., Boixo, J.C., Chamorro, C.A., de Paz, P., 2006. Ovum Pick-up in Sheep: a Comparison between Different Aspiration Devices for Optimal Oocyte Retrieval. Reproduction in Domestic Animals 41, 106-113.

Romaguera, R., Moll, X., Morato, R., Roura, M., Palomo, M.J., Catala, M.G., Jimenez-Macedo, A.R., Hammami, S., Izquierdo, D., Mogas, T., Paramio, M.T., 2011. Prepubertal goat oocytes from large follicles result in similar blastocyst production and embryo ploidy than those from adult goats. Theriogenology 76, 1-11.

Sasamoto, Y., Sakaguchi, M., Katagiri, S., Yamada, Y., Takahashi, Y., 2003. The effects of twisting and type of aspiration needle on the efficiency of transvaginal ultrasound-guided ovum pick-up in cattle. J Vet Med Sci 65, 1083-1086.

Silva, J.C.B., Okabe, W.K., Traldi, A.S., 2012. From cattle to sheep: a view of the difficulties and success of commercial in vitro production of sheep embryos. Animal Reproduction 9, 195-200.

Souza, J.M.G., Duffard, N., Bertoldo, M.J., Locatelli, Y., Corbin, E., Fatet, A., Freitas, V.J.F., Mermillod, P., 2013. Influence of heparin or the presence of *cumulus* cells during fertilization on the in vitro production of goat embryos. Animal Reproduction Science in press.

Takuma, T., Sakai, S., Ezoe, D., Ichimaru, H., Jinnouchi, T., Kaedei, Y., Nagai, T., Otoi, T., 2010. Effects of season and reproductive phase on the quality, quantity and developmental competence of oocytes aspirated from Japanese black cows. Journal of Reproduction and Development 56, 55-59.

Tan, W.L., Wan Khadijah, W.E., Abdullah, R.B., 2011. Effect of sperm insemination duration on in vitro fertilisation (IVF) performance on goats. Malaysian Journal of Science 30, 105-111.

Tervit, H.R., 1996. Laparoscopy/laparotomy oocyte recovery and juvenile breeding. Animal Reproduction Science 42, 227-238.

Vazquez, M.I., Abecia, J.A., Forcada, F., Casao, A., 2010a. Effects of exogenous melatonin on *in vivo* embryo viability and oocyte competence of undernourished ewes after weaning during the seasonal anestrus. Theriogenology 74, 618-626.

Vazquez, M.I., Forcada, F., Casao, A., Abecia, J.A., Sosa, C., Palacin, I., 2010b. Undernutrition and exogenous melatonin can affect the in vitro developmental competence of ovine oocytes on a seasonal basis. Reproduction in Domestic Animals 45, 677-684.

Viana, J.H., De Almeida Camargo, L.S., De Moraes Ferreira, A., De Sa, W.F., De Carvalho Fernandes, C.A., De Pinho Marques Junior, A., 2004. Short intervals

between ultrasonographically guided follicle aspiration improve oocyte quality but do not prevent establishment of dominant follicles in the Gir breed (Bos indicus) of cattle. Anim Reprod Sci 84, 1-12.

Wani, N.A., Wani, G.M., Khan, M.Z., Sidiqi, M.A., 1999. Effect of different factors on the recovery rate of oocytes for in vitro maturation and in vitro fertilization procedures in sheep. Small Ruminant Research 34, 71-76.

Younis, A.I., Zuelke, K.A., Harper, K.M., Oliveira, M.A., Brackett, B.G., 1991. In vitro fertilization of goat oocytes. Biology of Reproduction 44, 1177-1182.

PARTE III: BIOTECNOLOGIAS REPRODUTIVAS APLICADAS À CONSERVAÇÃO DE RUMINANTES AMEAÇADOS DE EXTINÇÃO – PASSADO, PRESENTE E FUTURO

Resumo

As preocupações com o futuro do meio ambiente têm crescido de forma intensa nos últimos anos. Dentre elas, destaca-se a possibilidade de extinção de várias espécies por causas não naturais, ao invés da ação lenta do processo evolutivo. Ecossistemas funcionais, que dependem da biodiversidade, são importantes para a manutenção da vida no planeta. Deste modo, o objetivo da preservação pode ser entendido como a garantia da sobrevivência e evolução da espécie e da população animal em seu habitat nativo. De uma forma simplificada, a diversidade biológica é a chave para manter a vida como nós a conhecemos. Desta forma, ações que objetivem a preservação de espécies e raças são importantes para que o material genético destes animais não seja perdido de forma definitiva. Felizmente, a importância de se preservar a biodiversidade do planeta tem sido amplamente reconhecida e diversas estratégias de conservação para manutenção do ecossistema global propostas por conservacionistas. A possibilidade de criação de bancos de recursos genéticos (conservação *ex situ*) tem sido sugerida como uma forma de atingir esse objetivo e pode ser também considerada uma atividade complementar para a conservação *in situ* de recursos genéticos de animais de produção. Esta medida pode promover o incremento do número de indivíduos cujas espécies e raças encontram-se ameaçadas, elevando o tamanho da população efetiva. Assim, o objetivo deste estudo é descrever as diversas tentativas que estão sendo realizadas para conservar genomas e genes individuais através do uso da tecnologia reprodutiva assistida em ruminantes.

Palavras-chave: Ameaçados de extinção, biodiversidade, reprodução assistida, risco de extinção.

A parte III dessa revisão de literatura corresponde ao artigo publicado na **Revista Portuguesa de Ciências Veterinárias**, 110, 577-580, 2011 (Qualis: B4).

Reproductive biotechnologies applied to the conservation of endangered ruminant – Past, Present and Future

Joanna Maria G. de Souza[*], Ribrio I.T.P. Batista, Luciana M. Melo, Vicente José de F. Freitas

Laboratory of Physiology and Control of Reproduction, Veterinary Faculty, Ceará State University (UECE). Universidade Estadual do Ceará. Av. Dedé Brasil, 1700, CEP: 60740-093, Fortaleza, CE, Brazil.

[*]joannavet@gmail.com; Phone: +55 85 3101 9861 / Fax: +55 85 3101 9840.

Summary

Intensely concerns about the future of the environment have grown intense in recent years. The possibility of extinction of various species by unnatural causes, instead of the slow action of the evolutionary process has become a reality. Thus, the objective of preservation can be understood as a guarantee of the survival and evolution of species and animal populations in their native habitat. Simplifying, biological diversity is the key to the sustainment of life as we know it. Therefore, all actions that aim to preserve species and breeds are important so that their genetic material is not permanently lost. Fortunately, the magnitude of preserving planet's biodiversity has been widely recognized and various conservation strategies to maintain the global ecosystem have been proposed by conservationists. The possibility of creating genetic resource banks (*ex situ* conservation) has been suggested as one way of achieving this goal and is considered an essential complementary activity to *in situ* conservation of genetic resources of farm animals. This measure would tend to encourage the growth of individual species and endangered breeds, affecting positively their population. The aim of this review is to report the work that is being done to conserve the genome and individual genes through the use of various assisted reproductive technologies in ruminants.

Keywords: Assisted reproduction, biodiversity, risk of extinction, threatened.

1. Introduction

In recent decades, the planet has experienced a significant reduction of genetic diversity due to the alteration or destruction of habitats, mainly as a result of anthropogenic forces. The importance of this phenomenon is illustrated by the IUCN "red list", that is widely recognized as the most comprehensive and global objective approach for evaluating the conservation status of animal species. Once the number of the population is determined, the species is given a term to describe its status, such as “endangered” or “threatened”, that is sometimes misunderstood**.** Endangered are those species that are in danger of extinction throughout all or a portion of their range, whereas threatened are those likely to become endangered within the near future. The loss of animal genetic resources is occurring at alarming rates across the globe. According to FAO, at least one livestock breed has become extinct per month over the past several years, resulting in its genetic characteristics being lost forever. For an unpredictable future, traditions, cultural values and safeguarding diversity are all driving forces in support of genetic conservation, which is a human responsibility. Nowadays it’s clear that the various forms of life on Earth only exist because of its integrated complex system between living species and even between physical environmental components such as water, atmosphere, rocks and soil. As human change the physical environment, the environment also changes human life. The larger the number of living organisms the more stable is the ecosystem. Air and water purification, food provision, stabilization of the Earth`s climate, erosion control, disease control and nutrient recycling are some important conditions that depend on biodiversity conservation (Holt *et al*., 1996b).

For the reasons presented above it is imperative to conserve and maintain animal genetic resources to ensure preserving biodiversity and keeping alternative and potentially useful genes available in the gene pool. *In situ* and *ex situ* methods have been proposed to conserve genetic resources. The first one is ideal as the conservation of live species occurs in their natural habitats. The disadvantage of this method is that it requires land and people which are limited in some regions. *Ex situ* conservation, by the other hand, deals with protection of biological diversity components outside their natural habitats (Glowka *et al*. 1994). It covers widely applied conservation techniques such as the establishment of genome banks, referring captive breeding of animals far

removed from their indigenous environment. It is noteworthy that *ex situ* and *in situ* conservation are not mutually exclusive. Frozen animal genetic resources or captive live populations can play an important role in the support of *in situ* program (Holt *et al*., 1996b). However, turning this idea into reality is a rather complex process, requiring interdisciplinary collaboration and clearly defined goals. Therefore, the aim of this review is to report the work that is being done to conserve genome and individual genes through the use of modern reproductive biotechnologies in endangered ruminants around the world.

2. Situation of domestic ruminants in the World and in Brazil

Of all ruminant livestock, cattle are the most numerous, followed by sheep, goats and buffalo. It is known that many ruminant breeds have already become extinct in the world: 3% of goat, 12% of sheep and 17% of cattle breeds. Nevertheless, goats have the largest percentage of breeds (28%) with a global unreported/unknown population size (Figure 1), hinting towards the possibility of that the number could be underestimated (Galal, 2005).

When Europeans discovered Brazil, about 500 years ago, the first ruminant specimens were brought by Portuguese settlers. Throughout the years, these animals resulted in the formation of "criollo", "local" or "naturalized" breeds that for centuries were responsible for livestock production in the country. Over the years, natural selection occurred and these breeds developed morphological and physiological characteristics adapted to specific Brazilian environmental conditions. Nevertheless, from the early 20^{th} century, commercial breeds that have been imported have been responsible to promote the gradual replacement of naturalized breeds to such an extent that the latter are in danger of extinction. To avoid further loss of this important genetic material, in 1983, the National Research Center for Genetic Resources and Biotechnology (Cenargen) of the Brazilian Agricultural Research Corporation (Embrapa) decided to include among its priorities the conservation of animal genetic resources (Mariante and Cavalcante, 2006; Mariante *et al.*, 2009). The *in situ* conservation of cattle, buffaloes, donkeys, goats and sheep is being carried out by Conservation Nuclei, located in the animal's original habitat. *Ex situ* conservation is centered at the Brazilian Animal Germplasm Bank (AGB) that is responsible for the

storage of semen and embryos of various breeds of threatened domestic animals. Presently the AGB has almost 60,000 doses of semen and more than 250 embryos, as well as over 7000 DNA samples (Mariante *et al*., 2009).

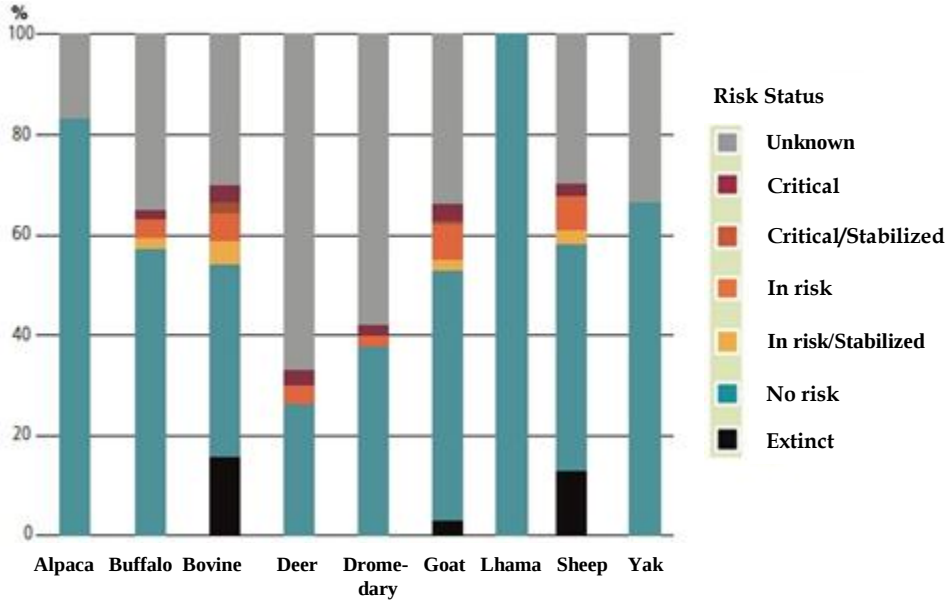

Figure 1 – Risk status (%) of ruminant species in January, 2006 (Commission on Genetic Resources - FAO, 2007).

3. Reproductive biotechnologies applied to *ex situ* conservation of ruminants

Modern reproductive technologies have allowed a large number of progeny to be produced from a single individual, and the transport has enabled the distribution of germplasm around the world rapidly and efficiently. The successful use of assisted reproductive technologies (ART) in domestic livestock suggests that they can also be used for the conservation of species and breeds in danger of extinction (Solti *et al*., 2000).

A good example of ART application was reported by Ptak *et al*. (2002), that used the European mouflon (*Ovis orientalis musimon*) to demonstrate for the first time the potential of establishing an integrated package of modern reproductive biotechnologies to rescue an endangered species with substantial yields of oocytes, embryos and pregnancy, independent of age, breeding season and donor treatment. *Ex situ in vitro* conservation programs of endangered ruminant genetic resources have focused efforts on cryopreservation of gametes, embryos and somatic cells as well as testis and ovarian

tissues, effectively lengthening the genetic lifespan of animals in a breeding program even after the death. Although significant progress has been made in both semen and embryo cryopreservation of several domestic species there still remains many difficulties in a few steps that will be detailed shortly.

3.1. Animal genetic resource bank (GRB)

Conservationists are looking for additional means to secure some portion of the biodiversity that are at risk of being lost. The establishment of biomaterial to constitute a GRB is rather complex and expensive, but it is essential to preserve genetic diversity through *ex situ* conservation in the variety of semen, oocytes, embryos, tissues or DNA of endangered animals (Andrabi and Mawxell, 2007). A disadvantage of this is that breed restoration may be extremely costly and time consuming. However, as a complementary conservation approach, cryopreservation provides a long-term insurance system to *in situ* conservation, since when combined with artificial insemination or embryo transfer ensures genetic variability (Holt and Pickard, 1999).

Otherwise, the formation of a GRB generates the possibility of re-establishing a particular population after disasters or epidemics episodes. Currently, there are two more realistic options, either cryopreserve embryos or gametes (i.e., spermatozoa and oocytes), which have advantages and disadvantages. A bovine breed could be saved with 1,000 sperm doses collected on 25 different males or 300 embryos (non-sexed) from 90 donors (Comizzoli *et al*., 2000). Boettcher *et al*. (2005) simulated a creation of a GRB for reconstruction of an extinct breed using different strategies: embryos-only, embryos in combination with semen, and semen-only. The strategy embryos-only required the shortest time to reach reconstruction, in the strategy embryos + semen the time increased with decreasing proportions of embryos, whereas in the semen-only, reconstruction time varied from 2 to 21 years. The risk of extinction was extremely high when a very small proportion of embryos (< 20%) was used. Decreasing the percentage of embryos further diminished costs. The authors emphasized that the combination of both embryos and semen would be more indicated as it would reduce costs allowing the conservation of more breeds. Similarly, Amstislavsky and Trukshin (2010) reported that embryo cryopreservation seems reliable and most simple, whereas the use of gametes requires the use of subsequent techniques. The authors also described that in recent

years the number of cryobanks increased worldwide, what may be considered as response to this need.

3.1.1. Cryopreservation – Semen

This biotechnique has been the most widely used for germplasm preservation of endangered species, due to its abundant availability and ease of application. Thus, several attempts have been reported for its optimization. Coloma *et al.* (2010) compared different extenders and observed that the seminal plasma removal improved the response of freezing-thawing semen in Spanish ibex (*Capra pyrenaica*). The effects of cooling rates, glycerol concentrations and diverse extenders on the post-thawing sperm viability of camel sperm (*Camelus bactrianus*) were compared in Iran (Niasari-Naslaji *et al*., 2007). Cheng *et al*. (2004) reported that optimal extenders for semen were obtained when working with Formosan Sika (*Cervus nippon taiouanua*) and Formosan Sambar (*C. unicolor swinhoe*), both endangered. All freezing protocols tested were also useful to the post-thawing viability of semen obtained in the endangered *Père David's* deer (*Elaphurus davidianus*; Soler *et al.*, 2003). In another trial, the performance of freezing the sperm-rich fraction showed better results than the whole ejaculate on the Iberian Red deer (*C. elaphus hispanicus;* Martínez-Pastor *et al.*, 2009).

The use of epididymal sperm cells in ART on endangered animals could be considered a useful source. According to the sperm maturation process, sperm from the cauda epididymis are of good quality and potentially fertile (Bedford, 1978). In this approach, *post-mortem* semen cryopreservation from the cauda epididymis was successfully performed in five endangered gazelles (*Gazella gazella*, *G. dorcas* and *G. gazella acaiae*) in Israel. This result was specifically important for the last species since there were only 12 individuals left in the wild (Saragusty *et al*., 2006). However, the protocols currently used to conserve semen are still suboptimal and cannot be easily applied across species.

3.1.2. Cryopreservation – Embryos

Cryopreservation of embryos and their subsequent storage generates enormous potential for protecting the population integrity and heterozygosity. However, it is a more complex and costly procedure than semen cryopreservation and the successful use of this technology for wildlife is dictated by the singularity of the species (Pukazhenthi and Wildt, 2004). Protocols for freezing bovine embryos have been reasonably effective

in studies that were conducted on non-domestic cattle such as gaur (*Bos gaurus*), eland (*Taurotragus oryx*) and bongo (*Tragelaphus eurycerus*; Revised by Loskutoff *et al.*, 1995). Similarly, Lopes Jr. *et al*. (2006) were also able to freeze embryos from Morada Nova (white variety), a domestic sheep breed that has a very small population in Brazil.

Traditional procedures to freeze embryos require much time and are quite onerous. This technique may be replaced by a relatively simpler one and less costly called vitrification. Vitrification is a physical phenomenon of amorphous solidification, achieved with extreme increase of cryoprotectants viscosity in temperatures below the melting point. The purpose is to prevent intracellular and extracellular ice crystals formation that is responsible for damage of membranes and cell organelles during the cryopreservation process (Vajta, 2000).

In this way, Thundathil *et al.* (2007) described for the first time that embryo production followed by vitrification in wood bison (*Bison bison athabascae*) could be able to recover its genetic material. Moreover, Bettencourt *et al.* (2009) conducted a study to compare the efficiency of three cryopreservation techniques: controlled slow freezing, conventional vitrification and open pulled straw (OPS) vitrification for the cryopreservation of *in vivo* produced Portuguese Black Merino ovine embryos. The authors concluded that all the three techniques were efficient in preserving and propagating genetic material. It is apparent that earlier and *in vivo* derived embryos can withstand cryopreservation better than later stage and *in vitro* produced embryos. Therefore, the current challenge is to develop a standardized protocol that can be applied to embryos of different species at various developmental stages.

3.1.3. Cryopreservation - Oocytes

Oocyte cryopreservation, despite its high impact on the preservation of genetic resources is has yet to be considered an established technology (Ledda *et al.*, 2001). However, important progress has been made in recent years and successful oocyte cryopreservation has been obtained in several species (reviewed by Andrabi and Maxwell, 2007). These studies have provided substantial progress and can also be used for the conservation of endangered species.

Undoubtedly, oocyte freezing is the best way to preserve the genetic material of *post-mortem* females. Conversely, few gametes resist cryopreservation or develop when fertilized. Vitrification is considered a promising technique for cryopreservation of

female gametes instead of classical slow freezing. However, vitrified prepubertal sheep oocytes showed a high sensitivity to both low temperature and cryoprotectants, leading to a low developmental competence after thawing (Succu *et al*., 2007). Therefore, new investigations should be made in order to improve their results.

3.1.4. Cryopreservation - Somatic Cells

The cryopreservation of blood and its products, tissues and DNA, allows them to be stored and used at a later time. Moreover, these cells provide rich material for the development of basic and applied research. The establishment of fibroblast banks has also been proposed as a practical approach to endangered species conservation. This technique associated with nuclear somatic cell transfer allows the restoration of extinct or endangered species with greater genetic diversity. Therefore, some studies have reported success in constituting fibroblasts banks from ruminant threatened species such as the Jining Black Goat (Li *et al.*, 2009).

3.2. Artificial Insemination (AI)

According to Durrant (2009), AI is the ART that is less complex, invasive and costly and is therefore the first logical choice for companion animals or non-domestic endangered animal species. One of the most interesting AI application in conservation is to avoid the genetic depression caused by group fragmentation into free species. Thus, in situations where a given species live in small groups, females can be captured for a short period of time, be inseminated using sperm from animals from zoos and then be released back into their habitat. It still may be possible to capture males living freely and to collect their semen to inseminate females in captivity (Pukazhenthi and Wildt, 2004).

There are a small amount of reports applying AI that produced endangered ruminants such as the Blackbuck (*Antilopre cervicapra;* Holt *et al*., 1988) and the oryx (*Oryx dammah;* Morrow *et al.*, 2000) antelopes and the eland (Bartels *et al*., 2001). Moreover, conceptions from AI were also reported in Adras gazelles (*Gazella dama mhor*; Holt *et al.*, 1996a) and Gazelle giraffe (*Litocranius walleri*; Penfold *et al.*, 2005). Santiago-Moreno *et al.* (2006) reported for the first time the birth of a Spanish ibex (*C. pyrenaica*

hispanica) after AI. This species inhabits the mountains in Spain and is in risk of extinction due mainly to inbreeding.

3.3. Multiple Ovulation and Embryo Transfer (MOET)

The use of the MOET technique is not yet widespread in wildlife populations. A key conservation strategy has been the interspecies MOET biotechnique, i.e., embryo transfer from endangered species to non-threatened recipients. Some examples of success are a gaur born to a Holstein cow (Stover *et al*., 1981), an Armenian Red sheep (*Ovis orientalis*) born to a domestic sheep (Coonrod *et al.*, 1994) and a Spanish ibex born to domestic goats (Fernandez-Arias *et al.*, 1996).

Regarding traditional MOET, Othen *et al.* (1999) proposed an experiment to refine estrous synchronization and superovulation steps following the administration of different hormones in bison. This study showed that the synchronization treatment commonly used in domestic cattle may be successfully applied to the bison, but new superovulatory protocols are required for an effective response in this species. In wildlife, scant knowledge exists about the kinetics of embryo development and maternal-fetal recognition. Thus, Demmers *et al.* (2000) administered recombinant interferon-τ in cervid recipients and observed a significant reduction in embryo loss after asynchronous transfer that had as an objective the reduction of asynchrony between transferred embryos and recipients.

Chagas e Silva *et al.* (2003) conducted a study to evaluate different treatments for estrous synchronization and superovulation in Saloia sheep, an endangered breed native of Portugal. The authors emphasized that the semilaparoscopic transfer allowed high rates of embryo survival and hence the birth of lambs. Recently, the MOET program was achieved in endangered Portuguese Black Merino ewes irrespective of the season of the year with a better response when using ovine FSH instead of porcine (Bettencourt *et al.*, 2008).

3.4. In vitro embryo production (IVP)

In vitro embryo production (IVP) involves collection and *in vitro* maturation (IVM) of the oocytes, *in vitro* fertilization (IVF) of matured oocytes and *in vitro* culture (IVC)

of probable embryos obtained up to a stage that is compatible with its transfer to the recipient uterus (Freitas and Melo, 2010). Its application has been proposed as a valuable strategy for the conservation of endangered species (Comizzoli *et al*., 2000).

The major studies involving IVP in threatened ruminants were performed in gaur (Johnston *et al.*, 1994), Armenian Red sheep (Coonrod *et al.*, 1994), Sika deer (*C. nippon*) Comizzoli *et al.*, 2001) and European mouflon (Ptak *et al.*, 2002). Live births were possible only in some of them, probably due to the scarcity of the physiological knowledge of the species of interest.

Recently, Wirtu *et al.* (2009) demonstrated the feasibility of oocytes retrieval and IVM in eland and bongo (*T. eurycerus isaaci)* antelope. IVP was successfully achieved when oocytes were obtained from slaughterhouse ovaries from Red deer (*C. elaphus*) or after successive laparoscopic follicular aspiration in Sika deer. Some factors affecting IVP efficacy after both methods of collection were tested such as the effect of EGF *vs.* FSH and follicular fluid supplementation in IVM, sperm concentrations and incidence of polyspermy on IVF (Comizzoli *et al.*, 2001). Locatelli *et al.* (2005) carried out an experiment with suggestions to improve IVM and IVF and studied a system of co-culture with sheep oviduct cells that allowed the production of 39% of viable blastocysts after IVC in Red deer. This viability was confirmed by pregnancies and births of normal offspring after transfer to recipients. Subsequently, the same laboratory was responsible for the first report of blastocyst in Sika deer produced after IVP (Locatelli *et al.*, 2006).

3.5. Somatic Cell Nuclear Transfer (SCNT)

Cloning or SCNT is a process by which the nucleus (DNA) is removed from a donor cell to an enucleated recipient cell to create an exact genetic match of the donor (Andrabi and Maxwell, 2007). The report of the first mammal produced by SCNT (Wilmut *et al.*, 1997) indicated that this technology could be adopted to increase the population size of threatened or extinct animals. Undoubtedly, the bucardo (*Capra pyrenaica pyrenaica*) is the best example of the use of SCNT for an extinct species recovery. The bucardo population was abundant in the Pyrenees, but decreased sharply due to hunting, leaving only three old females in 1989. By 1999, only one female about 12 years old remained alive. Skin samples from her were obtained, multiplied and

cryopreserved. This female died in 2000 and the Spanish government declared the species extinct. Experiments were conducted and it became possible the birth of a normal morphologically bucardo female. However, the newborn died few minutes after birth due to physical defects in the lungs. This was the first animal born of an extinct subspecies and the first interspecies nuclear transfer (SCNTi) from adult somatic cell in *Capra* with success (Folch *et al*., 2009).

Currently, the use of SCNT for reconstruction of endangered species occurs mainly by SCNTi. Often, as the oocytes availability in endangered species is low, the possibility of using oocytes from other species as donor cytoplasts has been the proposed method of SCNTi. Also problematic such as the low availability of oocytes, the accessibility of recipients to promote the development of a cloned embryo is a real obstacle (Andrabi and Maxwell, 2007). To overcome this difficulty, the strategy commonly used is to transfer embryos obtained for the closest available phylogenetically species.

4. Future application opportunities

4.1 Embryo Sexing

Embryo sexing has been recognized to control effectively the sex of offspring in the embryo transfer industry and it could be a useful conservation tool. Through a careful analysis about the population of a given species, this technique would allow us to achieve a balanced sex ratio of any population at risk. The accuracy of sex prediction was 100% in 58 bovine embryos when the blastomeres dissociated from a morula exceeds three (Zoheir & Allam, 2010). Mara *et al*. (2004) described a work with embryo sexing of *in vitro* produced sheep embryos by the duplex PCR that demonstrated the viability of transferring fresh sexed embryos on the same day of biopsy. However, when recipients are not accessible, sexed embryos could also be vitrified (Akiyama *et al.*, 2010) after gender selection. An accuracy rate of 100% was reached for sex determination with a single blastomere at the blastula stage isolated from 43 goat embryos (Tsai *et al.*, 2010). Thus, efforts to extend the sexing of embryos for endangered species should be studied in a near future, since the technique seems to be the same as for sexing bovine and ovine embryos.

4.2. Cryopreservation of ovarian tissue and manipulation of oocytes enclosed in preantral follicles

Cryopreservation of ovarian tissue and the manipulation of oocytes enclosed in preantral follicles are performed in only a few centers of animal genetic resources. These techniques could be of great interest in the conservation of endangered species in the future (Ledda *et al.*, 2001). The main objective is the storage of primordial follicles that are located in the ovarian cortex, which represents the immature oocyte reserve. The potential recovery of thousands of viable oocytes is extremely important for IVP on a large scale (Figueiredo *et al.*, 2007). In this context, freezing protocols have been improved over the past 20 years and high cell survival rates have been obtained (Dermici *et al.*, 2003). Muruvi *et al*. (2009) demonstrated that sheep primary follicles isolated from cryopreserved neonatal ovarian tissue could be successfully grown *in vitro* using a serum-free, attachment-based culture system. Different standardized cryopreservation protocols need to be developed and tested to apply them to diverse species. Thus, priority must be given to the basic research as many key points need to be clarified, so the use of this technique could become realistic in endangered species.

4.3. Xenografting of ovarian tissue

Ovary xenografting is a valuable tool that may enable the generation of good quality oocytes from ovarian tissues recovered from endangered wildlife species. Oocytes recovered from grafts can be used in IVF to produce offspring. A major advantage of this biotechnology is the possibility of ovarian tissue to be transplanted irrespective of age, reproductive cycle or even *post-mortem* (Snow *et al.*, 2001). Regardless of the differences between species, the recipient´s hormonal *milieu* allows the xenotransplantated ovarian tissue to reassume its normal function as well as its follicular development in a similar way of the donor species of ovarian tissue (Wolvekamp *et al.*, 2001).

5. Conclusions

The erosion of genetic resources is increasing and it is clear that the problem of the growing extinction of biological species is far from an adequate solution. The use of ART in endangered species is limited, especially because favorable results depend on the knowledge of reproductive physiology of the species in question and few studies are available.

Usually, the technologies applied to endangered species are adapted from domestic breeds and, as a consequence, some biotechnologies have adjusted well, producing great results, while others were unsuccessful and frustrating. However, the positive results which were obtained are important to ensure that the use of these biotechnologies is a major tool for the conservation of endangered and threatened species. Finally, it is noteworthy that the interaction between the breeding program and GRB should be dynamic and interactive, maximizing each strategy's potential.

Acknowledgments

This study was sponsored by research funds and scholarship from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brasília, Brazil), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (PNPD/CAPES, Brasília, Brazil) and Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico (FUNCAP, Fortaleza, Brazil). Special thanks go to Christiane Garcia Vilela Nunes for critical reading of the manuscript and helpful comments.

Bibliography

Akiyama K, Kobayashi J, Sato Y, Sata R, Ohashi M, Sasaki E, Oda Y, Ogawa Y, Ueda S, Nabenishi H, Matoba S (2010). Calf production from vitrified bovine sexed embryos following in-straw dilution. Animal Science Journal, 81(4), 461-464.

Amstislavsky SY, Trukshin IS (2010). Cryobanking mammalian embryos: priorities and the optimal choice of reproductive technologies. Russian Journal of Developmental Biology, 41(1), 13–23.

Andrabi SMH, Maxwell WMC (2007). A review on reproductive biotechnologies for conservation of endangered mammalian species. Animal Reproduction Science, 99, 223–243.

Bartels P, Friedmann Y, Lubbe K, Mortimer D, Rasmussen LA, Godke RA (2001). The live birth of an eland (*Taurotragus oryx*) calf following estrous synchronization and artificial insemination using frozen-thawed epididymal sperm. Theriogenology, 55, 381 [Abstract].

Bedford JM (1978). Influence of abdominal temperature on epididymal function in the rat and rabbit. American Journal of Anatomy, 152, 509-622.

Bettencourt EM, Bettencourt CM, Chagas e Silva J, Ferreira P, Manito CI, Matos CM, Romão RJ, Rocha A (2008). Effect of season and gonadotrophin preparation on superovulatory response and embryo quality in Portuguese Black Merinos. Small Ruminant Research, 74, 134–139.

Bettencourt EM, Bettencourt CM, Chagas e Silva J, Ferreira P, Matos CP, Romão RJ, Rocha A (2009). Fertility rates following the transfer of ovine embryos cryopreserved using three protocols. Small Ruminant Research, 82(2), 112-116.

Boettcher PJ, Stella A, Pizzi F, Gandini G (2005). The combined use of embryos and semen for cryogenic conservation of mammalian livestock genetic resources. Genetics Selection Evolution, 37, 657–675.

Chagas e Silva J, Lopes da Costa L, Cidadão R, Robalo Silva J (2003). Plasma progesterone profiles, ovulation rate, donor embryo yield and recipient embryo survival in native Saloia sheep in the fall and spring breeding seasons. Theriogenology, 60(3), 521-532.

Cheng FP, Wu JT, Chan JPW, Wang JS, Fung HP, Colenbrander B, Tung KC (2004). The effect of different extenders on post-thaw sperm survival, acrosomal integrity and longevity in cryopreserved semen of Formosan Sika deer and Formosan Sambar deer. Theriogenology, 61, 1605-1616.

Coloma MA, Toledano-Díaz A, López-Sebastián A, Santiago-Moreno J (2010). The influence of washing Spanish ibex (*Capra pyrenaica*) sperm on the effects of cryopreservation in dependency of the photoperiod. Theriogenology, 73, 900-908.

Comission on Genetic Resources – FAO. The State of the World's Animal Genetic Resources for Food and Agriculture. Rome: 2007. 524p.

Comizzoli P, Mermillod P, Cognié Y, Chai N, Legendre X, Mauget R (2001). Successful in vitro production of embryos in the red deer (*Cervus elaphus*) and the sika deer (*Cervus nippon*). Theriogenology, 55, 649–659.

Comizzoli P, Mermillod P, Mauget R (2000). Reproductive biotechnologies for endangered mammalian species. Reproduction Nutrition Development, 40, 493–504.

Coonrod SA, Flores-Foxworth G, Moreno JF, Westhusin M, Byrd SR, Kraemer DC (1994). Birth of Armenian red sheep (*Ovis orientalis*) lambs to domestic sheep (*Ovis aries*) following interspecific transfer of IVM-IVF derived embryos. Theriogenology, 41, 182. [Abstract].

Demirci B, Lornage J, Salle B, Poirel MT, Guerin JF, Franck M (2003). The cryopreservation of ovarian tissue: uses and indications in veterinary medicine. Theriogenology, 60, 999–1010.

Demmers KJ, Jabbour HN, Deakin DW, Flint APF (2000). Production of interferon by red deer (*Cervus elaphus*) conceptuses and the effects of roIFN-tau on the timing of luteolysis and the success of asynchronous embryo transfer. Journal of Reproduction and Fertility, 118, 387–395.

Durrant BS (2009). The importance and potential of artificial insemination in CANDES (companion animals, non-domestic, endangered species). Theriogenology, 71, 113-122.

Fernandez-Arias A, Folch J, Alabart JL, Ramon J (1996). Successful interspecific embryo transfer between Spanish ibex (*mpvrenaica*) and domestic goat (*Capra hircus*) using microosmotic pumps for FSH administration. Theriogenology, 45, 247. [Abstract].

Figueiredo JR, Celestino JJH, Rodrigues APR, Silva JRV (2007). Importância da biotécnica de MOIFOPA para o estudo da foliculogênese e produção *in vitro* de embriões em larga escala. Revista Brasileira de Reprodução Animal, 31(2), 143-152.

Folch J, Cocero MJ, Chesné P, Alabart JL, Domínguez V, Cognié Y, Roche A, Fernández-Arias A, Martí JI, Sánchez P, Echegoyen E, Beckers JF, Sánchez Bonastre A, Vignon X (2009). First birth of an animal from an extinct subspecies (*Capra pyrenaica pyrenaica*) by cloning. Theriogenology, 71, 1026-1034.

Freitas VJF, Melo LM (2010). *In vitro* embryo production in small ruminants. Revista Brasileira de Zootecnia, 39, 409-413.

Galal S (2005). Biodiversity in goats. Small Ruminant Research, 60, 75-81.

Glowka L, Burhenne-Guilmin F, Synge H (1994). *A guide to the convention on biological diversity*, *IUCN*. IUCN – the World Conservation Union (Gland, Switzerland).

Holt WV, Pickard AR (1999). Role of reproductive technologies and genetic resource banks in animal conservation. Reviews of Reproduction, 4, 143–150.

Holt WV, Abaigar T, Jabbour HN (1996a). Oestrous synchronization, semen preservation and artificial insemination in the Mohor gazelle (*Gazella dama mhorr*) for the establishment of a genome resource bank programme. Reproduction, Fertility and Development, 8, 1215–1222.

Holt WV, Bennett PM, Volobouev V (1996b). Genetic resource banks in wildlife conservation. Journal of Zoology, 238, 531-544.

Holt WV, Moore HDM, North RD, Hartman TD, Hodges JK (1988). Hormonal and behavioural detection of oestrus in blackbuck, *Antilopre cervicapra*, and successful artificial insemination with fresh and frozen semen. Journal of Reproduction and Fertility, 82, 717–725.

Johnston LA, Parrish JJ, Monson R, Leibfried- Rutledge L, Susko-Parrish JL, Northey DL, Rutledge JJ, Simmons LG (1994). Oocyte maturation, fertilization and embryo development in vitro and in vivo in the gaur (*Bos gaurus*). Journal of Reproduction and Fertility, 100, 131–136.

Ledda S, Leoni G, Bogliolo L, Naitana S (2001). Oocyte cryopreservation and ovarian tissue banking. Theriogenology, 55, 1359-1371.

Li XC, Yue H, Li CY, He XH, Zhao QJ, Ma YH, Guan WJ, Ma JZ (2009). Establishment and characterization of a fibroblast cell line derived from Jining Black Grey goat for genetic conservation. Small Ruminant Research, 87, 17-26.

Locatelli Y, Cognié Y, Vallet JC, Baril G, Verdier M, Poulin N, Legendre X, Mermillod P (2005). Successful use of oviduct epithelial cell coculture for in vitro production of viable red deer (Cervus elaphus) embryos. Theriogenology, 64, 1729-1739.

Locatelli Y, Vallet JC, Huyghe FP, Cognié Y, Legendre X, Mermillod P (2006). Laparoscopic ovum pick-up and in vitro production of sika deer embryos: Effect of season and culture conditions. Theriogenology, 66, 1334-1342.

Lopes Jr ES, Maia ELMM, Paula NRO, Teixeira DIA, Villaroel ABS, Rondina D, Freitas VJF (2006). Effect of age of donor on embryo production in Morada Nova

(white variety) ewes participating in a conservation programme in Brazil. Tropical Animal Health and Production, 38(7-8), 555-561.

Loskutoff NM, Bartels P, Meintjes M, Godke RA, Schiewe MC (1995). Assisted reproductive technology in nondomestic ungulates: a model approach to preserving and managing genetic diversity. Theriogenology, 43, 3–12.

Mara L, Pilichi S, Sanna A, Accardo C, Chessa B, Chessa F, Dattena M, Bomboi G, Cappal P (2004). Sexing of *in vitro* produced ovine embryos by duplex PCR. Molecular Reproduction and Development, 69, 35–42.

Mariante AS, Cavalcante N (2006). *Animals of the Discovery. Domestic breeds in the history of Brazil*. Clécia M. 2nd Ed. Brasília: Embrapa Recursos Genéticos e Biotecnologia.

Mariante AS, Albuquerque MSM, Egito AA, McManus C, Lopes MA, Paiva SR (2009). Present status of the conservation of livestock genetic resources in Brazil. Livestock Science, 120, 204–212.

Martínez-Pastor F, Martínez F, Álvarez M, Maroto-Morales A, García-Alvarez O, Soler AJ, Garde JJ, Paz, P, Anel L (2009). Cryopreservation of Iberian red deer (*Cervus elaphus hispanicus*) spermatozoa obtained by electroejaculation. Theriogenology, 71, 628–638.

Morrow CJ, Wolfe BA, Roth TL, Wildt DE, Bush M, Blumer ES, Atkinson MW, Monfort SL (2000). Comparing ovulation synchronization protocols for artificial insemination in the scimitar-horned oryx (*Oryx dammah*). Animal Reproduction Science, 59, 71–86.

Muruvi W, Picton HM, Rodway RG, Joyce IM (2009). In vitro growth and differentiation of primary follicles isolated from cryopreserved sheep ovarian tissue. Animal Reproduction Science, 112(1), 36–50.

Niasari-Naslaji A, Mosaferi S, Bahmani N, Gerami A, Gharahdaghi AA, Abarghani A, Ghanbari A (2007). Semen cryopreservation in Bactrian camel (*Camelus bactrianus*) using SHOTOR diluent: Effects of cooling rates and glycerol concentrations. Theriogenology, 68, 618-625.

Othen LS, Bellem AC, Gartley CJ, Auckland K, King WA, Liptrap RM, Goodrowe KL (1999). Hormonal control of estrous cyclicity and attempted superovulation in wood bison (*Bison bison athabascae*). Theriogenology, 52, 313-323.

Penfold LM, Monfort SL, Wolfe BA, Citino SB, Widlt DE (2005). Reproductive physiology and artificial insemination studies in wild and captive gerenuk

(*Litocranius walleri walleri*). Reproduction, Fertility and Development, 17, 707–714.

Ptak G, Clinton M, Barboni B, Muzzeddu M, Cappai P, Tischner M, Loi P (2002). Preservation of the wild european mouflon: the first example of genetic management using a complete program of reproductive biotechnologies. Biology of Reproduction, 66, 796–801.

Pukazhenthi BS, Wildt DE. (2004). Which reproductive technologies are most relevant to studying, managing and conserving wildlife? Reproduction, Fertility and Development, 16, 33–46.

Santiago-Moreno J, Toledano-Díaz A, Pulido-Pastor A, Gómez-Brunet A, López-Sebastián A (2006). Birth of live Spanish ibex (*Capra pyrenaica hispanica*) derived from artificial insemination with epididymal spermatozoa retrieved after death. Theriogenology, 66, 283-291.

Saragusty J, Gacitua H, King R, Arav A (2006). Post-mortem semen cryopreservation and characterization in two different endangered gazelle species (*Gazella gazella* and *Gazella dorcas*) and one subspecies (*Gazella gazelle acaiae*). Theriogenology, 66, 775-784.

Snow M, Cleary M, Cox SL, Shaw JM, Paris MCJ, Jenkin G (2001). Comparison of the effects of in vitro and in situ storage on the viability of mouse ovarian tissue collected after death. Reproduction, Fertility and Development, 13, 389–394.

Soler AJ, Astore V, Sestelo A, Rivolta M, Jácome LN, Garde JJ (2003). Effect of thawing procedure on cryosurvival of deer spermatozoa: work in progress. Theriogenology, 60, 511-520.

Solti L, Crichton EG, Loskutoff NM, Cseh S (2000). Economical and ecological importance of indigenous livestock and the application of assisted reproduction to their preservation. Theriogenology, 53, 149-162.

Stover J, Evans J, Dolensek EP. (1981). Inter species embryo transfer from the gaur to domestic Holestein. In: ANNUAL MEETING OF AMERICAN ASSOCIATION OF ZOO VETERINARIANS, Woodland Park Zoo, USA. Proceedings… 122–124.

Succu S, Leoni GG, Berlinguer F, Madeddu M, Bebbere D, Mossa F, Bogliolo L, Ledda S, Naitana S (2007). Effect of vitrification solutions and cooling upon in vitro matured prepubertal ovine oocytes. Theriogenology, 68, 107-114.

Thundathil J, Whiteside D, Shea B, Ludbrook D, Elkin B, Nishi J (2007). Preliminary assessment of reproductive technologies in wood bison (*Bison bison athabascae*): Implications for preserving genetic diversity. Theriogenology, 68, 93-99.

Tsai TC, Wu SH, Chen HL, Tung YT, Cheng WT, Huang JC, Chen CM (2011). Identification of Sex-Specific Polymorphic Sequences in Goat Amelogenin Gene for Embryo Sexing. Journal of Animal Science, (X), XX - XX.

Vajta G. (2000). Vitrification of the oocytes and embryos of domestic animals. Animal Reproduction Science, 60-61, 357-364.

Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KHS (1997). Viable offspring derived from fetal and adult mammalian cells. Nature, 385, 810–813.

Wirtu G, Pope CE, Paccamonti DL, Godke RA, Dresser BL (2009). Ultrasonographic-guided retrieval and *in vitro* maturation of eland (*Taurotragus oryx*) and bongo (*Tragelaphus eurycerus isaaci*) antelope oocytes. Animal Reproduction Science, 111(2-4), 160–172.

Wolvekamp MC, Cleary ML, Cox SL, Shaw JM, Jenkin G, Trounson AO (2001). Follicular development in cryopreserved Common Wombat ovarian tissue xenografted to Nude rats. Animal Reproduction Science, 65(1-2), 135–147.

Zoheir KM, Allam AA (2010). A rapid method for sexing the bovine embryo. Animal Reproduction Science, 119(1-2), 92-96.

3 JUSTIFICATIVA

Considerando a grande relevância econômica que a espécie caprina representa especialmente para o Nordeste brasileiro, aliado ao aproveitamento ainda incipiente de sua capacidade reprodutiva, é de extrema importância o desenvolvimento de um sistema eficiente de PIVE capaz de otimizar o número de embriões produzidos no fim do processo. Os dados disponíveis na literatura já permitem afirmar a viabilidade da técnica para caprinos. Contudo, existe ainda uma carência de estudos abordando seus vários aspectos quando se compara com bovinos. A avaliação do efeito de diferentes moléculas na maturação do oócito, sistemas de capacitação espermática e FIV são alguns exemplos. Neste sentido, algumas etapas da técnica ainda não se encontram bem estabelecidas. Na busca por um sistema ideal, é compreensível que estas pesquisas sejam conduzidas em sua maior parte utilizando oócitos oriundos de ovários de abatedouro, já que estes constituem uma fonte de baixo custo e relativa disponibilidade. Isto contribui para o estabelecimento das melhores condições *in vitro* e, consequentemente, incrementa as taxas de PIVE em caprinos.

Entretanto, a utilização da técnica como ferramenta de melhoramento genético requer a colheita de oócitos de fêmeas de elevado mérito genético ou econômico, o que deve ser obtido a partir de laparoscopia (COL). Desta forma, ambas as fontes oocitárias são igualmente importantes a serem estudadas. Contudo, estas condições *in vitro* consideradas ideais para oócitos de abatedouro são normalmente transpostas sem quaisquer alterações quando utilizam-se oócitos oriundos de COL. Torna-se então essencial a identificação das diferenças ou similaridades existentes entre as exigências de ambas as fontes oocitárias e, consequentemente, adequação das condições *in vitro* para cada sistema. Similarmente, a COL é essencial para a utilização da PIVE na conservação de raças ameaçadas. Felizmente, existe atualmente uma grande preocupação com o futuro do meio ambiente com relação à possibilidade de que muitas espécies/raças serão forçadas a entrar em extinção por causas não naturais, em vez de por meio de lento processo evolutivo. Até os dias de hoje, 3% das raças de caprinos já se tornaram extintas no mundo e algumas se encontram ameaçadas como, por exemplo, a raça Canindé. Desta forma, a otimização da técnica de PIVE pode ser uma forte aliada para a sua conservação.

No presente estudo foram empregadas diferentes metodologias durante a maturação e fecundação *in vitro* que efetivamente permitiram incrementar as taxas de

produção de embriões em oócitos caprinos a partir de ovários de abatedouro. Além disso, foi possível aplicar a técnica de COL seguida de PIVE na raça caprina Canindé, produzindo seus primeiros embriões *in vitro* no mundo e possibilitando a implantação de banco de embriões da raça. Com o estabelecimento de procedimentos de manipulação *in vitro,* a técnica tem potencial de atender um mercado com infraestrutura já estabelecida pela demanda de embriões bovinos.

4 HIPÓTESE CIENTÍFICA

Diante da existência de diferentes etapas de execução, a presente tese apresenta as seguintes hipóteses principais:

4.1 Capítulo 1

- Meios de maturação *in vitro* indefinido, semidefinido ou definido resultam em taxas similares para a PIVE a partir de oócitos oriundos de ovários de abatedouro;
- Oócitos oriundos de ovários de abatedouro ou coletados por laparoscopia possuem cinética de maturação *in vitro* e exigências diferentes;
- A competência ao desenvolvimento de oócitos oriundos de ovários de abatedouro ou coletados por laparoscopia é similar, dependendo das condições *in vitro* encontradas;

4.2 Capítulo 2

- A inclusão de heparina como agente capacitante no meio de fecundação *in vitro* possui efeitos benéficos sobre as taxas de produção de embriões;
- A presença das células do *cumulus* é importante durante a maturação e fecundação *in vitro*, a fim de elevar as taxas de produção de embriões;
- A utilização de oócitos desnudos no momento da colheita pode representar produção adicional interessante de embriões no fim do processo.

4.3 Capítulo 3

- Parâmetros reprodutivos e perfil plasmático de progesterona da raça Canindé são semelhantes entre categorias e em comparação a outras raças caprinas;
- Um protocolo simplificado de superestimulação ovariana é eficiente para colheita de oócitos por laparoscopia. Estes oócitos quando submetidos a maturação, fecundação e desenvolvimento *in vitro* são capazes de gerar embriões de boa qualidade.

5 OBJETIVOS

5.1 OBJETIVO GERAL

- Aprimorar a técnica de PIVE em caprinos por meio de melhorias substanciais nas etapas de maturação e fecundação *in vitro* a partir de oócitos oriundos de ovários de abatedouro;
- Caracterizar os parâmetros reprodutivos da raça Canindé e constituir um banco de embriões, contribuindo para sua conservação.

5.2 OBJETIVOS ESPECÍFICOS

- Avaliar o efeito de meio de maturação *in vitro* indefinido, semidefinido ou definido sobre a PIVE a partir de oócitos oriundos de ovários de abatedouro;
- Identificar a diferença da cinética de maturação *in vitro* em oócitos oriundos de ovários de abatedouro ou colheita por laparoscopia;
- Verificar a competência ao desenvolvimento de oócitos oriundos de ovários de abatedouro ou colheita por laparoscopia para a PIVE;
- Avaliar o efeito da inclusão de heparina no meio de fecundação *in vitro* na PIVE;
- Assessar a influência das células do *cumulus* quando separadas ou unidas ao oócito durante a maturação e/ou fecundação *in vitro* para a PIVE;
- Identificar o potencial de utilização de oócitos já desnudos no momento da colheita para a PIVE;
- Caracterizar os parâmetros reprodutivos e perfil plasmático de progesterona em cabras nulíparas e pluríparas da raça Canindé;
- Utilizar protocolo simplificado de superestimulação para colheita de oócitos por laparoscopia seguida de PIVE, possibilitando a formação de banco de embriões, contribuindo para a preservação da raça Canindé.

Nas páginas seguintes, serão apresentados três capítulos referentes aos artigos científicos que compõem esta tese. Vale salientar que um artigo já se encontra publicado, um aceito para publicação e o terceiro em processo de submissão.

6 CAPÍTULO 1

Produção *in vitro* de embriões em caprinos: oócitos oriundos de colheita por laparoscopia ou ovários de abatedouro possuem cinética da MIV e exigências diferentes para o desenvolvimento embrionário

Resumo

Um total de 3427 oócitos caprinos foi utilizado neste estudo com o objetivo de identificar possíveis diferenças durante a produção *in vitro* de embriões de oócitos oriundos de abatedouro ou coletados por laparoscopia (COL). No Experimento 1, foram comparados meio de maturação indefinido, semidefinido ou definido (MM) em oócitos de abatedouro. No Experimento 2, nós avaliamos o efeito da fonte do oócito (ovários de abatedouro ou COL) sobre a cinética da maturação (18 *vs.* 22 *vs.* 26 h) quando submetidos a MM semidefinido ou definido. No Experimento 3, nós determinamos as diferenças sobre o desenvolvimento embrionário entre oócitos de COL e abatedouro quando submetidos a ambos os MM e à FIV ou ativação partenogenética (AP). Embriões de todos os grupos foram vitrificados e sua viabilidade avaliada após o aquecimento. No Experimento 1, não houve diferença ($P > 0,05$) entre os tratamentos na taxa de maturação (88% em média), clivagem (72%), blastocistos a partir do número inicial de COC (46%) ou de clivados (63%), taxa de eclosão (69%) e o número total de blastômeros (187). No Experimento 2, 18 h ou 22 h de maturação alcançaram resultados similares para oócitos de abatedouro, contudo 22 h resultaram em maiores taxas ($P < 0,05$) que 18 h para oócitos de COL maturados em meio definido. Além disso, oócitos de abatedouro cultivados em meio definido maturaram significativamente mais rápido do que os de COL (18 h e 22 h, $P < 0,05$). No Experimento 3, a taxa de clivagem foi significativamente superior ($P < 0,001$) em todos os quatro grupos de embriões produzidos por AP em relação aos de FIV. Interessantemente, a AP promoveu taxas

similares em oócitos de abatedouro cultivados em ambos os meios, porém elevou a taxa de clivagem quando oócitos de COL foram maturados em meio semi definido. Oócitos de abatedouro obtiveram boas taxa de clivagem (~67%) enquanto os de COL apresentaram baixa taxa (~38%; $P < 0,05$). A porcentagem de blastocistos em relação aos clivados não foi afetada pelo tratamento. Desta forma, oócitos de abatedouro desenvolveram uma maior proporção de blastocistos que os de COL quando leva-se em consideração o número inicial de COC que entraram para MIV. Blastocistos vitrificados e posteriormente aquecidos apresentaram resultados similares nas taxas de sobrevivência e eclosão com relação à fonte do oócito, MM ou método de ativação. Em conclusão, oócitos oriundos de abatedouro ou COL podem ter diferenças na cinética da MIV e nas exigências durante a MIV e a FIV. Embora os sistemas de MIV e FIV ainda necessitem aprimoramento no sentido de aumentar a produção embrionária, a etapa de DIV é capaz de gerar embriões de boa qualidade a partir de oócitos de COL.

Palavras-Chave: Blastocisto, Caprino, PIV, LOPU, Maturação do oócito.

Periódico: Theriogenology, em processo de submissão (Qualis: A2, Fator de Impacto: 2,082).

In vitro embryo production in goats: Slaughterhouse and laparoscopic ovum pick up (LOPU) derived goat oocytes have different IVM kinetics and requirements for embryo development

Joanna Maria Gonçalves de Souza-Fabjan[1,2], Nicolas Duffard[1,3], Yann Locatelli[1,3], Emilie Corbin[1], Christine Perreau[1], Jean-Luc Touzé[1], Vicente José de Figueirêdo Freitas[2], Pascal Mermillod[1]

1 INRA, UMR7247, Physiologie de la Reproduction et des Comportements, INRA, CNRS, Université de Tours, Haras Nationaux. 37380 Nouzilly, France

2 Laboratory of Physiology and Control of Reproduction, Veterinary School, State University of Ceará, Av. Dedé Brasil, 1700, 60740-90, Fortaleza, CE, Brazil

3 Museum National d'Histoire Naturelle, Réserve de la Haute Touche, 36290, Obterre, France

ABSTRACT

A total of 3427 goat oocytes were used in this study in order to identify possible differences during *in vitro* embryo production of slaughterhouse or LOPU oocytes. In Experiment 1, undefined, semi defined or defined maturation media (MM) were compared in slaughterhouse oocytes. In Experiment 2, we checked the effect of oocyte origin (slaughterhouse ovaries or LOPU) on the kinetics of maturation (18 *vs.* 22 *vs.* 26 h) when submitted to semi defined or defined MM. In Experiment 3, we determined the differences on embryo development between slaughterhouse or LOPU

oocytes when submitted to both MM and IVF or parthenogenetic activation (PA). Embryos from all groups were vitrified and their viability evaluated after thawing. In Experiment 1, no difference ($P > 0.05$) was detected among treatments for maturation rate (88% on average), cleavage (72%), blastocyst from the initial number of COC (46%) or from the cleaved ones (63%), hatching rate (69%) and the total number of blastomeres (187). In Experiment 2, 18 h or 22 h of maturation reached similar results for slaughterhouse, but 22 showed greater rate ($P < 0.05$) than 18 h for LOPU oocytes matured in defined medium. Moreover, slaughterhouse oocytes cultured in defined medium matured significantly faster than LOPU oocytes at 18 and 22 h ($P < 0.05$). In Experiment 3, cleavage rate was significant greater ($P < 0.001$) in all four groups of embryos produced by PA than IVF. Interestingly, PA reached similar rates for slaughterhouse oocytes cultured in both media, but improved ($P < 0.05$) cleavage rate when LOPU oocytes were matured in semi defined medium. Slaughterhouse oocytes had acceptable (~67%) whereas LOPU oocytes had low cleavage rate (~38%). The percentage of blastocysts in relation to cleaved ones was not affected ($P > 0.05$). Therefore, slaughterhouse oocytes developed a greater proportion of blastocysts than LOPU oocytes, expressed as the percentage of total COC entering to IVM. Vitrified-thawed blastocysts presented similar results in survival and hatching rates between the oocyte origin, MM or method of embryo production. In conclusion, slaughterhouse and LOPU derived oocytes may have different IVM kinetics and require different IVF conditions. Although the IVM and IVF systems still need improvements in order to enhance embryo yield, the IVD step is able to generate good quality embryos from LOPU derived oocytes.

Keywords: Blastocyst, Caprine, IVP, LOPU, Oocyte maturation

1. Introduction

Goats are well adapted to many different environments and they are very versatile as producers of food and raw matter such as milk, meat and skin. Efficient reproductive biotechnologies are essential in order to sustain worldwide production. Over the recent years, researchers have been trying to determine which conditions are needed during *in vitro* maturation (IVM), fertilization (IVF) and *in vitro* development (IVD) processes to enhance embryo production. Regarding to maturation process, in mammals, primary oocytes enter meiosis in early prenatal life, progress to the diplotene stage of prophase I (germinal vesicle stage; GV), and remain arrested until shortly before the time of ovulation. Resumption of meiosis is mediated *in vivo* under the influence of hormonal stimuli and *in vitro* by releasing oocytes from the follicles and further culturing them in suitable conditions. However, it was well demonstrated that IVM oocytes are compromised in their developmental capacity compared with those matured *in vivo* [1, 2]. The lower potential of IVM oocytes is probably related to the heterogeneity of the oocytes obtained, in terms of differentiation status, and to inappropriate conditions used in IVM [3]. This results in a relatively low rate of oocytes finally reaching the blastocyst stage, which is one of the main limitations of IVP from immature oocytes in mammals. Therefore, remains a challenge to enhance IVM rates in order to obtain good quality IVP embryos.

In most of the studies, the basic medium is supplemented with hormones and different concentrations of serum [3, 4]. However, all complex supplements such as fetal calf serum, estrus goat/sheep serum or follicular fluid lead to a lack of reproducibility. For these reasons, there has been a trend to use defined or at least semi defined maturation media, but this information for goat oocyte is still incipient. In order to make IVM simpler and more repeatable, we proposed a maturation medium using

just defined compounds – TCM 199 supplemented with 10 ng/mL EGF and 100 μM cysteamine – and obtained good results in embryo development using slaughterhouse oocytes [5]. Currently, oocytes used for IVP are mainly collected from slaughterhouse ovaries or by LOPU from live animals. Slaughterhouse ovaries provide a cheap source of large number of oocytes from unknown females which are helpful for research and improvement of IVP conditions. However, the use of IVP for genetic improvement or diffusion requires collecting oocytes by repeated LOPU from given females with high economic or genetic merit. Therefore, both sources are equally important to be studied. Earlier trials performed in our laboratory suggest that oocytes requirements during maturation may differ according to their origin, i.e., slaughterhouse or LOPU derived and, thus, the same maturation media could have different effects on both sources [6].

The time required for IVM varies among different species. Earlier studies reported that IVM of goat oocyte should last at least 27 h [7] or even 32 h, in comparisons from 0 to 36 h [8]. The authors justified that this long time was related to the origin of oocyte (slaughterhouse ovaries that were not stimulated by gonadotropins). However, in the last decade we have been using 22 to 24 h of IVM for slaughterhouse oocytes reaching good results [3, 5, 9]. Interestingly, a recent study reported longer IVM for slaughterhouse oocytes (24 to 27 h) than for LOPU (18 to 21 h), probably since the latter were collected from stimulated goats [10]. However, no studies were performed to evaluate the kinetics of maturation in goat LOPU oocytes or, even further, a direct comparison between the IVM kinetics of both sources.

Besides the enhanced number of blastocysts in the end of the process, another challenge is to make sure these embryos are of good quality. The best way to assess embryo quality or viability is to check their capacity of establishing pregnancy and consequently give birth to normal offspring. However, embryo transfers being heavy

and costly in domestic species, some other reliable indicators of embryo viability are: the evaluation of the level of expression of specific gene sets, number of blastocyst cells (blastomeres) count, kinetic of development and the resistance to cryopreservation [11]. Vitrification has proven to be more effective than slow cooling methods to cryopreserve mammalian oocytes and it was tested in goats with good results [12].

Most studies carried out to identify the factors influencing IVM and subsequent embryo development of goat oocytes were performed using slaughterhouse ovaries. Therefore the aims of this study were to examine the i) effect of undefined, semi defined and defined maturation media on maturation, fertilization and embryo development for slaughterhouse oocytes, ii) IVM kinetics of slaughterhouse and LOPU derived oocytes when submitted to different maturation media, and iii) the developmental competence of slaughterhouse and LOPU derived oocytes when submitted to different maturation media by the use of IVF and parthenogenetic activation in order to produce good quality embryos.

2. Material and methods

Except where otherwise indicated, chemicals were purchased from Sigma Chemical Co. (Saint Louis, MO, USA).

All the experiments were conducted at the Experimental Unit UEPAO in Nouzilly (France, latitude 47°22’N, longitude 00°41’E). The procedures were approved by the agricultural and scientific research agencies and ethical committee of INRA. A series of three experiments were performed to examine the impact of different aspects of IVM on maturation, fertilization, embryo development and quality of goat oocytes obtained by LOPU or slaughterhouse ovaries.

2.1. Experiment 1

2.1.1. Experimental design

The objective of this experiment was to determine the effect of undefined, semi defined and defined maturation media on the maturation, fertilization, cleavage and embryo development rates. In total, 846 COC obtained by slaughterhouse ovaries were analyzed in four replicates.

2.1.2. Aspiration of oocytes

During the non-breeding season (March and April) ovaries from goats, regardless the stage of estrous cycle, were collected from a local slaughterhouse and transported to the laboratory in a thermos box containing saline solution at 30 °C within 4 h after collection. Ovaries were washed in pre-warmed fresh saline (30 °C), and oocytes were aspirated through a 19 gauge short needle from all visible follicles between 2 and 6 mm in diameter into a Falcon tube under controlled vacuum (30 mm Hg). The collection tube was previously filled with 3 to 5 mL of HEPES buffered tissue culture medium 199 (TCM 199) supplemented with 10 IU/mL heparin (Choay, Glaxo Wellcome Production, Notre Dame de Bondeville, France), 4 µg/mL gentamycin and 1 mg/mL BSA (A 9647).

2.1.3. IVM of oocytes

Cumulus oocyte complexes (COC) were isolated under a stereo zoom microscope (Nikon Corporation, Japan) and graded as Grade 1, 2 or 3. Only good quality (Grade 1 and 2), i.e., surrounded by at least one complete layer of unexpanded *cumulus* cells were considered acceptable and used in IVM [13]. The COC were washed four times and transferred to four well petri dishes (Nunc, Roskilde, Denmark) containing 45 to 50 oocytes in 500 µL of maturation medium. The maturation medium

consisted of TCM 199 supplemented either with: 1) 10 ng/mL epidermal growth factor (EGF) and 100 µM cysteamine (EGF medium; defined); 2) 10% fetal calf serum (FCS), 100 µM cysteamine and 50 ng/mL oFSH (FCS medium; undefined) or 3) 10 ng/mL EGF, 5 IU/mL hCG, 10 IU/mL eCG, 19 ng/mL IGF-1, 2.2 ng/mL FGF, 5 µg/mL Insuline, 5 µg/mL Transferine, 5 ng/mL Selenium, 90 µg/mL L-Cystein, 0.1 mM β-mercapto, 75 µg/mL vitamin C, 720 µg/mL Glycine, 0.1 mg/mL Glutamine and 110 µg/mL Pyruvate, MIX (semi defined). COC were incubated for 22 h at 38.8 °C in a humidified atmosphere of 5% CO_2 in air [1][3].

2.1.4. Determination of stage of nuclear maturation

After 22 h of IVM, part of the oocytes (approximately ¼) from each maturation treatment was placed into 15 mL Falcon tubes containing 2 mL of TCM 199 medium and BSA (2 µL/mL), and vortexed for 2 min (medium speed) to remove *cumulus* oophorus. These oocytes were recovered in 35 mm petri plates, washed, transferred within a 10 µL droplet, positioned on grease-free slide (Lames Porte-Objet, RS France) and overlaid with a cover slip. The slide was fixed in methanol for at least 3 h. Oocytes were stained in 2 µL/mL Hoechst 33342 (stains all cells blue). Fixed oocytes were stored at 4 °C until they were examined using fluorescent microscopy to the stage of nuclear division and the presence or absence of first polar body.

2.1.5. Sperm preparation and IVF of oocytes

Two straws of semen per replicate from the same ejaculate/buck were used throughout experiments. Motile sperm from frozen/thawed semen were separated by centrifugation (15 min at 700 *g*) on 2 mL of Percoll (Pharmacia, Uppsala, Sweden) discontinuous density gradient (45/90%). Viable sperm were diluted in the appropriate volume of fertilization medium, to achieve a final concentration of 2.0 x 10^6 sperm/mL (Day of IVF = Day 0).

COC were transferred to plates containing washing medium and washed. The washing medium used was synthetic oviduct fluid (SOF) medium (pH = 7.3, 280 mOsm), which contained 10% of heat-inactivated estrus sheep serum, 5 µg/mL heparin (Calbiochem 375 095) and 4 µg/mL gentamycin. Groups of 45 to 50 oocytes were transferred into four well petri dishes, containing 450 µL of fertilization medium and 50 µL of sperm suspension were added to each well. Sperm and oocytes were co-incubated for 18 h at 38.8 °C in a humidified atmosphere of 5% CO_2 in air [9].

2.1.6. Fertilization rate

At the end of IVF all COC were denuded by vortex using the same methodology as earlier described. Part of the oocytes (approximately ¼ from the initial number) from each maturation treatment were fixed in slides and stained with Hoechst, as previously detailed (*2.1.4*. section). Sperm penetration and pronuclear formation were assessed under an epifluorescence microscope. The presence of three or more pronuclei was designated as polyspermia.

2.1.7. IVD of embryos

The other presumptive zygotes (approximately ½ from the initial number) were washed four times in culture medium (SOF supplemented with 3 mg/mL BSA) to remove spermatozoa and transferred to four well petri dishes containing 25 µL drops of culture medium covered with 700 µL of mineral oil. The presumptive zygotes were incubated for seven days at 38.8 °C in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2. After 48 h post-insemination, 10% FCS was added to the culture droplets.

Embryos were examined morphologically and the efficiency of development was evaluated (i) as the percentage of cleaved embryos 2 days after fertilization, and the percentage of blastocysts on Day 8 expressed (ii) on the basis of the number of oocytes

entering into IVM (iii) or on the basis of the number of cleaved embryos at Day 2. On day 8, all expanded blastocysts were transferred to washing plates, washed to remove the mineral oil, fixed in slides and stained with Hoechst in order to count the number of blastomeres. Cell count was conducted under a fluorescence microscope.

2.2. Experiment 2

2.2.1. Experimental design

The objective of this experiment was to determine the effect of oocyte origin (slaughterhouse ovaries or LOPU) on the kinetics of maturation when submitted either to semi defined or defined maturation media. Oocytes from both origins were cultured for 18, 22 or 26 h. The effect of oocyte quality on maturation kynetic was also evaluated. No IVF was performed in this experiment. Consequently, this experiment was a factorial arrangement of 2 types of oocytes (LOPU *vs.* slaughterhouse) x 2 IVM media (EGF *vs.* MIX) x 3 maturation times (18 *vs.* 22 *vs.* 26 h). In total, 545 oocytes were used from slaughterhouse ovaries and 423 from LOPU, in three replicates.

2.2.2. Aspiration of oocytes – Slaughterhouse ovaries and LOPU in live goats

In the subsequent months (May and June), the same procedure as in Experiment 1 was adopted to recover the oocytes from slaughterhouse ovaries. LOPU sessions were organized in the same day of slaughterhouse-oocytes recovery and conducted in 32 pluriparous Saanen and Alpine goats (alternating 16 goats per week). The animals were housed in free stalls and provided with food and water *ad libitum*. Females received intravaginal sponges impregnated with 45 mg FGA (Chronogest CR, Intervet Schering-Plough Animal Health, Angers, France) for 11 days and a prostaglandin i.m. injection

(Cloprostenol, 50 µg; Intervet Schering-Plough Animal Health) that was administered eight days after the FGA sponge insertion. At the same moment, they were stimulated with a total of 16 mg FSH, which was given as twice-daily i.m. injections in five decreasing dose schedule (4/4/3/3/2 mg). All FSH used in the present study was highly purified porcine FSH, supplied by the Laboratory of Endocrinology, Faculty of Veterinary Medicine, University of Liege, Belgium. FGA sponges were removed at the moment of LOPU.

The goats were deprived of feed and water for 24 h prior to laparoscopy. Animal suffering was avoided by proceeding under general anaesthesia with xylazine (0.5 mg/10 kg of Kensol 2%, Konig, France) and ketamine (25 mg/10 kg of Ketalar Parke-Davis, France). The animal was placed in an inverted position on a cradle at a 45° angle. The aspiration was performed by the use of 5 mm laparoscope attached to a video system. The endoscope was inserted into the abdominal cavity through a trocar, cranial to the udder and to the left of the midline. The second trocar was inserted into the right side (opposite from the first one) of the abdomen for passing the non-traumatic grasping forceps. The last trocar was inserted in the midline for passing oocyte retrieval needle. The ovary was held by the grasper and all ovarian follicles bigger than 2 mm were individually aspirated using a 18-gauge needle (Cook Ireland Ltda, Limerick, Ireland) connected to an aspiration and flushing system. The vacuum pressure was set at 50 mmHg. The collection tube was previously filled with 3 to 5 mL of HEPES buffered tissue culture medium 199 (TCM 199) supplemented with 10 IU/mL heparin (Choay, Glaxo Wellcome Production, Notre Dame de Bondeville, France), 4 µg/mL gentamycin and 1 mg/mL BSA (A 9647). Once the aspiration was ended, each ovary was gently flushed with a heparinized saline solution (25 IU/mL) at 37 ºC for the prevention of

possible adhesions. Finally, the trocar orifices were treated with a local antibiotic healing solution.

2.2.3. Assessment of COC quality and IVM of oocytes

Part of the oocytes from the first goat (around 9 am) submitted to LOPU was found, washed and lasted in the washing medium until the last goat was aspirated (16^{th}, around 12 pm), which also had part of her oocytes separated and washed. These oocytes were denuded, fixed and stained with Hoechst. The same process was performed with a random ovary aspirated (slaughterhouse ovaries collected around 10 am and aspiration performed at 2 pm). The stages of oocyte nuclei upon aspiration (immature oocytes) were determined. By observing nuclear stages, the nuclei were classified into germinal vesicle (GV), GV breakdown (GVBD) or intermediary stages (metaphase, anaphase, telophase I).

Just after the aspiration of the second and subsequent goats, the collection tube was transported to the culture room, where the oocytes were found, selected and transferred to the washing plate. After aspirating approximately half of the goats of the day (the 8^{th} doe, around 10:30 am), a first LOPU group was pooled and transferred to the maturation media (interval from the first to the eight goat lasted maximum 2 h, approximately from 9 to 10:30 am). The aspiration continued and from the 9^{th} to 16^{th} goat, the second LOPU group was formed (once more, maximum 2 h between the 9^{th} to 16^{th} doe, approximately from 10:30 am to 12 pm). All good (Grade 1 and 2) and bad (Grade 3, denuded) quality oocytes were used, but Grade 3 oocytes were separated in a different group for each origin (LOPU or slaughterhouse). The oocytes were washed four times and placed in wells containing 45 to 50 oocytes in 500 µL of maturation medium. The maturation media used were either EGF or MIX, both described in Experiment 1. IVM lasted 18, 22 or 26 h, with a third of the oocytes removed each time,

from each maturation medium and quality (Grade 1 and 2 or Grade 3). It is noteworthy to make clear that 18, 22 and 26 h occurred at different moments for the three groups (first LOPU, second LOPU and slaughterhouse oocytes). At each moment, a third of the oocytes in each well (three different times of maturation, two different maturation media and two different quality oocytes) was recovered, washed, denuded, fixed and stained with Hoechst, as previously detailed in Experiment 1. In the end, the data of both LOPU groups were pooled and compared to slaughterhouse oocytes.

2.3. Experiment 3

2.3.1. Experimental design

The objective of this experiment was to determine the differences between the oocyte origin (slaughterhouse ovaries and LOPU) when submitted to semi defined and defined maturation media and either IVF or parthenogenetic activation. Embryo quality was evaluated through vitrification and warming analyses. Consequently, this experiment was a factorial arrangement of 2 types of oocytes (LOPU *vs.* slaughterhouse) x 2 IVM media (EGF *vs.* MIX) x 2 methods of embryo production (IVF *vs.* Parthenogenesis). A total of 1043 oocytes were used from slaughterhouse ovaries and 570 from LOPU, in four replicates.

2.3.2. Aspiration of oocytes – Slaughterhouse ovaries and LOPU in live goats

In the subsequent months (July to November), the same procedures as in Experiment 2 were adopted to recover the oocytes from slaughterhouse ovaries and LOPU. In the current experiment, other 32 pluriparous Saanen and Alpine goats were selected. The same stimulatory treatment and aspiration procedure were employed.

2.3.3. Assessment of COC quality and IVM of oocytes

COC from both origins were isolated and graded. The oocytes were washed four times and placed in wells containing 45 to 50 oocytes in 500 µL of maturation medium. The maturation media used were either EGF or MIX, both previously described in Experiment 1. Considering the proportion of good and bad quality oocytes obtained after the end of LOPU, oocytes were equally mixed between both maturation media and the same proportion was respected for slaughterhouse ovaries in all treatments. The use of Grade 1, 2 and 3 quality oocytes is not routinely practice in our laboratory, and so for comparison, treatments containing only in Grade 1 and 2 oocytes were provided as a control. IVM lasted on average 22 h in all six treatments that were following IVF (LOPU EGF, G1/2/3; LOPU MIX, G1/2/3; Slaughterhouse EGF, G1/2/3; Slaughterhouse MIX, G1/2/3, Slaughterhouse EGF, G1/2 and Slaughterhouse MIX, G1/2. Oocytes submitted to parthenogenetic activation were cultured for 24 h (LOPU EGF, G1/2/3; LOPU MIX, G1/2/3; Slaughterhouse EGF, G1/2/3 and Slaughterhouse MIX, G1/2/3).

2.3.4. IVF of oocytes

After 22 h of maturation, oocytes from each of the six treatments were washed and allocated to IVF medium. Semen preparation, the fertilization medium and IVF followed the same protocol as that used in Experiment 1.

2.3.5. Parthenogenetic Activation (PA)

After 24 h of maturation, oocytes from each of the four treatments were submitted to PA using a protocol earlier described for bovine. Briefly, all COC were denuded and transferred to four well plates, with each well containing 500 µL of medium. After vortexing for denuding, the oocytes were washed two times (5 min each) in TCM 199 added by 5% FCS and 1 µL/mL gentamycin medium. Then, activation process started when placing the oocytes for 5 min in the same medium supplemented

by 2 µL/mL ionomycin. Oocytes were washed again during 1 min, and two times for 5 min in washing medium. Finally, they were transferred to the same medium containing 5 µL/mL 6-DMAP during 4 h. After the activation process, oocytes were washed three more times and lastly washed in IVD medium before transference to IVD medium.

2.3.6. IVD of presumable zygotes

At the end of IVF all presumptive zygotes were denuded by vortex, washed four times in culture medium and incubated in microdroplets. All the procedure and media are detailed in Experiment 1. Embryos were examined morphologically for cleavage and embryo development rates as earlier described in Experiment 1 (*2.1.7.* section).

2.3.7. Vitrification and warming – Embryo evaluation

The ability of the blastocysts to survive cryopreservation was used as a tool to assess quality [14]. On day 8, all blastocysts were transferred to washing plates, washed to remove the mineral oil and vitrified according to previously described [15]. Embryos of the same stage of development were vitrified together in the same straw (young and expanded or hatched). Briefly, embryos were kept at room temperature for 5 min in PBS supplemented with 20% new-born calf serum (NBCS). Blastocysts were then vitrified in three steps at room temperature as follows: 10% glycerol for 5 min, 10% glycerol and 20% ethylene glicol for 5 min, and finally 25% glycerol and 25% ethylene glicol for 30 s in PBS– NBCS supplemented with 0.4 M sucrose. During the last step, embryos were quickly aspirated into the center of a 0.25 mL plastic straw (IVM, L'Aigle, France) within 20–30 µL of vitrification solution. Embryos were separated by two air bubbles from two surrounding segments of PBS–NBCS containing 0.8 M galactose (about 90 µL each). The straws were sealed and immediately plunged directly into liquid nitrogen.

All straws remained from 7 to 15 d in the liquid nitrogen before warming. For warming, straws were held 5 s in air followed by 15 s in a 22 °C water bath. The straw content was expelled into Petri plate, where embryos lasted 5 min and were stirred gently to facilitate the mixture of the two solutions. Afterwards, the embryos were gently placed into another plate containing PBS–NBCS and remained for 5 min in room temperature. They were subsequently washed in the same medium, but previously warmed (39 °C) and then placed in culture medium for 72 h. Survival rates were evaluated with morphological criteria, on the basis of the integrity of the embryo membrane and the zona pellucida (with the exception of hatched blastocysts), and re-expansion of the blastocoele. Furthermore, the percentages of embryos that resumed their development and reached a more advanced developmental stage after culture were recorded (development rate). The embryos were evaluated at 6 h, 24 h, 48 h and 72 h [16].

2.4. Statistical analysis

One-way analysis of variance (ANOVA) was performed for the comparison of the number of blastomeres in different treatments in Experiment 1. Differences between replicates were tested by Chi-square test within each treatment as well as differences among treatments at cleavage rate, embryo development and survival rates after vitrification/warming. A value of $P < 0.05$ was considered to be statistically significant. Statistical analyses were performed using Graph Pad Prism 5.0a software.

3. Results

3.1. Experiment 1

Greater *cumulus* expansion was subjectively noticed at the treatment MIX (Fig. 1). However, no significant difference was detected on maturation rate or earlier stages among the three treatments (Table 1). When the data were pooled regardless to the treatment, average maturation was 88 ± 2.0%, intermediary 8 ± 1.4% and germinal vesicle rate only 4 ± 1.0%.

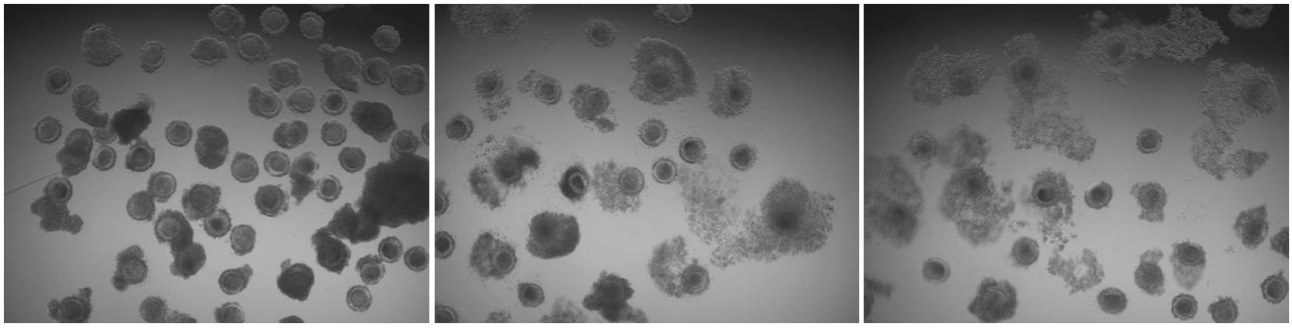

Fig. 1. *Cumulus* cell expansion of goat oocytes after 22 h of *in vitro* maturation in medium consisted of TCM 199 supplemented: A) 10 ng/mL epidermal growth factor (EGF) and 100 µM cysteamine; B) 10% fetal calf serum (FCS), 100 µM cysteamine and 50 ng/mL oFSH or C) MIX, 10 ng/mL EGF, 5 IU/mL hCG, 10 IU/mL eCG, 19 ng/mL IGF-1, 2.2 ng/mL FGF, 5 µg/mL Insuline, 5 µg/mL Transferine, 5 ng/mL Selenium, 90 µg/mL L-Cystein, 0.1 mM β-mercapto, 75 µg/mL vitamin C, 720 µg/mL Glycine, 0.1 mg/mL Glutamine and 110 µg/mL Pyruvate.

Table 1. Effect of different maturation media on the nuclear maturation of goat oocytes submitted to in *vitro* maturation for 22 h (Mean ± S.E.M.).

Treatments	n	GV[*] (%)	Intermediary[*] (%)	MII[*] (%)
EGF[**]	66	3 (4 ± 2.0) [a]	7 (11 ± 3.6) [a]	56 (85 ± 4.7) [a]
FCS[**]	74	2 (3 ± 2.1) [a]	4 (5 ± 2.1) [a]	68 (92 ± 3.6) [a]
MIX[**]	70	3 (4 ± 1.3) [a]	6 (9 ± 1.5) [a]	61 (87 ± 2.2) [a]

n: Number of *in vitro*-matured oocytes evaluated.

Within a column, values without a common superscript differ significantly by chi-square ($P < 0.05$).

* GV (germinal vesicle), Intermediary (metaphase I, anaphase I and telophase I), MII (metaphase II)

**EGF: 10 ng/mL epidermal growth factor and 100 µM cysteamine;

** FCS: 10% fetal calf serum, 100 µM cysteamine and 50 ng/mL oFSH

**MIX: 10 ng/mL EGF, 5 IU/mL hCG, 10 IU/mL eCG, 19 ng/mL IGF-1, 2.2 ng/mL FGF, 5 µg/mL Insuline, 5 µg/mL Transferine, 5 ng/mL Selenium, 90 µg/mL L-Cystein, 0.1 mM β-mercapto, 75 µg/mL vitamin C, 720 µg/mL Glycine, 0.1 mg/mL Glutamine and 110 µg/mL Pyruvate

Likewise, the different maturation media promoted similar rates of fertilization, averaging 72 ± 2.4% (Table 2).

Table 2. Effect of different maturation media on the *in vitro* fertilization pattern of goat oocytes (Mean ± S.E.M.).

Treatments	n	monospermic penetration (%)	polyspermic penetration (%)	non fertilized (%)	Fertilized* (%)
EGF**	76	28 (37 ± 7.1) [a]	28 (37 ± 5.9) [a]	20 (26 ± 4.0) [a]	56 (74 ± 3.1) [a]
FCS**	78	30 (39 ± 4.1) [a]	22 (28 ± 1.5) [a]	26 (33 ± 4.3) [a]	52 (67 ± 4.3) [a]
MIX**	78	29 (37 ± 6.5) [a]	29 (37 ± 3.6) [a]	20 (26 ± 5.2) [a]	58 (74 ± 5.2) [a]

n: Number of presumably zygotes evaluated.

Within a column, values with different superscripts differ significantly by chi-square ($P < 0.05$).

* Calculation of monospermic and polyspermic fertilized embryos

** EGF: 10 ng/mL epidermal growth factor and 100 µM cysteamine;

** FCS: 10% fetal calf serum, 100 µM cysteamine and 50 ng/mL oFSH

** MIX: 10 ng/mL EGF, 5 IU/mL hCG, 10 IU/mL eCG, 19 ng/mL IGF-1, 2.2 ng/mL FGF, 5 µg/mL Insuline, 5 µg/mL Transferine, 5 ng/mL Selenium, 90 µg/mL L-Cystein, 0.1 mM β-mercapto, 75 µg/mL vitamin C, 720 µg/mL Glycine, 0.1 mg/mL Glutamine and 110 µg/mL Pyruvate

High rates of polyspermic penetration were verified in the current study, averaging 34 ± 2.4% and monospermic penetration rate was 38 ± 3.2% (Fig. 2).

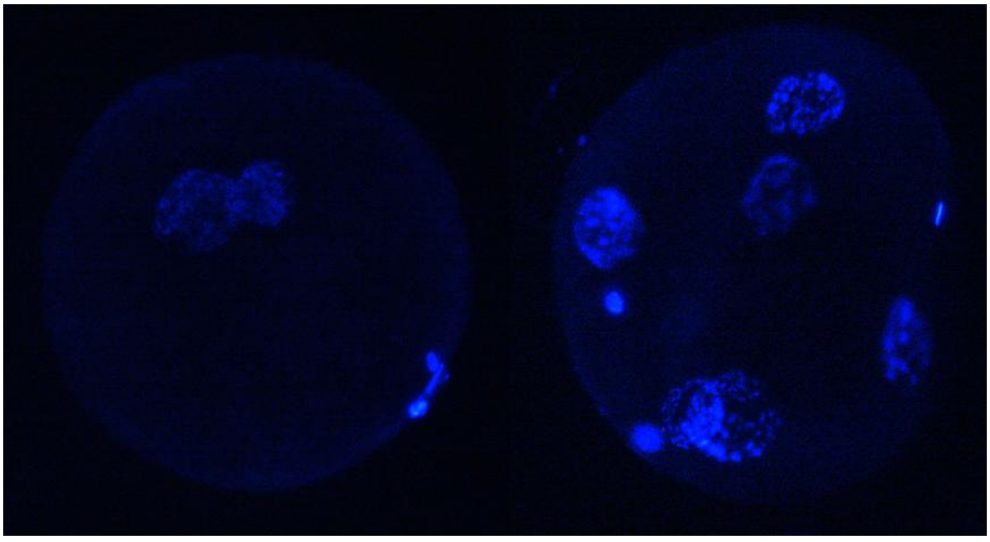

Fig. 2. *In vitro* fertilization of goat oocyte. A) Monospermic penetration, with two pronucleous; and B) Polyspermic penetration, with five visible pronucleous.

Embryo development is shown in Table 3. Similarly, the maturation medium influenced neither cleavage (72 ± 3.0% on average), blastocyst from the initial number

of COC entering to IVM (46 ± 1.1%) or from the cleaved ones (63 ± 2.1%), hatching rate (69 ± 3.3%) or the total number of blastomeres (187 ± 26.1) in expanded blastocysts produced from each maturation system.

Table 3. Effect of different maturation media on embryo developmental competence, i.e., percentages of cleavage, blastocysts (Bl), and hatched embryos in relation to the total blastocysts (Hbl/totBl) and number of blastocyst cells (Mean ± S.E.M.).

Treatments	n	Cleavage %	Bl/COC %	Bl/cleaved %	Hbl/totBl %	Total cells n*
EGF**	141	77 ± 3.4[a]	46 ± 0.5[a]	60 ± 2.2[a]	72 ± 6.4[a]	192 ± 13.7 (18)[a]
FCS**	132	72 ± 4.8[a]	45 ± 2.9[a]	63 ± 3.8[a]	67 ± 5.1[a]	179 ± 13.9 (19)[a]
MIX**	131	66 ± 4.8[a]	45 ± 1.4[a]	68 ± 3.0[a]	68 ± 4.6[a]	189 ± 11.4 (19)[a]

n: Number of *in vitro*-matured oocytes submitted to IVF and IVD.

Within a column, values with different superscripts differ significantly ($P < 0.05$) by chi-square.

* ANOVA test ($P > 0.05$)

**EGF: 10 ng/mL epidermal growth factor and 100 µM cysteamine;

** FCS: 10% fetal calf serum, 100 µM cysteamine and 50 ng/mL oFSH

**MIX: 10 ng/mL EGF, 5 IU/mL hCG, 10 IU/mL eCG, 19 ng/mL IGF-1, 2.2 ng/mL FGF, 5 µg/mL Insuline, 5 µg/mL Transferine, 5 ng/mL Selenium, 90 µg/mL L-Cystein, 0.1 mM β-mercapto, 75 µg/mL vitamin C, 720 µg/mL Glycine, 0.1 mg/mL Glutamine and 110 µg/mL Pyruvate

3.2. Experiment 2

Although not significant different, slaughterhouse immature oocytes were observed mostly in a more advanced stage, i.e., GVBD (19/26; 73%), than immature oocytes from LOPU, either in the first (7/13; 54%) or last goat (6/11; 55%). Consequently, the GV stage was numerically greater in LOPU, for the first (6/13; 46%) or last goat (5/11; 45%) than in slaughterhouse oocytes (7/26; 27%).

LOPU and slaughterhouse oocytes may have different maturation kinetics, since significant effect of culture time on nuclear maturation of good quality (Grade 1 and 2) oocytes were observed between both oocyte origins. At 18 h of maturation similar results were obtained in comparison to 22 h for slaughterhouse oocytes. However, LOPU oocytes matured in EGF showed greater rate ($P < 0.05$) when cultured for 22 than 18 h. Moreover, slaughterhouse oocytes cultured in EGF matured significantly faster than LOPU oocytes (18 and 22 h, $P < 0.05$), whereas no difference was observed when oocytes from both origins were matured in MIX. Although at 22 and 26 h of maturation slaughterhouse oocytes showed similar rates when comparing both maturation media, at 18 h EGF showed greater ($P < 0.05$) maturation rate than MIX (Table 4).

Interesting results were obtained when comparing good (Grade 1 and 2) with bad quality (Grade 3) oocytes. It is noteworthy that regarding to oocyte quality no difference was detected at maturation rate in LOPU oocytes at 18, 22, 26 h, respectively, for EGF (50, 54 and 44%) or MIX (62, 53 and 64%). Conversely, significant differences were found in slaughterhouse oocytes between good and bad quality oocytes, respectively, at 18 h (87 *vs.* 67%; EGF), 22 h (90 *vs.* 66%; EGF) and 26 h (90 *vs.* 70%; MIX). At the other moments, bad quality oocytes did not adversely influence maturation rates that reached 80% (26 h; EGF), 47% (18 h; MIX) and 88% (22 h; MIX). Only slaughterhouse oocytes cultured in EGF for 22 h lead to greater

($P < 0.05$) degenerated oocytes rate for bad quality in relation to good ones (17 *vs.* 2%) and all the other 11 groups had similar degenerated rates. No difference was observed at VG rate in any group. Bad quality oocytes demonstrated similar behavior at maturation rates from both origins, where slaughterhouse had greater ($P < 0.05$) maturation rate than LOPU oocytes, respectively, at 26 h for EGF (44 *vs*. 80%) or 22 h for MIX (53 *vs.* 88%). Interestingly, for slaughterhouse oocytes cultured for 22 h, MIX promoted greater ($P < 0.05$) maturation rate than EGF.

Table 4. Effect of culture time on nuclear maturation of good quality goat (Grade 1 and 2) oocytes obtained after laparoscopic ovum pick up (LOPU) or slaughterhouse ovaries submitted to different maturation media (Mean ± S.E.M.)

Oocyte origin	IVM media	Culture time (h)	n	Degenerated (%)	GV* (%)	Intermediary* (%)	M II* (%)
LOPU	EGF**	18	57	3 (5 ± 2.9) ^{a,x,A}	12 (21 ± 7.4) ^{a,x,A}	12 (21 ± 3.1) ^{a,xA}	30 (53 ± 7.2) ^{a,x,A}
		22	50	1 (2 ± 2.1) ^{a,x,A}	7 (14 ± 3.9) ^{a,x,A}	6 (12 ± 7.2) ^{a,x,A}	36 (72 ± 1.8) ^{b,x,A}
		26	52	1 (2 ± 2.1) ^{a,x,A}	8 (15 ± 4.4) ^{a,x,A}	9 (17 ± 2.3) ^{a,x,A}	34 (65 ± 4.6) ^{a,b,x,A}
	MIX**	18	57	6 (11 ± 5.5) ^{a,x,A}	6 (11 ± 2.7) ^{a,x,A}	10 (18 ± 2.0) ^{a,x,A}	35 (61 ± 6.3) ^{a,x,A}
		22	50	2 (4 ± 1.9) ^{a,x,A}	1 (2 ± 1.6) ^{a,y,A}	14 (28 ± 6.4) ^{a,y,A}	33 (66 ± 8.7) ^{a,x,A}
		26	50	1 (2 ± 1.2) ^{a,x,A}	1 (2 ± 1.2) ^{a,y,A}	10 (20 ± 6.0) ^{a,x,A}	38 (76 ± 6.4) ^{a,x,A}
Slaughterhouse	EGF**	18	46	1 (2 ± 4.2) ^{a,x,A}	0 (0 ± 0.0) ^{a,x,B}	5 (11 ± 7.4) ^{a,x,A}	40 (87 ± 3.2) ^{a,x,B}
		22	48	1 (2 ± 1.8) ^{a,x,A}	1 (2 ± 1.8) ^{a,x,B}	3 (6 ± 3.2) ^{a,x,A}	43 (90 ± 0.4) ^{a,x,B}
		26	56	3 (5 ± 0.7) ^{a,x,A}	2 (4 ± 2.9) ^{a,x,B}	7 (12 ± 8.4) ^{a,x,A}	44 (79 ± 4.8) ^{a,x,A}
	MIX**	18	42	2 (5 ± 2.8) ^{a,x,A}	1 (2 ± 1.4) ^{a,x,A}	13 (31 ± 1.4) ^{a,y,A}	26 (62 ± 2.8) ^{a,y,A}
		22	54	3 (6 ± 0.4) ^{a,x,A}	2 (4 ± 2.9) ^{a,x,A}	6 (11 ± 7.1) ^{b,x,B}	43 (80 ± 3.7) ^{a,b,x,A}
		26	51	4 (8 ± 1.8) ^{a,x,A}	0 (0 ± 0.0) ^{a,x,A}	1 (2 ± 2.8) ^{b,y,B}	46 (90 ± 1.0) ^{b,x,A}

n: Number of oocytes evaluated.

Within a column, values with different superscripts differ significantly by chi square ($P < 0.05$).

a,b differ between the time of maturation (18 *vs.* 22 *vs.* 26 h) within the same medium and origin of the oocyte.

x,y differ between the maturation medium (EGF *vs.* MIX) at the same time of maturation and origin of the oocyte.

A,B differ between the origin of oocytes (LOPU *vs.* Slaughterhouse) at the same time of maturation and medium.

* GV (germinal vesicle), Intermediary (Metaphase I, anaphase I and telophase I), MII (metaphase II)

** EGF: 10 ng/mL epidermal growth factor and 100 µM cysteamine;

** MIX: 10 ng/mL EGF, 5 IU/mL hCG, 10 IU/mL eCG, 19 ng/mL IGF-1, 2.2 ng/mL FGF, 5 µg/mL Insuline, 5 µg/mL Transferine, 5 ng/mL Selenium, 90 µg/mL L-Cystein, 0.1 mM β-mercapto, 75 µg/mL vitamin C, 720 µg/mL Glycine, 0.1 mg/mL Glutamine and 110 µg/mL Pyruvate

3.3. Experiment 3

Cleavage and blastocyst development rates are shown in Table 5. Cleavage rate was significant greater ($P < 0.001$) in all four groups of embryos produced by PA than IVF. Interestingly, PA reached similar rates for slaughterhouse oocytes cultured in both media, but improved cleavage rate when LOPU oocytes were matured in MIX in comparison with EGF. This behavior was not observed in any group that IVF was performed. As in Experiment 2, LOPU and slaughterhouse oocytes showed differences, whereas slaughterhouse oocytes had acceptable cleavage rates (~67%), LOPU oocytes had low (~38%), regardless to the maturation medium used. The percentage of blastocysts in relation to cleaved ones was affected neither by the oocyte origin, maturation medium or method of embryo production. Therefore, slaughterhouse oocytes developed a greater proportion of blastocysts than LOPU oocytes, expressed as the percentage of total COC entering to IVM, obviously due to cleavage rate reflect (Table 5). Our control groups using only Grade 1 and 2 slaughterhouse oocytes, *in vitro* fertilized, showed no differences to correlated groups formed by Grade 1, 2 and 3 oocytes. Similar results were obtained between EGF and MIX, respectively, for cleavage [69 ± 2.4 (129/187) and 71 ± 1.4% (130/183)], percentage of blastocysts from cleaved [64 ± 1.6 (83/129) and 63 ± 6.3% (82/130)], percentage of blastocysts from initial COC (44 ± 2.1 and 45 ± 4.2%) and hatching rate [65 ± 3.9 (54/83) and 66 ± 5.4% (54/82)].

Blastocyst re-expansion and hatching rates after vitrification, warming and 72 h of *in vitro* culture are summarized in Table 6. Although differences were detected between expanded and hatched embryos from the same groups, similar results were obtained in survival and hatching rates between the oocyte origin, maturation medium

or method of embryo production (Table 6). Our control groups had similar survival and hatching rates when Grade 3 oocytes were mixed (data not shown).

Table 5. Effect of oocyte origin, maturation media and method to produce goat embryos (*in vitro* fertilization, IVF or submitted to parthenogenetic activation, PA) from a mixed group of oocytes (Grade 1, 2 and 3) on embryo developmental competence, i.e., percentages of cleavage, blastocysts (Bl), and hatched embryos in relation to the total blastocysts (Hbl/totBl) (Mean ± S.E.M.)

Oocyte origin	IVM system	Method	n	Cleavage %	Bl/COC %	Bl/cleaved %	Hbl/totBl %
LOPU	EGF*	IVF	150	39 ± 3.4 ^{a,x,A}	28 ± 2.4 ^{a,x,A}	71 ± 4.0 ^{a}	55 ± 4.8 ^{a}
		PA	120	83 ± 4.5 ^{b,x,A}	55 ± 1.9 ^{b,x,A}	67 ± 5.9 ^{a}	65 ± 4.7 ^{a}
	MIX*	IVF	151	38 ± 4.0 ^{a,x,A}	25 ± 4.8 ^{a,x,A}	64 ± 6.5 ^{a}	57 ± 4.4 ^{a}
		PA	149	93 ± 3.5 ^{b,y,A}	61 ± 5.2 ^{b,x,A}	65 ± 4.0 ^{a}	69 ± 2.1 ^{a}
Slaughterhouse	EGF*	IVF	169	68 ± 2.6 ^{a,x,B}	47 ± 2.4 ^{a,x,B}	70 ± 4.1 ^{a}	65 ± 6.5 ^{a}
		PA	170	89 ± 3.1 ^{b,x,A}	61 ± 4.9 ^{b,x,A}	68 ± 4.2 ^{a}	62 ± 3.3 ^{a}
	MIX*	IVF	170	66 ± 6.6 ^{a,x,B}	43 ± 3.9 ^{a,x,B}	65 ± 6.1 ^{a}	68 ± 3.7 ^{a}
		PA	164	91 ± 2.3 ^{b,x,A}	59 ± 5.6 ^{b,x,A}	64 ± 5.5 ^{a}	63 ± 5.3 ^{a}

n: Number of blastocysts evaluated.

Within a column, values with different superscripts differ significantly by chi square ($P < 0.05$).

a,b differ between the method of embryo production (IVF *vs.* PA) at the same origin of oocytes and maturation medium.

x,y differ between the maturation medium (EGF *vs.* MIX) at the same method of embryo production and origin of the oocyte.

A,B differ between the origin of oocytes (LOPU *vs.* Slaughterhouse) at the same method of embryo production and maturation medium.

* EGF: 10 ng/mL epidermal growth factor and 100 µM cysteamine;

* MIX: 10 ng/mL EGF, 5 IU/mL hCG, 10 IU/mL eCG, 19 ng/mL IGF-1, 2.2 ng/mL FGF, 5 µg/mL Insuline, 5 µg/mL Transferine, 5 ng/mL Selenium, 90 µg/mL L-Cystein, 0.1 mM β-mercapto, 75 µg/mL vitamin C, 720 µg/mL Glycine, 0.1 mg/mL Glutamine and 110 µg/mL Pyruvate

Table 6. Differences in re-expansion rates of vitrified and warmed blastocysts derived from laparoscopic ovum pick-up (LOPU) or slaughterhouse ovaries in goats submitted to different maturation media and fertilized or submitted to parthenogenetic activation (PA) control (Mean ± S.E.M.)

Oocyte origin	IVM system	Method	Blastocyst development stage (n)	Post-warming blastocyst survival, %				Hatched blastocysts* %
				6 h	24 h	48 h	72h	
LOPU	EGF**	IVF	Expanded (11)	27 ± 12.7 ^{a,x,A,X}	64 ± 16.7 ^{a,x,A,X}	82 ± 9.6 ^{a,x,A,X}	73 ± 9.6 ^{a,x,A,X}	73 ± 9.6 ^{a,x,A}
			Hatched (20)	40 ± 6.9 ^{a,x,A,X}	50 ± 12.0 ^{a,x,A,X}	65 ± 20.0 ^{a,x,A,X}	65 ± 20.0 ^{a,x,A,X}	-
		PA	Expanded (18)	50 ± 11.7 ^{a,x,A,X}	50 ± 11.7 ^{a,x,A,X}	67 ± 14.3 ^{a,x,A,X}	61 ± 8.8 ^{a,x,A,X}	61 ± 8.8 ^{a,x,A}
			Hatched (33)	33 ± 5.8 ^{a,x,A,X}	55 ± 6.1 ^{a,x,A,X}	85 ± 3.9 ^{a,x,A,X}	70 ± 6.4 ^{a,x,A,X}	-
	MIX**	IVF	Expanded (13)	46 ± 8.4 ^{a,x,A,X}	69 ± 10.3 ^{a,x,A,X}	69 ± 10.3 ^{a,x,A,X}	77 ± 12.9 ^{a,x,A,X}	69 ± 10.3 ^{a,x,A}
			Hatched (20)	50 ± 11.0 ^{a,x,A,X}	70 ± 14.4 ^{a,x,A,X}	80 ± 9.6 ^{a,x,A,X}	85 ± 8.0 ^{a,x,A,X}	-
		PA	Expanded (20)	55 ± 21.3 ^{a,x,A,X}	70 ± 23.7 ^{a,x,A,X}	70 ± 23.7 ^{a,x,A,X}	70 ± 23.7 ^{a,x,A,X}	55 ± 21.3 ^{a,x,A}
			Hatched (54)	30 ± 8.2 ^{a,x,A,Y}	56 ± 3.5 ^{a,x,A,X}	78 ± 7.4 ^{a,x,A,X}	70 ± 6.7 ^{a,x,A,X}	-
Slaughterhouse	EGF**	IVF	Expanded (15)	40 ± 3.9 ^{a,x,A,X}	80 ± 7.9 ^{a,x,A,X}	73 ± 10.5 ^{a,x,A,X}	73 ± 17.8 ^{a,x,A,X}	53 ± 13.1 ^{a,x,A}
			Hatched (52)	25 ± 5.0 ^{a,x,A,X}	65 ± 15.1 ^{a,x,A,X}	73 ± 13.7 ^{a,x,A,X}	62 ± 7.2 ^{a,x,A,X}	-
		PA	Expanded (25)	40 ± 10.1 ^{a,x,A,X}	48 ± 20.4 ^{b,x,A,X}	52 ± 19.7 ^{a,x,A,X}	52 ± 19.7 ^{a,x,A,X}	48 ± 20.4 ^{a,x,A}
			Hatched (56)	27 ± 10.2 ^{a,x,A,X}	46 ± 7.0 ^{a,x,A,X}	57 ± 7.1 ^{a,x,B,X}	57 ± 12.0 ^{a,x,A,X}	-
	MIX**	IVF	Expanded (20)	35 ± 11.9 ^{a,x,A,X}	75 ± 19.0 ^{a,x,A,X}	75 ± 19.0 ^{a,x,A,X}	70 ± 18.9 ^{a,x,A,X}	50 ± 22.6 ^{a,x,A}
			Hatched (47)	36 ± 6.5 ^{a,x,A,X}	47 ± 13.4 ^{a,x,A,Y}	57 ± 13.0 ^{a,x,A,X}	62 ± 12.7 ^{a,x,A,X}	-
		PA	Expanded (29)	38 ± 14.5 ^{a,x,A,X}	66 ± 18.9 ^{a,x,A,X}	72 ± 21.0 ^{a,x,A,X}	59 ± 18.5 ^{a,x,A,X}	48 ± 14.0 ^{a,x,A}
			Hatched (60)	23 ± 4.7 ^{a,x,A,X}	50 ± 8.8 ^{a,x,A,X}	68 ± 6.8 ^{a,x,A,X}	73 ± 3.5 ^{a,x,A,X}	-

n: Number of blastocysts evaluated.

[*] The hatching rate was calculated based on the number of vitrified blastocysts, just in the groups that were expanded before vitrification

Within a column, values with different superscripts differ significantly by chi square ($P < 0.05$).

a,b differ between the method of embryo production (IVF *vs.* PA) at the same blastocyst stage, origin of oocytes and maturation medium.

x,y differ between the maturation medium (EGF *vs.* MIX) at the same blastocyst stage, method of embryo production and origin of the oocyte.

A,B differ between the origin of oocytes (LOPU *vs.* Slaughterhouse) at the same blastocyst stage, method of embryo production and maturation medium.

X,Y differ between the blastocyst stage (Expanded *vs.* Hatched) at the same origin of oocytes, maturation medium and method of embryo production.

[**] EGF: 10 ng/mL epidermal growth factor and 100 µM cysteamine;

[**]MIX: 10 ng/mL EGF, 5 IU/mL hCG, 10 IU/mL eCG, 19 ng/mL IGF-1, 2.2 ng/mL FGF, 5 µg/mL Insuline, 5 µg/mL Transferine, 5 ng/mL Selenium, 90 µg/mL L-Cystein, 0.1 mM β-mercapto, 75 µg/mL vitamin C, 720 µg/mL Glycine, 0.1 mg/mL Glutamine and 110 µg/mL Pyruvate

4. Discussion

The main objectives of the present study were to investigate different approaches regarding IVM of goat oocytes recovered from slaughterhouse ovaries or by LOPU in live goats. Four main conclusions can be drawn from our study. First, it is possible to achieve high maturation, fertilization, cleavage rates and embryo development when using undefined, semi defined and defined maturation media in slaughterhouse oocytes. Second, slaughterhouse and LOPU derived oocytes have different IVM kinetics, with the latter requiring more time when submitted to EGF medium. Third, LOPU and slaughterhouse oocytes have similar intrinsic quality, reaching similar developmental competence after PA. Fourth, the embryos produced by IVF or PA have similar survival rates, demonstrating that although the IVM and IVF systems still need improvements in order to enhance embryo yield, the IVD step is able to generate good quality embryos from LOPU derived oocytes.

The use of undefined, semi defined or defined maturation media promoted similar maturation, fertilization and cleavage rate, embryo development and quality in slaughterhouse derived oocytes. The average maturation rate (88%) obtained is considerably high, since often lower rates are reported in the literature, even when using serum (66%) [2]. As a measure of IVF success, usually only cleavage rate is evaluated. However, in some cases, the sperm penetration rate is different from cleavage as for example in comparison between young and adult females [17]. The current work demonstrated that sperm penetration (72%) was similar to cleavage rate (72%) in all three IVM media tested. It is noteworthy that high rates of polyspermic penetration were verified in the current study (~34% in average regardless to the treatment). Even greater polyspermic rate (45%) was reported in prepubertal goats [18], implying that polyspermia in goats is a recurrent event.

Embryo quality was evaluated in base of the number of blastomeres in expanded blastocysts at Day 8, which did not differ significantly among treatments. Our finding corroborated with an earlier report in ovine [19] but were contrary to those previously reported in cattle, when the IVM medium supplemented with serum resulted in blastocysts with a larger number of cells [20]. We obtained an average of 187 cells, greater than 120 [21] or 130 [22] and lesser than 243 cells, but counted on Day 9 [23], all reports in goats. It was previously described that morphological appearance did not necessarily indicate the true developmental status of the goat embryo, however, all expanded blastocysts we evaluated in the current study were compatible to the expected [21]. These data indicate that it is possible to produce high quality embryos with similar results when using undefined, semi defined and defined maturation media in slaughterhouse oocytes. In general, the basic medium is supplemented with hormones and serum [3, 4]. However, all complex supplements such as serum lead to a lack of reproducibility and should be strongly avoided and for further comparisons in the current study only semi defined and defined media were used.

Regarding kinetics comparison between both oocyte origins, under our system, slaughterhouse immature oocytes in the beginning of IVM were found mostly in GVBD (73%) than LOPU oocytes (~54%, NS), suggesting that the latter would need longer time to be matured. Confirming this hypothesis, at 18 h of IVM similar results were obtained in comparison to 22 h for slaughterhouse oocytes, submitted to either maturation medium; conversely, LOPU oocytes matured in EGF showed greater rate when cultured for 22 than 18 h. Moreover, slaughterhouse cultured in EGF matured significantly faster than LOPU oocytes (at both 18 and 22 h). These data generate a conflict to the theory that slaughterhouse oocytes needed longer time of IVM than LOPU [10], being actually the opposite depending on the used medium. We observed a

numerical fall at 26 h in the percentage of matured oocytes in some groups, but an increase in degenerated oocytes was not observed. This difference could be due to the difference in media or culture system, as earlier suggested [8]. In order to facilitate, we believe that 22 h is an optimal time for IVM for both oocyte origins, although oocytes cultured in MIX for 26 h had an increase of ~10% in maturation rate (NS). Despite of 15-20% greater maturation rate for the slaughterhouse oocytes groups, LOPU treatments were able to achieve reasonable rates when matured for 22 h in MIX (66%) or EGF (72%), greater than 56% [24] and 49% [25] but still lower than 85% [26]. Similar maturation rates were obtained between good and bad quality oocytes recovered by LOPU but significant differences were detected in some groups for slaughterhouse oocytes and mostly the degenerated rates were similar between both qualities.

In order to check the competence to development of both sources of oocytes, we conducted further experiments comparing either IVF or PA. Our group recently demonstrated it was possible to enhance the number of blastocysts by some alterations on IVF system, reaching up to 54% of embryos. However, this experiment was performed using only slaughterhouse oocytes [9]. The same IVF conditions were used in the present study. Under our experimental conditions, while both semi defined and defined maturation medium were suitable for IVM and IVF conditions were appropriate for slaughterhouse oocytes, LOPU derived oocytes showed very low cleavage rate after IVF (~39%), significantly different from slaughterhouse oocytes (~67%). The high cleavage rate obtained after PA in both maturation media (83 to 93%) indicate that oocytes from LOPU have similar competence to development as slaughterhouse ones. Thus, we infer this discrepancy in cleavage to unsuitable IVF conditions for LOPU oocytes, due to possible different requirements. During embryo development to the blastocyst stage, parthenotes are comparable to IVF embryos [27], or even more

effective than IVF as reported in goats [28]. Corroborating to this argument, in fact, cleavage rate was significant greater in all four groups of embryos produced by PA. It is noteworthy that if we already had some signs that both oocyte origins have different requirements during IVM [6], the current study is the first report which proposes that the conditions in IVF should also be adapted for LOPU oocytes. The developmental potential of cleaved embryos was not influenced by IVM or IVF treatments, ranging from 64 to 71% in all eight groups. As a consequence, the rate of blastocysts from initial number of COC was only a reflex of cleavage rate.

The ability of the blastocyst to quickly restore its functionality in terms of blastocoelic cavity re-expansion after vitrification and warming can be considered as a reliable marker for embryo quality and developmental potential [26]. Therefore, we evaluated the quality of the embryos produced in the different systems by looking at the resistance to cryopreservation. The survival rates of vitrified thawed embryos influenced neither, oocyte origin, maturation medium or method of embryo production and were similar to 63% previously reported in goat embryos [26]. It has been clearly demonstrated in cattle that while the rate of success of IVP in terms of blastocysts yield rely on oocyte intrinsic quality and maturation conditions, the quality of the resulting blastocysts (cryosurvival, viability) relies on the conditions encountered during earlier steps of development [29]. Thus, it is possible to assume that oocytes that were able to mature and be fertilized, independently of their origin, had a good support to develop into good quality embryos with similar results.

5. Conclusions

In conclusion, under our experimental conditions, similar maturation, fertilization and embryo development rates were obtained when using undefined, semi

defined and defined maturation media in slaughterhouse oocytes. Slaughterhouse and LOPU derived oocytes may have different IVM kinetics, with the latter requiring more time depending of the IVM medium. LOPU and slaughterhouse oocytes have similar intrinsic quality, reaching similar developmental competence in our control system using PA. Finally, the embryos produced by IVF or PA have similar survival rates, demonstrating that although the IVM and IVF systems still need improvements in order to enhance embryo yield, the IVD step is able to generate good quality embryos from LOPU derived oocytes. The present study has demonstrated a substantive advance in goat IVP but further research towards identifying specific requirements during IVM and IVF are yet necessary for goat LOPU derived oocytes.

Acknowledgments

The authors would like to thank the invaluable help of all staff of the veterinary hospital at INRA. J.M.G. Souza-Fabjan was supported by CAPES and Ceará State University. The authors wish to thank CAPES-COFECUB bilateral framework for financial support of collaboration between the Ceará State University and INRA on goat IVP (Grant 728/11, 2011-2013). This work was supported by a grant from Région Centre, France (PIVER program, #200800030493, 2008-2011). The authors thank Thierry Delpuech, Bruno Mirey and Jean-Noël Couet for ovary collection at slaughterhouse.

References

[1] Cognié Y, Baril G, Poulin N, Mermillod P. Current status of embryo technologies in sheep and goat. Theriogenology 2003;59: 171-188.

[2] Khatun M, Bhuiyan MM, Ahmed JU, Haque A, Rahman MB, Shamsuddin M. *In vitro* maturation and fertilization of prepubertal and pubertal black Bengal goat oocytes. Journal of veterinary science 2011;12: 75-82.

[3] Cognié Y, Poulin N, Locatelli Y, Mermillod P. State-of-the-art production, conservation and transfer of *in-vitro*-produced embryos in small ruminants. Reproduction, fertility, and development 2004;16: 437-445.

[4] Paramio MT. *In vivo* and *in vitro* embryo production in goats. Small Ruminant Research 2010;89: 144-114.

[5] Rodriguez-Dorta N, Cognié Y, Gonzalez F, Poulin N, Guignot F, Touze JL, Baril G, Cabrera F, Alamo D, Batista M, Gracia A, Mermillod P. Effect of coculture with oviduct epithelial cells on viability after transfer of vitrified *in vitro* produced goat embryos. Theriogenology 2007;68: 908-913.

[6] Locatelli Y, Poulin N, Baril G, Touze JL, Fatet A, Beckers JF, Mermillod P. *In vitro* maturation treatment affects developmental competence of laparoscopic ovum pickup-derived oocytes in follicle-stimulating hormone-stimulated goats. Reproduction Fertility And Development 2008;20: 182-183.

[7] Rho GJ, Hahnel A, Betteridge KJ. Comparisons of oocyte maturation times and of three methods of sperm penetration for their effects on the production of goat embryos *in vitro*. Theriogenology 2001;56: 503-516.

[8] Taru Sharma G, Majumdar AC, Bonde SW. Chronology of maturational events in goat oocytes cultured *in vitro*. Small Ruminant Research 1996;22: 25-30.

[9] Souza JMG, Duffard N, Bertoldo MJ, Locatelli Y, Corbin E, Fatet A, Freitas VJF, Mermillod P. Influence of heparin or the presence of *cumulus* cells during

fertilization on the *in vitro* production of goat embryos. Animal Reproduction Science 2013;138: 82-89.

[10] Tan WL, Wan Khadijah WE, Abdullah RB. Effect of sperm insemination duration on *in vitro* fertilisation (IVF) performance on goats. Malaysian Journal of Science 2011;30: 105-111.

[11] Mermillod P, Dalbies-Tran R, Uzbekova S, Thelie A, Traverso JM, Perreau C, Papillier P, Monget P. Factors affecting oocyte quality: who is driving the follicle? Reproduction in domestic animals = Zuchthygiene 2008;43 Suppl 2: 393-400.

[12] Isachenko V, Alabart JL, Dattena M, Nawroth F, Cappai P, Isachenko E, Cocero MJ, Oliveira J, Roche A, Accardo C, Krivokharchenko A, Folch J. New technology for vitrification and field (microscope-free) warming and transfer of small ruminant embryos. Theriogenology 2003;59: 1209-1218.

[13] Guler A, Poulin N, Mermillod P, Terqui M, Cognie Y. Effect of growth factors, EGF and IGF-I, and estradiol on *in vitro* maturation of sheep oocytes. Theriogenology 2000;54: 209-218.

[14] Lonergan P, Rizos D, Kanka J, Nemcova L, Mbaye AM, Kingston M, Wade M, Duffy P, Boland MP. Temporal sensitivity of bovine embryos to culture environment after fertilization and the implications for blastocyst quality. Reproduction 2003;126: 337-346.

[15] Guignot F, Bouttier A, Baril G, Salvetti P, Pignon P, Beckers JF, Touze JL, Cognie J, Traldi AS, Cognie Y, Mermillod P. Improved vitrification method allowing direct transfer of goat embryos. Theriogenology 2006;66: 1004-1011.

[16] Dinnyes A, Carolan C, Lonergan P, Massip A, Mermillod P. Survival of frozen or vitrified bovine blastocysts produced *in vitro* in synthetic oviduct fluid. Theriogenology 1996;46: 1425-1439.

[17] Duby RT, Damiani P, Looney CR, Fissore RA, Rolb JM. Prepubertal calves as oocyte donors: promises and problems. Theriogenology 1996;45: 121-130.

[18] Rodriguez-Gonzalez E, Lopez-Bejar M, Velilla E, Paramio MT. Selection of prepubertal goat oocytes using the brilliant cresyl blue test. Theriogenology 2002;57: 1397-1409.

[19] Shirazi A, Ardali MA, Ahmadi E, Nazari H, Mamuee M, Heidari B. The Effect of Macromolecule Source and Type of Media During *in vitro* Maturation of Sheep Oocytes on Subsequent Embryo Development. Journal of REproduction and Infertility 2012;13: 13-19.

[20] Korhonen K KK, Ketoja E, Matomäki J, Halmekytö M, Peippo J. Effects of serum-free *in vitro* maturation of bovine oocytes on subsequent embryo development and cell allocation in two developmental stages of day 7 blastocysts. Reproduction in Domestic Animals 2010;45: 42-49.

[21] Koeman J, Keefer CL, Baldassarre H, Downey BR. Developmental competence of prepubertal and adult goat oocytes cultured in semi-defined media following laparoscopic recovery. Theriogenology 2003;60: 879-889.

[22] Romaguera R, Casanovas A, Morató R, Izquierdo D, Catalá M, Jimenez-Macedo AR, Mogas T, Paramio MT. Effect of follicle diameter on oocyte apoptosis, embryo development and chromosomal ploidy in prepubertal goats. Theriogenology 2010;74: 364-73.

[23] Hammami S, Morato R, Romaguera R, Roura M, Catala MG, Paramio MT, Mogas T, Izquierdo D. Developmental Competence and Embryo Quality of Small Oocytes from Pre-pubertal Goats Cultured in IVM Medium Supplemented with Low Level of Hormones, Insulin–Transferrin–Selenium and Ascorbic Acid. Reproduction in Domestic Animals 2013;48: 339-344.

[24] Graff KJ, Meintjes M, Han Y, Reggio BC, Denniston RS, Gavin WG, Ziomek C, Godke RA. Comparing follicle stimulating hormone from two commercial sources for oocyte production from out-of-season dairy goats. Journal of Dairy Science 2000;83: 484-487.

[25] Avelar SRG, Moura RR, Sousa FC, Pereira AF, Almeida KC, Melo CHS, Teles-Filho ACA, Baril G, Melo LM, Teixeira DIA, Freitas VJF. Oocyte production and *in vitro* maturation in Canindé goats following hormonal ovarian stimulation. Animal Reproduction 2012;9: 1-7.

[26] Leoni GG, Succu S, Satta V, Paolo M, Bogliolo L, Bebbere D, Spezzigu A, Mardeddu M, Berlinguer F, Ledda S, Naitana S. *In vitro* production and cryotolerance of prepubertal and adult goat blastocysts obtained from oocytes collected by laparoscopic oocyte-pick-up (LOPU) after FSH treatment. Reproduction, Fertility and Development 2009;21: 901-908.

[27] Paffoni A, Brevini TAL, Gandolfi F, Ragni G. Parthenogenetic Activation: Biology and Applications in the ART Laboratory. Placenta 2008;S: 121-125.

[28] Onger EM, Bormann CL, Bulter RE, Melican D, Gavin WG, Echlelard Y, Krisher RL, Behboodi E. Development of goat embryos after *in vitro* fertilization and parthenogenetic activation by different methods. Theriogenology 2001;55: 1933–1945.

[29] Rizos D, Ward F, Duffy P, Boland MP, Lonergan P. Consequences of bovine oocyte maturation, fertilization or early embryo development *in vitro* versus *in vivo*: implications for blastocyst yield and blastocyst quality. Mol Reprod Dev 2002;61: 234-248.

7 CAPÍTULO 2

Influência da heparina e presença das células do *cumulus* durante a fecundação para produção *in vitro* de embriões caprinos

Resumo

Número considerável de pesquisas têm focado na produção *in vitro* de embriões caprinos com o intuito de aumentar sua eficiência. No Experimento 1, o objetivo foi avaliar o efeito das células do *cumulus* comparando oócitos de abatedouro desnudos de propósito (DOP) antes da FIV com COC intactos, e o efeito da heparina durante a FIV. No Experimento 2, oócitos que já se encontravam desnudos na coleta (DOC), DOP e COC intactos foram estudados. Três tratamentos utilizaram oócitos desnudos na coleta: oócitos DOC foram cultivados sozinhos durante a MIV e FIV; DOC e COC foram cultivados juntos durante a MIV e FIV ou os DOC foram maturados sozinhos e depois misturados com COC para FIV. Em outros tratamentos, os COC foram alocados em quatro tratamentos de FIV: COC intactos; COC foram desnudados antes da FIV; COC foram desnudados e as células do *cumulus* adicionadas no mesmo poço; COC foram desnudados e misturados na FIV com COC intactos, gerando dois sub-tratamentos: oócitos desnudos que foram fecundados com COC; e COC que foram fecundados na presença de oócitos desnudos. Após a FIV, todos os presumíveis zigotos foram cultivados por oito dias. No Experimento 1, a produção de blastocistos a partir do número total de oócitos foi maior ($P < 0,05$) para os COC que foram fecundados na presença de heparina (54%) do que em sua ausência (42%) ou oócitos desnudos com ou sem heparina (41%; 38%; respectivamente). No Experimento 2, o potencial de desenvolvimento de oócitos desnudos na coleta foi reduzido (taxas de clivagem e blastocistos calculadas a partir do total de oócitos: 34%; 11%, respectivamente) em

comparação aos COC (77%; 59%, $P < 0,05$). Entretanto, quando quantidades iguais de ambos foram misturadas no começo da MIV, as taxas não foram significativamente diferentes dos COC sozinhos (68%; 45%), contudo quando ambos foram misturados somente para a FIV, as taxas foram inferiores (57%; 40%, $P < 0,05$). Oócitos desnudos cocultivados com células do *cumulus* não diferiram significativamente dos COC intactos (76%; 55%). O efeito da adição de COC durante a FIV aos oócitos desnudos depois da MIV foi similar à adição de células do *cumulus* ao mesmo tipo de oócito. Em conclusão, o uso de heparina e a associação dos oócitos com as células do *cumulus*, tanto em contato direto ou não, durante a MIV e/ou FIV melhorou significativamente a PIV de embriões caprinos. Além disso, alguns oócitos que já foram encontrados desnudos na coleta desenvolvem-se satisfatoriamente até blastocistos quando maturados e fecundados junto com COC intactos.

Palavras-Chave: Blastocisto, Caprino, Células do *Cumulus*, Heparina, PIV, Oócito.

Periódico: Animal Reproduction Science 138:82-89, 2013 (Qualis: A2, Fator de Impacto: 1,897)

Influence of heparin or the presence of *cumulus* cells during fertilization on the *in vitro* production of goat embryos

Joanna Maria Gonçalves de Souza[1,2*], Nicolas Duffard[1,3], Michael J. Bertoldo[1], Yann Locatelli[1,3], Emilie Corbin[1], Alice Fatet[1], Vicente José de Figueiredo Freitas[2], Pascal Mermillod[1]

1 INRA, UMR7247, Physiologie de la Reproduction et des Comportements, INRA, CNRS, Université de Tours, Haras Nationaux. 37380 Nouzilly, France

2 Laboratory of Physiology and Control of Reproduction, Veterinary School, State University of Ceará, Av. Dedé Brasil, 1700, 60740-90, Fortaleza, CE, Brazil

3 Museum National d'Histoire Naturelle, Réserve de la Haute Touche, 36290, Obterre, France

* Corresponding author: Tel.: +33 608 279 759; E-mail address: joannavet@gmail.com (J.M.G. Souza)

ABSTRACT

Considerable research has been focused on *in vitro* production (IVP) of goat embryos to improve its efficiency. In Experiment 1, the effect of the *cumulus* cells by comparing slaughterhouse-oocytes denuded on purpose (DOP) prior to IVF to intact COC, and the effect of heparin during IVF were assessed. In Experiment 2, oocytes that were already denuded at collection (DOC), DOP and intact COC were studied. Three treatments used oocytes denuded at collection: DOC oocytes were cultured alone for both IVM and IVF;

DOC and COC were cultured together for both IVM and IVF or DOC were IVM alone and then mixed with COC for IVF. In other treatments, COC were allocated to four IVF treatments: Intact COC; COC were denuded prior to IVF; COC were denuded and IVF with added *cumulus* cells; COC were denuded and IVF mixed with intact COC giving two sub-treatments: Denuded oocytes that were IVF with COC; and COC that were IVF with denuded oocytes. After fertilization, all presumptive zygotes were cultured for 8 days. In Experiment 1, the yield of blastocysts as a proportion of total oocytes was greater ($P < 0.05$) for COC that were IVF in the presence of heparin (54%) than without heparin (42%) or oocytes already denuded at collection that were IVF with or without heparin (41%; 38%; respectively). In Experiment 2, the developmental potential of oocytes denuded at collection was reduced (cleavage and blastocyst rates calculated from total oocytes: 34%; 11%, respectively) as compared to COC (77%; 59%, $P < 0.05$). However, when equal numbers of both were mixed at the start of IVM, the rates were not significantly different to COC alone (68%; 45%), but when both were mixed equally only for IVF, the rates were reduced (57%; 40%, $P < 0.05$). Denuded oocytes co-cultured with *cumulus* cells were not significantly different to intact COC (76%; 55%). The effect of adding COC during IVF to oocytes denuded after IVM was similar to adding *cumulus* cells to the same type of oocytes. In conclusion, both the use of heparin and the association of oocytes with *cumulus* cells, either detached or in intimate contact, during IVM and/or IVF significantly improve IVP of goat embryos. Furthermore, some oocytes that are already denuded at collection will develop satisfactorily to blastocysts when matured and fertilized with intact COC.

Keywords*:* Blastocyst, Caprine, *Cumulus* cells, Heparin, IVP, Oocyte

1. Introduction

The goat industry has been experiencing a cycle of global growth and is now in need of efficient reproductive biotechnologies in order to sustain worldwide production. In small ruminants, *in vivo* embryo recovery usually requires surgical procedures that impair repeated embryo production from individual donors. *In vitro* embryo production (IVP) has some advantages over *in vivo* recovery such as reliability (Baldassarre and Karatzas, 2004), reproducibility (Stangl et al., 2009), the possibility of collecting oocytes from hormonally stimulated females (Morton et al., 2005), the use of pre-pubertal donors (Baldassarre et al., 2004; Morton, 2008), senile and pregnant females or even in *post-mortem* cases (Baldassarre et al., 2007). Beyond the potential use of IVP in breeding schemes and for patrimonial conservation of genetic diversity, this technique is also required for the establishment of new biotechnologies such as cloning and transgenesis.

Over recent years, considerable research into IVP technology has been undertaken in an attempt to determine which conditions are needed during *in vitro* maturation (IVM), fertilization (IVF) and *in vitro* development (IVD) processes to enhance embryo production. During fertilization, different methods have been described, but specific questions remain to be answered. It has been demonstrated that different supplements such as gonadotropins added to IVM medium may also affect IVF success (Younis et al., 1991), but their benefits alone or in association still need to be clarified. The importance of capacitating agents such as heparin for the success of IVF has been documented in many species. Heparin has been shown to increase fertilization rate in cattle (Parrish et al., 1988) and is thus widely used for sperm capacitation in this species. Similarly, there are some studies demonstrating the role of heparin in goats (Younis et al., 1991; Cox and Alfaro, 2007). However, some reports

indicate an adverse effect on fertilization (Malik et al., 1997) or lesser embryo development and quality (Cognié et al.,1995) making its use questionable for goat species.

The appropriate time of *cumulus* cells removal from oocytes during IVP also remains controversial. In mammals, the effect of *cumulus* cells on fertilization seems to be species dependent. Partial removal of *cumulus* cells before IVF decreases sperm penetration in cattle (Zhang et al., 1995), pigs (Suzuki et al., 2000) and the fertilization rate in humans (Tao Tao et al., 2004). Conversely, in different mouse strains *cumulus* removal did not affect fertilization rates (Vergara et al., 1997). In goats there are reports where oocytes were denuded immediately before IVF (Morton et al., 2005; Katska-Ksiazkiewicz et al., 2007; Rodriguez-Dorta et al., 2007; Freitas and Melo, 2010; Vazquez et al., 2010), just after IVF (Cox and Alfaro, 2007; Shirazi et al., 2010; Romanguera et al., 2011), or even *cumulus* was partially removed before IVF and oocytes where then completely denuded after IVF (Karami Shabankareh et al., 2011). However, without direct comparison of these procedures, it is difficult to precisely analyze the role and benefits of *cumulus* cells during fertilization. Furthermore, it is noteworthy to investigate whether any beneficial effect of *cumulus* cells during fertilization could be mediated by secreted factors, or requires direct contact of these cells with the oocyte (Fatehi et al., 2005).

In general, a great challenge in goat IVP research laboratories throughout the world is the number of goats slaughtered at the same time and consequently the availability of a substantial number of acceptable quality oocytes (Rahman et al., 2007). After retrieval from growing follicles, the oocytes are usually found as *cumulus* oocyte complexes (COC), within varying numbers of *cumulus* cells layers. A grade classification (I, II and III) based on the number of *cumulus* layers and ooplasm

morphology has been proposed (Baldassarre et al., 2003). A similar classification system is currently used by many laboratories, and typically only COC with at least one complete layer of *cumulus* cells are selected for IVM. Oocytes that are found denuded at collection are considered not suitable for IVP and thus routinely discarded. Therefore, if a particular strategy could be applied to the use of denuded oocytes in labor-intensive processes such as ovum pickup from animals of high genetic merit, it would be a benefit to the goat industry.

No previous attempt has been made in goats to analyze the effect of *cumulus* cell presence, (attached or not to the oocyte) during IVF, nor to evaluate the possibility of using oocytes found denuded at collection for IVP. Therefore, the aims of the present study were to examine the i) effect of including heparin in the fertilization medium ii) influence of *cumulus* cells both separately or attached to the oocyte during maturation and/or fertilization, and iii) the potential for using oocytes denuded at collection in the *in vitro* production of goat embryos.

2. Material and methods

Except where otherwise indicated, chemicals were purchased from Sigma Chemical Co. (Saint Louis, MO, USA).

2.1. Experiment 1

2.1.1. Experimental design

The objective of this experiment was to determine the effect of the presence or absence of *cumulus* cells by comparing oocytes denuded on purpose prior to IVF to intact COC, and to determine the effect of the use of heparin in the fertilization medium as well as its possible interactions with the presence of *cumulus* cells. Consequently,

this experiment was a factorial arrangement of two types of oocytes (denuded on purpose x COC) x two IVF media (supplemented with heparin or not) x six replicates.

2.1.2. Aspiration of oocytes

During the breeding season (September and October) ovaries from goats, regardless the stage of estrous cycle, were collected from a local slaughterhouse and transported to the laboratory (Latitude: 46'N) in a thermos box containing saline solution at 30 °C within 3 h after collection. Ovaries were washed in pre-warmed fresh saline (30 °C), and oocytes were aspirated through a 19 gauge short needle from all visible follicles between 2 and 5 mm in diameter into a Falcon tube under controlled vacuum (30 mm Hg). The collection tube was previously filled with 3 to 5 mL of HEPES buffered tissue culture medium 199 (TCM 199) supplemented with 10 IU/mL heparin (Choay, Glaxo Wellcome Production, Notre Dame de Bondeville, France) and 40 µg/mL gentamycin.

2.1.3. IVM of oocytes

Cumulus oocyte complexes were isolated under a stereo zoom microscope (Nikon Corporation, Japan) and graded as good, fair or poor. Only good and fair oocytes, i.e., surrounded by at least one complete layer of unexpanded *cumulus* cells were considered acceptable and used in IVM (Guler et al., 2000). A total of 1447 COC were used. The COC were washed four times and transferred to maturation medium consisting of TCM 199 supplemented with 10 ng/mL epidermal growth factor (EGF) and 100 µM cysteamine in four well petri dishes (Nunc, Roskilde, Denmark) with each well containing 45 to 50 oocytes in 500 µL of maturation medium. COC were incubated for 22 h at 38.8 °C in a humidified atmosphere of 5% CO_2 in air (Cognié et al., 2003).

2.1.4. Sperm preparation and IVF of oocytes

A batch of semen pooled from two bucks was used throughout experiments. Motile sperm from frozen/thawed semen were separated by centrifugation (15 min at 700 *g*) on 2 mL of Percoll (Pharmacia, Uppsala, Sweden) discontinuous density gradient (45/90%). Viable sperm were diluted in the appropriate volume of fertilization medium and 75 µL were added to each fertilization well, to achieve a final concentration of 1.5 x 10^6 spermatozoa/mL (Day of *in vitro* fertilization = Day 0).

At the end of IVM, half of the oocytes were placed into 15 mL Falcon tubes containing 2 mL of SOF medium (synthetic oviduct fluid) supplemented with Hepes (24 µg/mL) and BSA (2 µL/mL), and vortexed for 2 min (medium speed) to remove *cumulus* oophorus before IVF. These denuded oocytes (denuded on purpose, DOP) were recovered in 35 mm petri plates and then transferred to plates containing washing medium. The remaining COC (*cumulus* oocyte complex group; COC) were also transferred to similar plates and washed. The washing medium used was SOF medium (pH = 7.3, 280 mOsm), which contained 40 µg/mL gentamycin and 10% of heat-inactivated estrus sheep serum. The COC and the DOP were randomly divided between two fertilization conditions in IVF medium supplemented or not with 5 µg/mL heparin (Calbiochem 375 095). Groups of 45 to 50 oocytes were transferred into four well petri dishes, containing 425 µL of fertilization medium and 75 µL of sperm suspension were added to each well. Sperm and oocytes were co-incubated for 20 h at 38.8 °C in a humidified atmosphere of 5% CO_2 in air (Cognié et al., 2003).

2.1.5. IVD of embryos

After fertilization, the COC fertilized with or without heparin were denuded by vortex. The presumptive zygotes from the four groups were washed four times in

culture medium (SOF supplemented with 3 mg/mL BSA) to remove spermatozoa and transferred to four well petri dishes containing 25 µL drops of culture medium covered with 700 µL of mineral oil. The presumptive zygotes were incubated for seven days at 38.8 °C in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2. After 48 h post-insemination, 10% fetal calf serum (FCS) was added to the culture droplets.

2.2. Experiment 2

2.2.1. Experimental design

The experimental design is shown in Fig. 1. The experiment used oocytes that were already denuded at collection (DOC), oocytes denuded on purpose (DOP) and *cumulus*-oocyte complexes (COC). Different treatments used the various types of oocytes either separately or in combination only during the IVM (M) or in both the IVM and IVF (F) phases of culture. In one treatment the oocytes were IVF with separate *cumulus* cells (CC). Three treatments used oocytes denuded at collection: 1. DOC oocytes were cultured alone for both IVM and IVF 2. DOC and COC were cultured together (25 + 25 per well) for both IVM and IVF; 3. DOC were IVM alone and then mixed with COC (25 + 25 per well) for IVF. The other treatments used COC subjected to standard IVM and then allocated to four IVF treatments; 4. Intact COC; 5. COC were denuded prior to IVF; 6. COC were denuded and IVF with added *cumulus* cells (CC); 7. COC were denuded and IVF mixed with intact COC (25 + 25) giving two sub-treatments: 7a. Denuded oocytes that were IVF with COC; and 7b. COC that were IVF with denuded oocytes. Thus, there were eight IVF treatments each replicated five times with 45 to 50 oocytes per group per replicate (culture well).

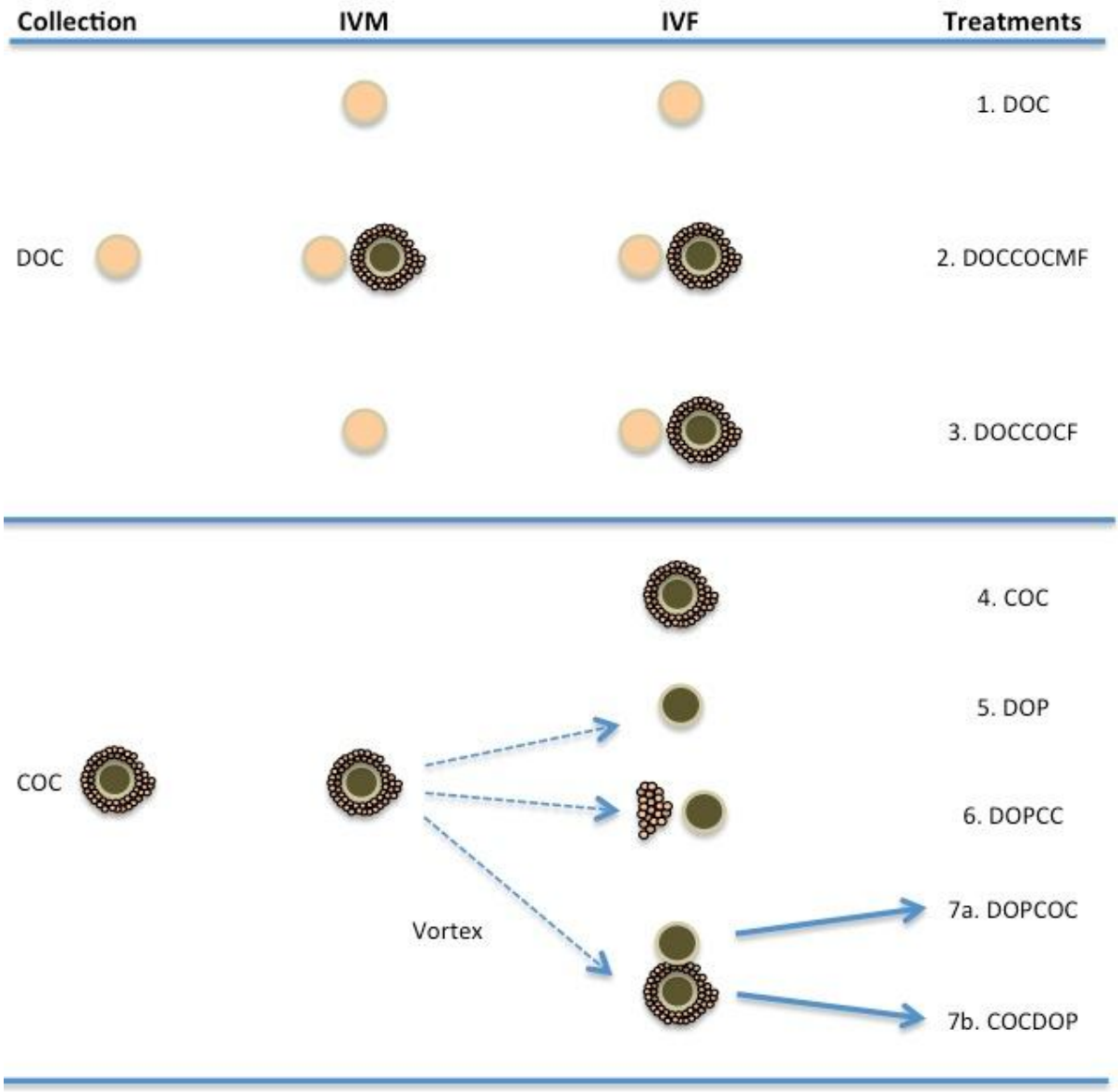

Fig. 1. The effect of different types of oocytes and their culture on the *in vitro* development of goat embryos. Diagram illustrating the treatments used in experiment 2. Oocytes were already denuded when collected (DOC) or *cumulus*-oocyte complexes (COC) were intact. DOC oocytes were 1. IVM and IVF alone (DOC), 2. IVM and IVF with an equal number (25 + 25) of COC (DOCCOCMF). 3. IVM alone then IVF with an equal number (25 + 25) of COC (DOCCOCF). All COC intact at collection remained intact for IVM and then were: 4. IVF as intact COC (COC), 5. COC were denuded prior to IVF (DOP), 6. COC denuded after IVM and then IVF in the presence of *cumulus* cells (DOPCC), 7. COC were denuded after IVM and then IVF in the presence of an

equal number (25 + 25) of COC giving two sub-treatments 7a. DOP that were IVF in the presence of COC (DOPCOC) and 7b. COC that were IVF in the presence of DOP (COCDOP). COC and DOP oocytes from treatments 7a and 7b were cultured separately following IVF.

2.2.2. Aspiration of oocytes

In the subsequent months, November and December, the same procedure was conducted to recover the oocytes. However, for Experiment 2, all oocytes with a homogenous ooplasm were used, even the ones totally denuded at collection, i.e., quality III (poor) oocytes but with a homogeneous ooplasm. A total of 1697 oocytes were used (1316 COC and 381 already found denuded at collection).

2.2.3. IVM of oocytes

The COC were washed four times, pooled and then placed, with each well containing 45 to 50 oocytes in 500 µL of maturation medium. The denuded group was submitted to the same process described for COC. A mixed group was formed at this time with half COC ($n = 25$) and half oocytes denuded at collection ($n = 25$). The maturation medium used was EGF + CYST for all conditions.

2.2.4. IVF of oocytes

Semen preparation and the fertilization medium were the same as in Experiment 1, but heparin was added in all treatments (5 µg/mL). Before IVF, all COC were pooled, then allocated at random to treatments and the mechanical manipulation appropriate to the treatment performed prior to IVF. Some of the matured COC were denuded on

purpose by vortexing. These denuded oocytes were fertilized alone, or with COC (25/25), or with separate *cumulus* cells. The COC and oocytes denuded on purpose that were fertilized together were subsequently cultured separately with one culture well containing 25 oocytes denuded on purpose and another with 25 COC. The oocytes that were already denuded at collection either remained alone for both IVM and IVF or were cultured with COC (25/25) for both IVM and IVF, or only for IVF.

Where *cumulus* cells were added to oocytes denuded on purpose after IVM (treatment DOPCC), the medium remaining after vortexing was centrifuged at 350 x *g* for 10 min and the supernatant removed. The pellet of *cumulus* cells was suspended in 100 µL of IVF medium and was transferred into the IVF well that contained the oocytes in 325 µL of IVF medium thus making the total volume of medium 425 µL as for the other treatments. Fertilization followed the same protocol as that used in Experiment 1.

2.2.5. IVD of presumable zygotes

After fertilization, the groups that were already denuded were transferred to the washing plate and received gentle pipetting in order to remove spermatozoa. The other groups were vortexed separately to remove *cumulus* cells, and transferred to the washing plates.

2.3. Assessment of embryo development and statistical analysis

Embryos were examined morphologically and the efficiency of development was evaluated (i) as the percentage of cleaved embryos 2 days after fertilization, and the percentage of blastocysts on Day 8 expressed (ii) on the basis of the number of oocytes entering into IVM (iii) or on the basis of the number of cleaved embryos at Day 2. Data

were collected over six (Experiment 1) and five (Experiment 2) replicates. After the use of Kolmogorof-Smirnov test for Gaussian distribution of the data, an arcsine transformation was applied to normalize the data, when necessary. However, means and S.E.M. are presented untransformed. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison posttests were performed. A value of $P < 0.05$ was considered to be statistically significant. Statistical analyses were performed using Prism 5.0a software.

3. Results

3.1. Experiment 1

When the data for oocytes fertilized (IVF) in the presence or absence of heparin were combined, the cleavage rate was similar for COC (69 ± 2.5%) and oocytes denuded on purpose (66 ± 2.2%) but the COC developed a greater proportion of blastocysts than denuded whether this was expressed as a percentage of cleaved oocytes (70 ± 3.0% compared with 60 ± 2.8%; $P < 0.05$) or total oocytes (48 ± 2.3% compared with 39 ± 2.1%; $P < 0.05$). The overall effect of including heparin in the IVF medium compared to no heparin was to increase cleavage rate (70 ± 2.2% compared with 65 ± 2.4%; $P < 0.05$), and the proportions of blastocysts formed from cleaved oocytes (68 ± 2.9% compared with 61 ± 2.9%; $P < 0.05$) or total oocytes (48 ± 2.1% compared with 40 ± 2.1%; $P < 0.01$). The results for the four treatments are presented in Table 1. COC that were IVF in medium supplemented with heparin had the greatest cleavage rate (72 ± 2.8%; NS), and the percentage of blastocysts produced from cleaved oocytes (75 ± 3.5%; NS) and total oocytes (54 ± 2.2%; $P < 0.05$).

Table 1

Effect of culturing goat oocytes during IVF as intact *cumulus*-oocyte complexes (COC) or denuding them prior to IVF (DOP), and the absence (-) or presence (+) of heparin in the IVF medium on the *in vitro* cleavage at 2 days post insemination and development at 8 days post insemination of goat embryos (Mean ± S.E.M.)

Conditions	Heparin	Oocytes (n)	Cleavage (%)	Blastocyst production (%) from	
				cleaved oocytes	total oocytes
COC	-	365	66 ± 4.1 [a]	63 ± 4.1 [ab]	42 ± 2.9 [a]
	+	364	72 ± 2.8 [a]	75 ± 3.5 [a]	54 ± 2.2 [b]
DOP	-	360	64 ± 2.5 [a]	59 ± 4.4 [b]	38 ± 3.0 [a]
	+	358	68 ± 3.5 [a]	61 ± 3.7 [ab]	41 ± 2.7 [a]

Within a column, values with different superscripts differ ($P < 0.05$)

3.2. Experiment 2

Cleavage and blastocyst development rates are shown in Table 2. Overall, cleavage rate was greater ($P < 0.01$) for the five treatments with intact COC during IVM (75 ± 1.8%) than for the three treatments using oocytes denuded at collection (DOC 54 ± 3.9%). Similarly, these groups of treatments differed ($P < 0.01$) in blastocyst development both as a proportion of cleaved (74 ± 1.8% compared with 57 ± 5.0%) or initial (53 ± 2.0% compared with 33 ± 4.2%) number of oocytes. Oocytes denuded at collection (treatment DOC) had a lesser ($P < 0.05$) cleavage (34 ± 1.5%) and blastocyst production (11 ± 1.9% of total oocytes) rates than all other treatments. The control treatment (intact COC), however, had the greatest cleavage and blastocyst production rates (Table 2).

Table 2

Cleavage rate and *in vitro* development at 8 days post insemination of goat embryos matured and fertilized in different conditions (Experiment 2)

Treatments [a]	Oocytes (n)	Cleavage (%)	Blastocyst production (%) from	
			cleaved oocytes	total oocytes
1. DOC	121	34 ± 1.5 [c]	32 ± 4.9 [b]	11 ± 1.9 [c]
2. DOCCOCMF	245	68 ± 1.4 [a,b]	67 ± 4.5 [a]	45 ± 3.6 [a,b]
3. DOCCOCF	275	57 ± 2.3 [b]	69 ± 2.7 [a]	40 ± 1.2 [b]
4. COC	289	77 ± 3.5 [a]	77 ± 3.1 [a]	59 ± 4.4 [a]
5. DOP	230	70 ± 4.3 [a,b]	68 ± 3.0 [a]	47 ± 3.9 [a,b]
6. DOPCC	227	76 ± 4.7 [a]	72 ± 3.1 [a]	55 ± 4.3 [a]
7a. DOPCOC	147	69 ± 2.8 [a,b]	76 ± 6.4 [a]	52 ± 5.0 [a]
7b. COCDOP	163	80 ± 3.0 [a]	74 ± 4.0 [a]	59 ± 3.5 [a]

Within a column, values with different superscripts differ ($P < 0.05$)

[a] Oocytes were already denuded when collected (DOC) or *cumulus*-oocyte complexes (COC) were intact. DOC oocytes were 1. IVM and IVF alone (DOC), 2. IVM and IVF with an equal number (25 + 25) of COC (DOCCOCMF). 3. IVM alone then IVF with an equal number (25 + 25) of COC (DOCCOCF). All COC intact at collection remained intact for IVM and then were: 4. IVF as intact COC (COC), 5. COC were denuded prior to IVF (DOP), 6. COC denuded after IVM and then IVF in the presence of *cumulus* cells (DOPCC), 7. COC were denuded after IVM and then IVF in the presence of an equal number (25 + 25) of COC giving two sub-treatments 7a. DOP that were IVF in the presence of COC (DOPCOC) and 7b. COC that were IVF in the presence of DOP (COCDOP). COC and DOP oocytes from treatments 7a and 7b were cultured separately following IVF (Mean ± S.E.M.)

When oocytes denuded at collection were IVM alone but mixed with equal numbers of intact COC during IVF (treatment DOCCOCF), the overall cleavage rate (57 ± 2.3%) was greater than DOC alone and less than COC alone (77 ± 3.5%; $P < 0.05$) being mid-way between the other two values. The cleavage rate of DOC oocytes mixed with COC during both IVM and IVF (68 ± 1.4%), however, was more similar to that of the control treatment COC (NS). Blastocyst production rate from cleaved oocytes for both these mixed treatments were similar and less (NS) than for COC. The proportion of blastocysts as a percentage of the initial number of oocytes was less for those only IVF with COC than for control (40 ± 1.2% compared with 59 ± 4.4%; $P < 0.05$) but not different than for oocytes both IVM and IVF with COC (45 ± 3.6%).

Oocytes that were intact during IVM but denuded on purpose (DOP) prior to IVF had a cleavage rate of 70 ± 4.3%, and blastocyst production rates of 68 ± 3.0% of cleaved and 47 ± 3.9% of total oocytes. All these values were less (NS) than for the control treatment COC but greater ($P < 0.05$) than for oocytes denuded at collection (DOC). In the treatment where *cumulus* cells (CC) were added during IVF to oocytes denuded after IVM, the cleavage (76 ± 4.7%) and overall blastocyst production (55 ± 4.3%) rates were greater than for oocytes denuded at collection (DOC; NS) and slightly less than those of the control treatment COC (NS). The effect of adding COC during IVF to oocytes denuded after IVM was similar to adding CC to the same type of oocytes (Table 2). In both cases, the blastocyst production rate was greater than for oocytes denuded during IVF but the differences were not significant. COC that were IVF in the presence of denuded oocytes had similar cleavage and blastocyst production rates to the control treatment (COC).

4. Discussion

The use of heparin as a capacitating agent can help embryo development. The hypothesis for the present experiments was that the presence of *cumulus* cells during fertilization could improve fertilization rate and help the oocyte during early post fertilization events. For the first time it is reported in goats that using oocytes with *cumulus* cells during IVF improved developmental potential, as compared to oocytes denuded before.

Greater numbers of blastocysts were obtained from COC fertilized in the presence of heparin than those for COC fertilized without heparin, suggesting that the addition of heparin to the fertilization medium improves sperm capacitation of frozen-thawed goat sperm. These results support those of previous studies demonstrating the role of heparin in goat IVP (Younis et al., 1991; Cox and Alfaro, 2007). Conversely, an adverse effect on fertilization (Malik et al., 1997) or a lesser viability of embryos after transfer (Cognié et al., 1995) was also reported when using this capacitating agent. Poulin et al. (1996) attributed the limited success of IVM/IVF technique in producing live goat offspring to date to the use of heparin for buck sperm. It is noteworthy to describe that in the current study, no positive effect for heparin was observed when oocytes were denuded (DOP) before IVF and that was the procedure used earlier by our group for previous studies (Poulin et al., 1996). Results of the present study showed that the effects of the presence of *cumulus* cells and the inclusion of heparin in the IVF medium were additive and resulted in improved development of oocytes to blastocysts. The fertilization rate was rapidly improved by adding heparin to the IVF medium at values that varied between 2.5 and 10 μg/mL, depending on the male (Cox et al., 1995). In the present study, a similar concentration was used (5 μg/mL) and the mean cleavage rate was 75% in the COC + heparin groups in both experiments, 21% greater than

previously described in goats (Katska-Ksiazkiewicz et al., 2004). However, a much greater concentration of heparin (50 µg/mL) was used, that may have negatively influenced the results in this previous study.

Information available on the role of *cumulus* oophorus during fertilization and the effect of these cells on subsequent embryo development in goat is currently limited. The appropriate time of *cumulus* cell removal from oocytes during IVP also remains controversial. We have previously reported that the *cumulus* oophorus was routinely removed before IVF (Cognié et al., 2004; Rodriguez-Dorta et al., 2007). However as demonstrated in the present study, the presence of *cumulus* cells and heparin during fertilization allowed better oocyte development than when oocytes were denuded before IVF, even though the cleavage rate was not affected. In a previous study, employing a similar methodology in oocytes of cattle, removal of *cumulus* cells from COC before IVF decreased the cleavage rate compared with intact COC. As in the present study, on the basis of the total number of oocytes at the onset of culture, the percentage of blastocysts that were formed at Day 9 was less when denuded oocytes were fertilized compared with oocytes fertilized within COC (Fatehi et al., 2005). It is possible that the presence of *cumulus* cells during fertilization could help the oocyte during early post fertilization events, such as sperm nucleus remodeling, and, in turn, affect developmental competence. *Cumulus* cells maintain the oocyte under meiotic arrest preventing oocyte ageing (Eppig, 1989), participate in the induction of meiosis by transducing the LH signal to the oocyte (Mattioli and Barboni, 2000), are important for efficient cytoplasmatic maturation of the oocyte (Mori et al., 2000) and protect oocytes against oxidative stress during oocyte maturation (Tatemoto et al., 2000). *Cumulus* cells also participate to the maintenance of greater concentrations of reduced glutathione in oocyte. This glutathione is necessary for correct processing of sperm chromatin

configuration changes after IVF (de Matos et al., 2002). All these positive effects of *cumulus* cells are probably responsible for the greater blastocyst rates achieved by the oocytes denuded on purpose in comparison to the oocytes already denuded at collection, because the latter underwent IVM without *cumulus* cells support.

After it was established in the present study, the importance of *cumulus* cells, it was ascertained whether the possible beneficial effect of *cumulus* cells during fertilization could result from factors secreted by the cells into the media or if the maintenance of gap junction communication between *cumulus* cells and oocytes was important for optimal fertilization. The understanding of the nature and diversity of compounds that transfer between the *cumulus* cells and the oocyte via gap junctions is limited (Gilchrist and Thompson, 2007). It is reasonable to assume that a positive effect occurs when denuded oocytes undergo fertilization with *cumulus* cells added in the same IVF well, because the group of oocytes denuded on purpose cultured with *cumulus* cells has similar cleavage and blastocyst rates to intact COC when compared to oocytes denuded on purpose. These findings were contrary to those previously reported in cattle, when denuded oocytes fertilized together with *cumulus* cells exhibited a greater cleavage rate, but the blastocyst rate was not different from that of denuded oocytes fertilized in control medium without *cumulus* cells support (Fatehi et al., 2005).

In general, a great challenge in research aimed at improving IVP in goats is the lack of adequate numbers of ovaries to obtain good quality oocytes (Rahman et al., 2007). Oocytes that are found denuded at collection are usually considered not suitable for IVM and thus discarded. In cases of recovering oocytes from live and valuable does by ovum pick up, a labor-intensive process, denuded oocytes that are often discarded could represent a loss of valuable genetic material. Therefore, an experiment was designed to evaluate the possibility of using these oocytes to produce some additional

blastocysts from a given female. Blastocyst rate from the COC group reached 59%, whereas only 11% of oocytes found denuded at collection reached the blastocyst stage. Hence, "mathematically" it can be projected that if 50% of each type of oocytes are used an average blastocyst rate of 35% could be obtained if the two groups were mixed equally. However, 45% of oocytes reached the blastocyst stage when COC and oocytes denuded at the collection underwent IVM and IVF together and 40% when COC and denuded oocytes at the collection were matured separately and mixed equally in IVF. Furthermore, when oocytes were matured separately a lesser development rate compared to intact COC was occurred. This indicates that co-culturing denuded oocytes with COC for maturation purposes could be an important tool to improve the quality of matured oocytes and the ability to develop to blastocyst after IVF. However, further research is still necessary to affirm that is possible to rescue the developmental competence of denuded oocytes when using similar methodologies and which steps are determinant and what are the mechanisms involved. A preliminary success was reported for producing goat embryos with ICSI using oocytes of heterogeneous and lesser quality (Rahman et al., 2007). During retrieval of oocytes for IVM, it may be reasonable to propose a new category for grading oocytes because the denuded oocytes with a homogeneous ooplasm may be mixed to COC from IVM, to produce some additional blastocysts.

5. Conclusions

It is concluded that i) the inclusion of heparin in the fertilization medium improves the *in vitro* production of goat embryos ii) the association of oocytes with *cumulus* cells, either separately or in intimate contact, during maturation and/or fertilization improves *in vitro* production of goat embryos iii) some oocytes that are

already denuded at collection will develop satisfactorily to blastocysts when matured and fertilized in the presence of intact *cumulus*-oocyte complexes. The present study has demonstrated a substantive advance in goat IVF and high rates of blastocyst were achieved depending on the treatment.

Acknowledgments

J.M.G. Souza was supported by CAPES and Ceará State University. The authors wish to thank CAPES-COFECUB bilateral framework for financial support of collaboration between the Ceará State University and INRA on goat IVP (Grant 728/11, 2011-2013). This work was supported by a grant from Région Centre, France (PIVER program, #200800030493, 2008-2011). The authors thank Thierry Delpuech, Bruno Mirey and Jean-Noël Couet for ovary collection at slaughterhouse.

References

Baldassarre, H., Karatzas, C.N., 2004. Advanced assisted reproduction technologies (ART) in goats. Anim. Reprod. Sci. 82-83, 255-266.

Baldassarre, H., Rao, K.M., Neveu, N., Brochu, E., Begin, I., Behboodi, E., Hockley, D.K., 2007. Laparoscopic ovum pick-up followed by *in vitro* embryo production for the reproductive rescue of aged goats of high genetic value. Reprod. Fertil. Dev. 19, 612-616.

Baldassarre, H., Wang, B., Kafidi, N., Gauthier, M., Neveu, N., Lapointe, J., Sneek, L., Leduc, M., Duguay, F., Zhou, J.F., Lazaris, A., Karatzas, C.N., 2003. Production of transgenic goats by pronuclear microinjection of in vitro produced zygotes derived from oocytes receovered by laparoscopy. Theriogenology 59, 831-839.

Baldassarre, H., Wang, B., Pierson, J., Neveu, N., Sneek, L., Lapointe, J., Cote, F., Kafidi, N., Keefer, C.L., Lazaris, A., Karatzas, C.N., 2004. Prepubertal propagation of transgenic cloned goats by laparoscopic ovum pick-up and in vitro embryo production. Clon. Stem Cell. 6, 25-29.

Cognié, Y., Baril, G., Poulin, N., Mermillod, P., 2003. Current status of embryo technologies in sheep and goat. Theriogenology 59, 171-188.

Cognié, Y., Poulin, N., Locatelli, Y., Mermillod, P., 2004. State-of-the-art production, conservation and transfer of *in-vitro*-produced embryos in small ruminants. Reprod. Fertil. Dev. 16, 437-445.

Cognié, Y., Poulin, N., Pignon, P., Sulon, J., Beckers, J.F., Guerin, Y., 1995. Does heparin affect developmental ability of IVP goat oocytes?, 11th, AETE, Hannover, Germany, p. 146.

Cox, J.F., Alfaro, V., 2007. *In vitro* fertilization and development of OPU derived goat and sheep oocytes. Reprod. Domest. Anim. 42, 83-87.

Cox, J.F., Saravia, F., Briones, M., Santa María, A., 1995. Dose-dependent effect of heparin on fertilizing ability of goat spermatozoa. Theriogenology 44, 451-460.

de Matos, D.G., Gasparrini, B., Pasqualini, S.R., Thompson, J.G., 2002. Effect of glutathione synthesis stimulation during in vitro maturation of ovine oocytes on embryo development and intracellular peroxide content. Theriogenology 57, 1443-1451.

Eppig, J.J., 1989. The participation of cyclic adenosine monophosphate (cAMP) in the regulation of meiotic maturation of oocytes in the laboratory mouse. J. Reprod. Fertil. 38, 3-8.

Fatehi, A.N., Roelen, B.A.J., Colenbrander, B., Schoevers, E.J., Gadella, B.M., Bevers, M.M., van den Hurk, R., 2005. Presence of *cumulus* cells during in vitro fertilization protects the bovine oocyte against oxidative stress and improves first cleavage but does not affect further development. Zygote 13, 177-185.

Freitas, V.J.F., Melo, L.M., 2010. *In vitro* embryo production in small ruminants. Rev. Bras. Zootec. 39, 409-413.

Gilchrist, R.B., Thompson, J.G., 2007. Oocyte maturation: Emerging concepts and tecnhnologies to improve developmental potential in vitro. Theriogenology 67, 6-15.

Guler, A., Poulin, N., Mermillod, P., Terqui, M., Cognié, Y., 2000. Effect of growth factors, EGF and IGF-I, and estradiol on in vitro maturation of sheep oocytes. Theriogenology 54, 209-218.

Karami Shabankareh, H., Sarsaifi, K., Mehrannia, T., 2011. *In vitro* maturation of ovine oocytes using different maturation media: effect of human menopausal serum. J. Assist. Reprod. Genet. 28, 531-537.

Katska-Ksiazkiewicz, L., Opiela, J., Rynska, B., 2007. Effects of oocyte quality, semen donor and embryo co-culture system on the efficiency of blastocyst production in goats. Theriogenology 68, 736-744.

Katska-Ksiazkiewicz, L., Rynska, B., Gajda, B., Smorag, Z., 2004. Effect of donor stimulation, frozen semen and heparin treatment on the efficiency of in vitro embryo production in goats. Theriogenology 62, 576-586.

Malik, R.K., Lohan, I.S., Dhanda, O.P., Tuli, R.K., 1997. Test for the acrosomal reaction of goat spermatozoa treated with heparin. Small Rumin. Res. 26, 163-166.

Mattioli, M., Barboni, B., 2000. Signal transduction mechanism for LH in the *cumulus*-oocyte complex. Mol. Cell. Endocrinol. 161, 19-23.

Mori, T., Amano, T., Shimizu, H., 2000. Roles of Gap Junctional Communication of *Cumulus* Cells in Cytoplasmic Maturation of Porcine Oocytes Cultured *In Vitro*. Biol. Reprod. 62, 913-919.

Morton, K.M., 2008. Developmental capabilities of embryos produced in vitro from prepubertal lamb oocytes. Reprod. Domest. Anim. 43, 137-143.

Morton, K.M., de Graaf, S.P., Campbell, A., Tomkins, L.M., Maxwell, W.M., Evans, G., 2005. Repeat ovum pick-up and in vitro embryo production from adult ewes with and without FSH treatment. Reprod. Domest. Anim. 40, 422-428.

Parrish, J.J., Susko-Parrish, J., Winer, M.A., First, N.L., 1988. Capacitation of bovine sperm by heparin. Biol. Reprod. 38, 1171-1180.

Poulin, N., Guler, A., Pignon, P., Cognié, Y., 1996. *In vitro* production of goat embryos: heparin in IVF medium affect developmental ability. 6th Int. Conf. on Goats, Pekin, 2: 838–40.

Rahman, A.N.M.A., Abdullah, R.B., Embong, W.K.W., 2007. Goat Embryo Development from in vitro Matured Oocytes of Heterogeneous Quality Through Intracytoplasmic Sperm Injection Technique. Biotechnology 6, 373-382.

Rodriguez-Dorta, N., Cognié, Y., Gonzalez, F., Poulin, N., Guignot, F., Touze, J.L., Baril, G., Cabrera, F., Alamo, D., Batista, M., Gracia, A., Mermillod, P., 2007. Effect of coculture with oviduct epithelial cells on viability after transfer of vitrified in vitro produced goat embryos. Theriogenology 68, 908-913.

Romaguera, R., Moll, X., Morato, R., Roura, M., Palomo, M.J., Catala, M.G., Jimenez-Macedo, A.R., Hammami, S., Izquierdo, D., Mogas, T., Paramio, M.T., 2011.

Prepubertal goat oocytes from large follicles result in similar blastocyst production and embryo ploidy than those from adult goats. Theriogenology 76, 1-11.

Shirazi, A., Shams-Esfandabadi, N., Ahmadi, E., Heidari, B., 2010. Effects of Growth Hormone on Nuclear Maturation of Ovine Oocytes and Subsequent Embryo Development. Reprod. Domest. Anim. 45, 530-536.

Stangl, M., Kuhholzer, B., Besenfelder, U., Brem, G., 1999. Repeated endoscopic ovum pick-up in sheep. Theriogenology 52, 709-716.

Suzuki, K., Eriksson, B., Shimizu, H., Nagai, T., Rodriguez-Martinez, H., 2000. Effect of hyaluronan on monospermic penetration of porcine oocytes fertilized *in vitro*. Int. J. Androl. 23, 13-21.

Tao Tao, A.R., Nadeau, S., Savoie, R., Gallant, B., Ouellette, R., 2004. Effect of *cumulus* cell removal on the fertilization and the day 3 embryo quality in human IVF. Int. Congr. 1271, 135-138.

Tatemoto, H., Sakurai, N., Muto, N., 2000. Protection of porcine oocytes against apoptotic cell death caused by oxidative stress during *in vitro* maturation: role of *cumulus* cells. Biol. Reprod. 63, 805-810.

Vazquez, M.I., Abecia, J.A., Forcada, F., Casao, A., 2010. Effects of exogenous melatonin on in vivo embryo viability and oocyte competence of undernourished ewes after weaning during the seasonal anestrus. Theriogenology 74, 618-626.

Vergara, G.J., Irwin, M.H., Moffatt, R.J., Pinkert, C.A., 1997. In vitro fertilization in mice: Strain differences in response to superovulation protocols and effect of *cumulus* cell removal. Theriogenology 47, 1245-1252.

Younis, A.I., Zuelke, K.A., Harper, K.M., Oliveira, M.A., Brackett, B.G., 1991. In vitro fertilization of goat oocytes. Biol. Reprod. 44, 1177-1182.

Zhang, L., Jiang, S., Wozniak, P.J., Yang, X., Godke, R.A., 1995. *Cumulus* cell function during bovine oocyte maturation, fertilization, and embryo development *in vitro*. Mol. Reprod. Dev. 40, 338-344.

8 CAPÍTULO 3

Avaliação dos parâmetros reprodutivos, colheita de oócitos por laparoscopia e os primeiros embriões produzidos *in vitro* em cabras da raça Canindé ameaçadas de extinção (*Capra hircus*)

Resumo

Canindé é uma raça caprina (*Capra hircus*) atualmente considerada em risco de extinção. Os objetivos deste estudo foram: (i) caracterizar o comportamento de estro, dinâmica ovulatória e perfil de progesterona além de (ii) avaliar a produção *in vitro* (PIV) de embriões na raça. No Experimento 1, fêmeas nulíparas (n = 10) ou pluríparas (n = 7) receberam tratamento de sincronização e seus parâmetros reprodutivos foram avaliados. No Experimento 2, oócitos foram obtidos por laparoscopia de fêmeas estimuladas hormonalmente (n = 15) e utilizadas para PIV de embriões. Os resultados são descritos como média ± EP. Não houve diferença (P > 0,05) entre nulíparas ou pluríparas para: resposta ao estro (40,0% *vs.* 85,7%), intervalo para o estro (62,0 ± 15,5 *vs.* 50,7 ± 19,2 h), duração do estro (25,0 ± 16,1 *vs.* 30,0 ± 15,1 h), porcentagem de cabras ovulando (60,0% *vs.* 85,7%), número de ovulações (1,2 ± 0,4 *vs.* 1,3 ± 0,8) e diâmetro do maior folículo (5,8 ± 0,5 *vs.* 6,1 ± 0,3 mm). Concentrações de progesterona foram similares (P > 0,05) entre ambas as categorias. Durante a recuperação laparoscópica, 12,2 foliculos aspirados e 9,1 oócitos foram registrados por cabra, resultando em altas taxas de recuperação (74,3%, 182/245). Um total de 78 embriões foram produzidos, obtendo-se satisfatória taxa de blastocistos (51,0%). O número médio de blastômeros contado em blastocistos expandidos no dia 7 do cultivo *in vitro*

foi de 170,3 ± 12,5. Em conclusão, cabras Canindé nulíparas e pluríparas demonstraram parâmetros reprodutivos similares. Pela primeira vez foi possível produzir embriões *in vitro* da raça Canindé, permitindo iniciar a formação de um banco de embriões, útil para a preservação da raça.

Palavras-Chave: Estro; Ovulação; Recuperação oocitária; PIV; Extinção.

Periódico: Reproductive Biology, aceito com correções (Qualis: B1, Fator de Impacto: 1,921).

Assessment of the reproductive parameters, laparoscopic oocyte recovery and the first embryos produced in vitro from endangered Canindé goats (*Capra hircus*)

Joanna Maria Gonçalves de Souza-Fabjan[a,c], Alexsandra Fernandes Pereira[a], Carlos Henrique Sousa de Melo[a], Deisy Johana Diaz Sanchez[a], Eunice Oba[b], Pascal Mermillod[c], Luciana Magalhães Melo[a], Dárcio Italo Alves Teixeira[a], Vicente José de Figueirêdo Freitas[a*]

[a] *Laboratory of Physiology and Control of Reproduction, School of Veterinary Medicine, Ceará State University, Av. Dedé Brasil 1700, Fortaleza, CE, 60714-903, Brazil*

[b] *Department of Animal Reproduction and Veterinary Radiology, São Paulo State University, Distrito Rubião Jr s/n, Botucatu, SP,18618000, Brazil*

[c] *INRA, UMR7247, Physiologie de la Reproduction et des Comportements, Université de Tours, Haras Nationaux, Nouzilly, 37380, France*

* Corresponding author: Vicente José de F. Freitas

Laboratory of Physiology and Control of Reproduction

School of Veterinary Medicine, Ceará State University

Av. Dedé Brasil, 1700, Fortaleza, CE, 60740-903, Brazil

Phone: +55 85 3101-9861

Fax: +55 85 3101-9840

E-mail: vicente.freitas@uece.br

ABSTRACT

Canindé is a goat (*Capra hircus*) breed currently considered endangered.

The aims of this study were to: (i) characterize estrus behavior, ovulatory dynamics and progesterone profile and (ii) evaluate embryo *in vitro* production (IVP) in this breed.

In Experiment 1, nulliparous ($n = 10$) or pluriparous ($n = 7$) females received a synchronization treatment and its reproductive parameters were evaluated. In Experiment 2, oocytes were obtained by laparoscopy from hormonally stimulated females ($n = 15$) and used for embryo IVP. Results are described as mean ± SEM.

There was no difference ($P > 0.05$) between nulliparous or pluriparous for: estrus response (40.0% *vs.* 85.7%), time to estrus onset (62.0 ± 15.5 *vs.* 50.7 ± 19.2 h), duration of estrus (25.0 ± 16.1 *vs.* 30.0 ± 15.1 h), percentage of ovulating goats (60.0% *vs.* 85.7%), number of ovulations (1.2 ± 0.4 *vs.* 1.3 ± 0.8) and diameter of the largest follicle (5.8 ± 0.5 *vs.* 6.1 ± 0.3 mm). Progesterone concentrations were similar ($P > 0.05$) between both categories. During laparoscopic recovery, 12.2 aspirated follicles and 9.1 oocytes were recorded per goat, resulting in high recovery rate (74.3%, 182/245). A total of 78 embryos were produced leading to 51.0% of blastocyst rate. Blastocysts at day 7 of *in vitro* culture showed a mean blastocyst cell numbers of 170.3 ± 12.5.

In conclusion, nulliparous and pluriparous Canindé goats showed similar reproductive profile. It was possible to produce embryos *in vitro* which allowed initiating an embryo bank to be used for breed preservation.

Keywords: Estrus; Ovulation; Oocyte recovery; IVP; Extinction.

1. Introduction

When Europeans discovered Brazil, about 500 years ago, Portuguese settlers brought the first ruminant specimens. Since then, these animals formed breeds which are called "naturalized" and for centuries were responsible for the country´s livestock production. Over the years, natural selection occurred and these breeds developed morphological and physiological characteristics that are adapted to specific Brazilian environmental conditions. Nevertheless, beginning in the early 20th century onwards, imported commercial breeds have gradually been replacing naturalized breeds to such an extent that the latter are now in danger of extinction [1]. The endangered Canindé goat is within this naturalized breed group and is found mainly in Northeastern Brazil. These animals are more frequently raised in an extensive way receiving almost no human handling and their total number is not known. Studies with Canindé goats utilizing molecular markers are already under way [1-2]. A greater effective of goats of this breed could have substantial importance on families that live in a subsistence culture basis by providing both milk and meat, especially due to their adaptation to this specific dry environment. Therefore, all actions that aim to preserve it are important so their genetic material is not permanently lost. Thus, recently, our group initiated a project to preserve this breed. However, the literature is scarce regarding its peculiarities in terms of reproductive physiology. Hence, it is necessary to know better their behavior and to verify the response of the animals when submitted to reproductive biotechnologies as embryo IVP.

The viability of IVP embryos is lower than their *in vivo* counterparts generated by multiple ovulation and embryo transfer (MOET embryos). Moreover, the latter may have higher resistance to cryopreservation than IVP embryos. However, in small ruminants, *in vivo* embryo recovery requires surgical procedures that impair repeated

embryo production from individual donors. In addition, some goats do not respond to superovulation protocols but are eligible to IVP. Therefore, both technologies have either advantages and could be combined. The application of IVP has been proposed as a valuable strategy for the conservation of endangered species, reviewed by [3]. The use of embryo IVP to preserve an endangered breed requires collecting oocytes, since obviously they do not go often to slaughterhouse. In order to recover high numbers of oocytes, the goats are treated with gonadotropin. Current treatments mostly consist in multi-injection FSH regimes, but the protocols are very labor intensive and rather stressful to the animals because of excessive handling. For those reasons, there is a demand for a simplification of the protocols [4]. Therefore, the one-shot regime, in which a combination of FSH and eCG is given as a single treatment administered ~ 36 h prior to laparoscopic ovum pick up (LOPU) is a good option [5], which is less expensive and requires less labor input. Recently, our group demonstrated for the first time that it was possible to use both protocols (five injections of FSH or a single dose of eCG and FSH) to stimulate Canindé goats [6]. However, in that study the oocytes were subjected just to *in vitro* maturation (IVM) and molecular analyses and it was not possible to produce the embryo *in vitro*.

Thus, the objectives of this study were to characterize the estrus response, ovulatory pattern and progesterone profile on the subsequent estrous cycle of endangered nulliparous and pluriparous Canindé goats. In order to avoid the effect of exogenous gonadotropin, it was used only a progestagen treatment associated with a luteolytic injection. Moreover, to assess the efficacy of a simplified stimulatory protocol (a single dose of FSH and eCG) to recover good quality oocytes, and to verify maturation, fertilization and development systems in order to produce embryos *in vitro*.

Afterwards, the vitrification of part these embryos allowed us to constitute a bank of embryos, contributing to the preservation of the Canindé breed (*Capra hircus*).

2. Material and methods

2.1. Animal care

Animal use and care were approved by the Animal Ethics Committee of the Ceará State University (CEUA/UECE, n° 09144595-7/50). All trials were conducted in accordance with the guidelines for animal care [7].

2.2. Chemicals and media

Unless indicated otherwise, all chemicals and reagents were from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Location and experimental animals

This study was carried out in the Laboratory of Physiology and Control of Reproduction (LFCR; School of Veterinary Medicine, Ceará State University), Fortaleza, CE, Brazil (latitude 3°47’38’’S and longitude 38°33’29’’W). Female goats reared at this latitude are usually non-seasonal breeders, showing estrus during the entire year. Six months before beginning of this experiment, the goats arrived in our lab

coming from another herd where they were raised in an extensive system with almost no human contact and handling.

All animals were maintained in a semi-intensive system, receiving Tifton (*Cynodon dactylon*) hay, and daily access to 4 h of pasture on this grass. In addition, the animals were supplemented with 0.2 kg/d commercial concentrate (minimum of 20% crude protein) and had free access to water and mineralized salt. All females were clinically and ultrasound (Falco 100; Pie Medical, Maastricht, Netherlands) examined to determine pregnancy or abnormalities of the reproductive tract.

2.4. Experimental steps

Two independents trials were carried out to characterize the estrus and ovulation responses, and to evaluate the LOPU efficiency for the IVP of viable goat embryos, as described below. In Experiment 1, we characterized the estrus response, ovulatory pattern and progesterone profile on the subsequent estrous cycle of endangered nulliparous and pluriparous Canindé does submitted to an estrus synchronization protocol. In Experiment 2, using oocytes from goats submitted a simplified stimulatory protocol (a single dose of FSH and eCG), we evaluated the percentage of recovered good quality oocytes, and we tested the maturation, fertilization and development systems in order to produce embryos *in vitro*. During the study, we also constituted a bank of Canindé goat embryos, contributing to the preservation of this breed.

2.5. Estrus and ovulatory responses – Experiment 1

2.5.1. Hormonal treatment

Seventeen Canindé goats, which were nulliparous (n = 10) or pluriparous (n = 7) were selected. They presented an average ± SEM of 27.6 ± 3.8 kg (body weight), 3.0 ± 0.2 (body condition score, BCS; 1-5 scale) [8] and aged between 1-4 years old. All females have already reached puberty. All does received intravaginal sponges containing 60 mg medroxyprogesterone acetate (MAP; Progespon, Syntex, Buenos Aires, Argentina) for six days and 75 µg D-cloprostenol (Prolise, Pfizer Animal Health, São Paulo, Brazil) 24 h before sponge removal. After sponge removal, estrus was monitored by observing the reactions of the females with the use of four fertile Canindé bucks every 4 h during 96 h. Goats were recorded as being in estrus if they accept to be mounted. After this moment, estrus detection was performed twice daily until either the subsequent estrus or the following 21 days.

2.5.2. Ovarian ultrasound examination

Ovarian transrectal ultrasound examination using Falcon 100 (Pie-Medical, Maastricht, Netherlands) scanner was performed in all goats every 12 h after sponge removal until ovulation detection or up to 120 h (in case no ovulation was detected) by the same operator. Six days after estrus detection, another ultrasound examination was performed in order to confirm the number and side of ovulations. A 6.0/8.0 MHz linear-array probe (60 mm; reference number: 410054) was adapted to a hard support to be manipulated externally in the rectum. Animals were kept in a standing position. Fecal pellets were manually removed and carboxymethylcellulose gel (15-20 mL) was administered with a syringe into the rectum. The number, diameter and position of ovarian follicles ≥ 3 mm were recorded. The procedure to locate the ovaries was the

same as previously described [9]. The day of ovulation was defined as the day of disappearance of the largest follicle, identified before. The preovulatory follicle diameter was the last measurement obtained before ovulation.

2.5.3. Plasma progesterone concentration

Blood collection was performed in all goats by jugular veinpuncture using 4 mL tubes containing EDTA (BD Vacutainer, Becton Dickinson and Company, Nebraska, USA) to determine plasma progesterone concentrations. Samples were obtained daily (08:00 am) after sponge removal (Day 6) to the second subsequent estrus or up to 21 days after the first estrus (around Day 31 of the protocol). Tubes were immediately placed and kept on ice until centrifugation at 2000 *g* for 15 min. Plasma was aliquoted and stored at –20°C until hormone assay. P4 was determined using a commercial kit (Coat-a-Count progesterone kit, DPC, Diagnostic Products Corporation, Los Angeles, USA) of solid phase radioimmunoassay (RIA) according to manufacturer's instructions. The mean intra- and inter-assay coefficients of variation were 8.8% and 9.7%, respectively. The analytical detection limit was 0.08 ng/mL and the mean concentration was 12.7 ng/mL.

2.5.4. Laparoscopy examination

The percentage of ovulating goats as well as side and numbers were determined by laparoscopy, six days after synchronized estrus, immediately after the last ultrasound examination. Females were starved for 24 h prior to laparoscopy. Anesthesia consisted in 0.3 mg / kg of xylazine hydrochloride (Dorcipec, Vallée, Montes Claros, Brazil) and

0.04 mg / kg atropine (Atropina 1%, Fagra, Mairiporã, Brazil). In addition, local anesthesia consisting of 2% clorhydrate lidocain (Anestésico L Pearson, Eurofarma, São Paulo, Brazil) was applied into the puncture sites of the trocars. The donor goats were restrained on a standard laparoscopy table. The procedure was performed by using a 5-mm laparoscope (Karl Storz Endoscopes GmbH & Co, Tuttlingen, Germany) attached to a video system. The laparoscope was inserted into the abdominal cavity through a trocar (cranial to the udder and to the left side of the midline). An atraumatic grasper was inserted into the right side of the abdomen to hold the ovary, making possible to locate and count the corpora lutea. Finally, the trocar wounds were treated with a local antibiotic healing solution (Terra-cortril spray, Pfizer Animal Health, São Paulo, Brazil).

2.6. Embryo *in vitro* production and vitrification – Experiment 2

2.6.1. Hormonal treatment for ovarian stimulation

Fifteen Canindé goats, which were nulliparous (n =9) or pluriparous (n = 6) ageing 2 to 4 years old were selected as oocytes donors. Five goats were used twice whereas the other 10 just once. All females received intravaginal sponges containing 60 mg MAP for 11 days and 75 µg D-cloprostenol at day 8 of progestagen treatment. For ovarian stimulation, goats received a single dose of 70 mg pFSH (Folltropin-V, Vetrepharm, Ontario, Canada) plus 200 IU eCG (Novormon, Syntex, Buenos Aires, Argentina) 36 h before sponge removal.

2.6.2. Anesthesia and laparoscopic ovum pick up (LOPU)

At the sponge removal, LOPU was performed. The females were deprived of feed and water for 36 h and 24 h, respectively, prior to laparoscopy. Thus, animals were subjected to anesthesia, which was performed a induction by administration of 20 mg/kg of thiopental (Tiopentax 2.5%, Cristália, São Paulo, Brazil) by intravenously via and the anesthesia was maintained with continuous infusion of 3% isoflurane (Isofrine, Cristália, São Paulo, Brazil) using the inhalational system with medical oxygen (HB Hospitalar, São Paulo, Brazil). Furthermore, local anesthesia was applied as earlier described.

The LOPU procedure was performed just after sponge removal by the same system as previously described. The ovary was held by the grasper and ovarian follicles were individually aspirated using a 22-gauge needle connected to an aspiration and flushing system (WTA, Cravinhos, Brazil). The vacuum pressure was set at -30 mmHg, generating a fluid flow of 7 to 7.5 mL/min. All follicles larger than 2 mm were aspirated and the total of small (< 3 mm), medium (3 to 4 mm) and large (> 4 mm) follicles were counted. This classification was performed taking into account the fenestrated grasping forceps width which measures 4 mm. The collection medium used was TCM 199 with 10 mM HEPES, 0.022 µg/mL sodium pyruvate, 10,000 IU penicillin, 10,000 µg/mL streptomycin sulfate, 25 µg/mL amphotericin B, 10% fetal calf serum (FCS) and 20 IU/mL heparin sulfate. Once LOPU was completed, each ovary was gently flushed with a heparinized saline solution (25 IU/mL) at 37 ºC for the prevention of possible adhesions. Finally, the trocar orifices were treated with a local antibiotic healing solution.

2.6.3. Assessment of cumulus-oocyte complexes (COC) quality and IVM

Assessment of the quality of the COCs was based on cellular vestments and cytoplasmic uniformity [6], allocating different grades (1 to 4) using a stereomicroscope (SMZ 800, Nikon, Tokyo, Japan). Grades 1, 2 and 3 were classified as good quality COCs for IVM. The remaining oocytes classified as grade 4 were discarded. The COCs were washed four times and transferred to maturation medium consisting of TCM 199 supplemented with 0.022 µg/mL sodium pyruvate, 10,000 IU penicillin, 10,000 µg/mL streptomycin sulfate, 25 µg/mL amphotericin B, 10% FCS, 10 ng/mL EGF, 5 µg/mL FSH, 10 µg/mL LH, 1 µg/mL 17β-estradiol and 100 µM cysteamine. COCs were incubated for 24 h at 38.5°C in a humidified atmosphere of 5% CO_2 in air.

2.6.4. Sperm preparation and in vitro fertilization (IVF) of oocytes

It was used fresh semen collected from Canindé bucks of known fertility. Motile sperm was obtained by centrifugation (15 min at 900 *g*) on 2 mL of Percoll discontinuous density gradient (45/90%). Viable spermatozoa were diluted in the appropriate volume of fertilization medium and it was added to each fertilization drop, to achieve a final concentration of 2.0×10^6 spermatozoa/mL. At the end of IVM, the COCs were transferred into fertilization medium drops [10]. Sperm and oocytes were co-incubated for 18 h at 38.5°C in a humidified atmosphere of 5% CO_2 in air.

2.6.5. In vitro embryo culture (IVC) conditions, evaluation of embryo development and vitrification

After fertilization, the COCs were denuded by vortex. The presumptive zygotes were washed four times in culture medium containing modified SOFaa medium [11] supplemented with 0.4% BSA and 2% FCS to remove spermatozoa and transferred to drops containing 50 µL of culture medium covered with mineral oil. The presumptive zygotes were incubated for seven days at 38.5°C in a humidified atmosphere of 5% CO_2/air. After 48 h post-insemination, 50% of the medium was replaced with fresh medium. Cleavage was evaluated on day 2, and the number of blastocysts on Day 7 post IVF. Although the kinetics of embryo development represents interesting data, it was not the focus of the current study. We preferred to open the incubator just once to evaluate the potential highest embryo rates obtained. Moreover, in Day 7, in order to assess the quality of produced embryos, blastocysts were classified according to their stage of development in accordance with the Manual of International Embryo Transfer. Additionally, the quality of expanded blastocysts (Day 7) was assessed subsequently by a simple staining. Briefly, 12 embryos were fixed in PBS with 4% paraformaldehyde (F1635) for approximately 15 min at 25°C. For counting the total cell numbers, blastocysts were incubated in 10 µg/mL bisbenzimide (Hoechst 33342, B2261) for 15 min at 25°C. Thereafter, samples were placed in a glycerol droplet on a glass slide and carefully covered with a coverslip. Samples were examined under a fluorescence microscope (TE200, Nikon, Japão) equipped with a UV filter. Other blastocyst and hatched embryos were vitrified according to Vajta et al. [12].

2.7. Variables and statistical analysis

The following end points were recorded for the first experiment 1: estrus response (%) – number of goats in estrus/number of treated females x 100; time to

estrus onset (h) – interval from device removal to first mounting acceptance; duration of estrus (h) – interval from the first to last mounting acceptance; interval from device removal to ovulation (h); interval from estrus onset to ovulation (h); percentage of ovulating goats by ultrasound (%) – number of goats with confirmed ovulation/number of goats evaluated by ultrasound x 100; number of ovulations by ultrasound – average number of ovulations determined by ultrasound examination per goat; diameter of the largest follicle (mm); percentage of ovulating goats by laparoscopy (%) – number of goats with CL/number of goats evaluated by laparoscopy x 100; number of ovulations by laparoscopy – average number of ovulations determined by laparoscopy per goat; ovulation on right ovary (%), ovulation on left ovary (%), plasma progesterone concentration (ng/mL). Comparisons were done on the basis of reproductive status (nulliparous *vs.* pluriparous). Data were submitted either to Mann-Whitney-Wilcoxon test or Fisher's exact test when appropriate. Statistical analyses were performed using Prism 5.0a software.

The following end points were recorded for the second experiment: total of visualized follicles; total of aspirated follicles; total of small (< 3 mm), medium (3 to 4 mm) and large (> 4 mm) follicles; mean aspirated follicles and oocytes recovered per goat in all sessions; oocyte recovery rate (%): number of recovered oocytes/number of aspirated follicles x 100; oocytes quality at the collection; cleavage rate (%): percentage of cleaved embryos 2 days after fertilization; blastocyst rate (%): percentage of blastocysts at day 7 expressed on the basis of the number of oocytes entering into IVM and on the basis of the number of cleaved embryos at day 2; embryo quality evaluated by stage classification in day 7 and mean blastocyst cell number per expanded blastocyst. All results are described as mean ± SEM.

3. Results

3.1 Estrus response, ovulatory dynamics and progesterone profile – Experiment 1

From the 17 synchronized goats, 10 presented estrus followed by ovulation and two ovulated without displaying estrus. One goat showed estrus at 108 h after sponge removal and was not considered in analysis. There was no difference ($P > 0.05$) between nulliparous and pluriparous females for: estrus response [40.0% (4/10) *vs*. 85.7% (6/7), respectively], time to estrus onset (62.0 ± 15.5 *vs*. 50.7 ± 19.2 h), duration of estrus (25.0 ± 16.1 *vs*. 30.0 ± 15.1 h), interval from onset of estrus to ovulation (49.0 ± 10.0 *vs*. 59.3 ± 18.1 h), interval from sponge removal to ovulation (106.0 ± 11.8 *vs.* 110.0 ± 11.8 h), percentage of ovulating goats [60.0% (6/10) *vs.* 85.7% (6/7)], number of ovulations (1.2 ± 0.4 *vs.* 1.3 ± 0.8), ovulation on right ovary [71.4% (5/7) *vs.* 50.0% (4/8)], ovulation on left ovary [28.6% (2/7) *vs.* 50.0% (4/8)] and diameter of the largest follicle (5.8 ± 0.5 *vs.* 6.1 ± 0.3 mm). One nulliparous had double ovulation with the second largest follicle measuring 5.5 mm and one pluriparous goat had triple ovulation with the second largest follicle measuring 5.7 mm and third one 5.5 mm. None of the goats showed estrus while the sponges were in place.

The percentage of ovulating goats by ultrasound examination and by laparoscopy was the same. During laparoscopic inspection of the ovaries, two goats (pluriparous) out of 12 that ovulated (16.7%) had only abnormal CL (avascular and pale; indicating premature luteolysis) and one presented simultaneously one active and one abnormal CL. Both animals returned to estrus four to five days after the first estrus. Two pluriparous and two nulliparous goats returned to estrus exactly 20-21 d after the synchronization of estrus. Plasma progesterone concentrations revealed no difference

between nulliparous and pluriparous females. Average progesterone in metaestrus was 0.5 ± 0.1 ng/mL whereas in diestrus was 5.2 ± 0.2 ng/mL. Progesterone concentrations of four selected goats according to their behavior are shown in Fig. 1.

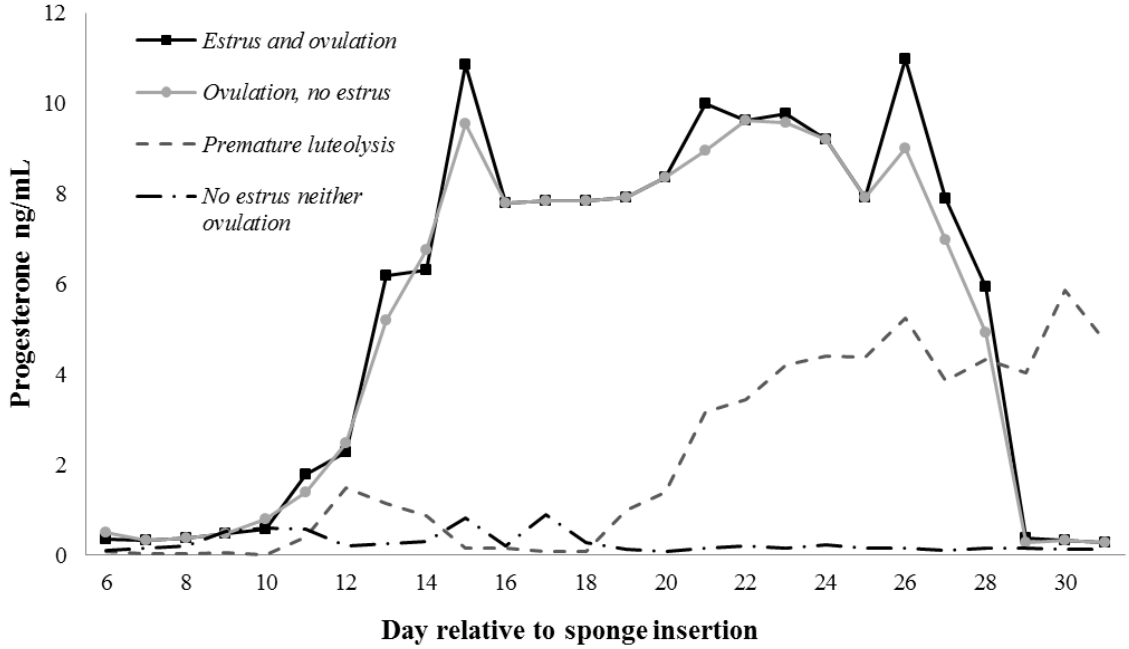

Figure 1. Plasma progesterone concentrations in four Canindé goats showing different behaviors when submitted to estrus synchronization.

3.2. Retrieval of COCs obtained i*n vivo* by LOPU and embryo IVP – Experiment 2

After four replications, a total of 310 follicles was visualized and 245 of them aspirated; 130 from the left and 115 from the right ovary. A mean of 12.2 aspirated follicles and 9.1 recovered oocytes were recorded from each donor goat in all sessions, resulting in an overall oocyte recovery rate of 74.3% (182/245). From these 245 aspirated, 77 (31.4%) were recovered from small, 88 (35.9%) from medium and 80 (32.7%) from large follicles. Regarding the quality, most part of the oocytes collected

had good quality [24 (grade 1), 124 (grade 2) and 10 (grade 3)] and only 24 were discarded (grade 4). A total of 153 COCs were subsequently used to embryo IVP.

Cleavage rate was 58.8% (90/153). A total of 78 embryos were produced leading to 86.7% of blastocysts rate (Fig. 2A) from cleaved oocytes or 51.0% from the total number of oocytes entering to IVM and distributed in early blastocyst (12.8%), blastocyst (35.9%), expanded (16.7%) and hatched blastocyst (34.6%). Twelve expanded blastocyst (Fig. 2B and 2C) were fixed, counted nuclei and showed a mean blastocyst cell numbers of 170.3 ± 12.5. Other part of blastocyst and hatched embryos was vitrified, thus, forming the beginning of an embryo bank for the Canindé breed. Early blastocysts were discarded since they could be arrested and thus not viable anymore.

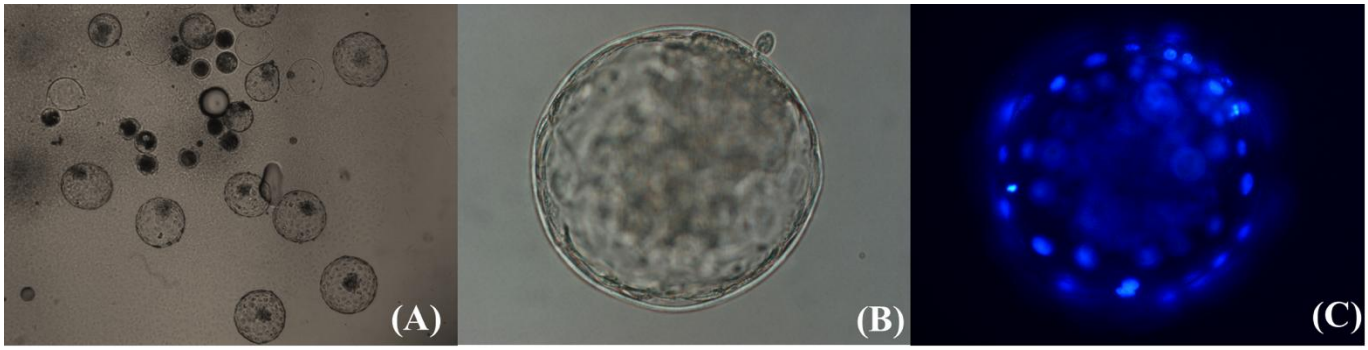

Figure 2. Canindé *in vitro* produced embryos cultured in SOFaa modified medium. (A) Blastocysts on Day 7 of *in vitro* culture (100x). **(B)** Expanded blastocyst on Day 7 (400x). **(C)** The cell nuclei of an expanded blastocyst on Day 7 under UV light after stained (400x).

4. Discussion

The present study is the first successful report that was possible the production of embryos *in vitro* in the endangered Canindé goat. Moreover, for the first time it was described in this breed the reproductive parameters during estrous cycle (estrus, ovulation and progesterone profile). As no statistical difference was detected in any reproductive end point between nulliparous and pluriparous, averages were assessed.

Estrus response rate was on average 58.8%. Although not significant difference, a greater number of pluriparous (85.7%) was detected in estrus in comparison with nulliparous (40.0%). This numerically difference could be associated to the maturation of the hormonal reproductive system of pluriparous goats. Moreover, different hormone profiles detected in nulliparous and pluriparous may contribute to the lower reproductive performance of nulliparous females [13]. The duration of estrus was on average 28 h in our study, similar to the 25 h observed in the Alpine [14] or the 31 h in the Saanen breed [15], but shorter than 59 h reported in Matou goats [16].

The interval from device removal to the onset of estrus was on average 55 h (range from 32 to 88 h), which was similar to 53 h, reported for Boer breed [17]. The time to estrus onset is very variable as verified after hormonal synchronization treatments in several studies [18-19]. Previously, working with ovariectomyzed goats, Freitas et al. [20] demonstrated that it is unlikely to improve the synchrony with treatments based on progestagen administration, what corroborates with the affirmation that disperse estrus is an inherent feature in goats. Injection of eCG is known to reduce the interval between progestagens removal and onset of estrus and to affect the duration of estrus [21]. However, in the present study, the use of eCG was eliminated in order to

obtain the most reliable response of the breed without an effect of external gonadotropin.

In the present study, overall number of goats ovulating was on average 70.5%. Although not significant difference, a greater number of pluriparous (85.7%) ovulated in comparison with nulliparous (60.0%). As earlier hypothesized regarding the estrus response rate, this difference could be associated to a better development of the hormonal reproductive system of pluriparous goats. Simões et al. [22] reported that significant differences occurred in the number and time of ovulations between nulliparous and pluriparous Serrana goats. The overall number of ovulations in the present study (1.3) was similar to Alpine (1.4), Saanen (1.2) [23] and to Nubian breed (1.4) [24], all studies without gonadotropin support. The number of ovulations is a good predictor of prolificacy. Thus, it is reasonable to assume that the Canindé breed has similar prolificacy as the most known and widespread breeds.

The slightly higher ovulation frequency of the right ovary (60.0% *vs* 40.0%) noted in this study corroborates earlier findings in dairy goats [25]. Chávez et al. [26] noted this phenomenon in rats when they showed the asymmetry of the information carried between right and left vagal nerve to ovaries. The uni or bilateral section of the vagal nerve produced different results depending on the *in situ* ovary. Ovulation rate in rats unilaterally ovariectomized was smaller in the left ovary when compared to the right one (42 *vs.* 84%). The authors concluded that, in the model used, regulatory compensatory systems are more likely to occur in the right ovary than in the left one.

The determination of percentage of ovulating goats by ultrasound and by laparoscopy was the same, demonstrating the reliability of the ultrasound technique to predict this parameter and thus is the technique suggested for being less invasive.

However, during the laparoscopic inspection of the ovaries, two does out of 12 (16.7%) had abnormal CLs suggesting premature luteolysis, what was not identified in ultrasound examination. The progesterone profile of one female with abnormal CL is shown in Fig. 1. It is interesting to notice in this figure that this female manifested estrus on day 8 (two days after sponge removal and estrus lasted up to day 10) and again on day 15 (five days after the end of the first estrus). It is possible to observe that both goats that had normal ovulation presented high concentrations of progesterone on day 15 in comparison to the one with premature luteolysis. Two does ovulated without displaying estrus, what is in accordance to previous reports in goats [17, 27]. It is noteworthy that these females had very similar progesterone profile to the animals presenting estrus from the sponge removal to the last day of the next cycle after the synchronized estrus, suggesting that the silent ovulation was not related to lower levels of progesterone.

Progesterone concentrations remained at basal levels throughout the estrus as previously observed [28-29]. In Markhoz goats, the progesterone levels gradually increased during the metaestrus and reached its maximum on day 12 during luteal phase, ranging from 5 to 12 ng/mL [29]. In Damascus goats, the progesterone concentrations during luteal phase ranged from 2.6 to 5.4 ng/mL [28], similar to our findings in the present study.

Regarding to the hormonal treatment for ovarian stimulation to collect oocytes, it was earlier reported that there were no significant differences between the protocol associating eCG and FSH in comparison to standard treatment that use several doses of FSH concerning the number of ova and embryos recovered in sheep [4]. Likewise, the simplified stimulation treatment appeared to be well suited to the Canindé breed since it lead to averages of aspirated follicles (12.3) and recovered oocytes (9.1) per donor

similar to those previously described in other goat breeds after hormonal treatment and LOPU [30-31].

In a previous study we obtained a maturation rate of 46% when using a similar ovarian stimulatory protocol in the same breed [6] with the oocytes being denuded before fertilization. We consider that *cumulus* cells may have an essential role during the fertilization, since they resulted in significantly improved development of oocytes to blastocysts [32]. Thus, in the current study they remained attached to the oocytes during this step and, therefore, it was not possible to assess the maturation rate. However, it is reasonable to assume that the maturation rate was higher now, since the cleavage rate was higher and just matured oocytes should be able to be fertilized. The overall cleavage rate was 58.8%, lower than 82% previous reported by Cox and Alfaro [33] and slightly greater than 51% [30], both studies also using oocytes recovered by LOPU. We consider that it may be possible to improve this rate in further studies evaluating IVM and IVF systems more suitable for these oocytes requirements.

The blastocyst rate from the number of oocytes cleaved (86.7%) or from the total number of oocytes entering to IVM (51.0%) was similar or higher than other studies in goats [10,33], what suggests that the *in vitro* culture system does not need to be modified. The developmental potential of an embryo in terms of blastocyst yield depends on the developmental potential of the oocyte from which it originates – its intrinsic quality – but also on the conditions they are submitted [34]. Moreover, the mean blastocyst cell number observed in this study was similar than other studies in goats [30,35], even in different conditions of IVC. All expanded blastocysts were submitted to cell counting. The other part of blastocyst and hatched embryos was vitrified. Most part of hatching and hatched embryos survive cryopreservation, showing

higher survival rates compared with those at earlier stages [36]. Thus, early blastocysts were discarded since they could be arrested and thus not viable anymore.

In summary, the present study will serve as a basis to upcoming works with this breed, regarding the correct use of efficient reproductive biotechnologies. This may be an important tool for their conservation. Moreover, it was possible to produce for the first time embryos *in vitro* and to start forming a bank of frozen embryos of the breed from oocytes obtained by follicular puncture assisted by laparascopy. Further studies are necessary to evaluate the *in vivo* viability of these vitrified IVP embryos.

Acknowledgments

This work was supported by FUNCAP, CNPq e CAPES. J.M.G. Souza-Fabjan is a recipient of a scholarship from CAPES. V.J.F. Freitas is CNPq senior investigator. The authors thank Mr. Joãozito Andrade *(in memorian)* for providing the animals used in this study and especially for their interest and enthusiasm with Canindé breed.

REFERENCES

[1] Mariante, AS, Albuquerque, MSM, Egito, AA, McManaus, C, Lopes, MA, Paiva, SR. Present status of the conservation of livestock genetic resources in Brazil. Livestock Science 2009;120(3):204–212.

[2] Ribeiro, MN, Bruno-de-Sousa, C, Martinez-Martinez, A, Ginja, C, Menezes, MPC, Pimenta-Filho, EC, Delgado JV, Gama, LT. Drift across the Atlantic:

genetic differentiation and population structure in Brazilian and Portuguese native goat breeds. Journal of Animal Breeding and Genetics 2012;129:79-87.

[3] Souza, JMG, Batista, RITP, Melo, LM, Freitas, VJF. Reproductive biotechnologies applied to the conservation of endangered ruminant - Past, Present and Future. Revista Portuguesa em Ciências Veterinárias 2011;110(577-580):31–38.

[4] Forcada, F, Ait Amer-Meziane, M, Abecia, JA, Maurel, MC, Cebrián-Pérez, JA, Muiño-Blanco, T, Asenjo, B, Vázquez, MI, Casao, A. Repeated superovulation using a simplified FSH/eCG treatment for *in vivo* embryo production in sheep. Theriogenology 2011;75(4):769–776.

[5] Baldassarre, H, Karatzas, CN. Advanced assisted reproduction technologies (ART) in goats. Animal Reproduction Science 2004;82-83:255–266.

[6] Avelar, SRV, Moura, RR, Sousa, FC, Pereira, AF, Almeida, KC, Melo, CHS, Teles Filho, ACA, Baril, G, Melo, LM, Teixeira, DIA, Freitas, VJF. Oocyte production and *in vitro* maturation in Canindé goats following hormonal ovarian stimulation. Animal Reproduction 2012;9(1):27–32.

[7] ASAB. Guidelines for the treatment of animals in behavioral research and teaching. Animal Behaviour 2006;71:245–253.

[8] Suiter, J. Body condition scoring in sheep and goats. Farmnonte 69/94. Available at: <http://www.agric.wa.gov.au/content/aap/sl/m/fn069_1994.htm>, 1994. Acess at 23rd of Setember, 2010.

[9] Ginther, OJ, Kot, K. Follicular dynamics during the ovulatory season in goats. Theriogenology 1994;42(6):987–1001.

[10] Berlinguer, F, Leoni, GG, Succu, S, Spezzigu, A, Madeddu, M, Satta, V, Bebbere, D, Contreras-Solis, I, Gonzalez-Bulnes, A, Naitana, S. Exogenous melatonin positively influences follicular dynamics, oocyte developmental competence and blastocyst output in a goat model. Journal of Pineal Research 2009;46(4):383–391.

[11] Takahashi, Y. and First, N.L. In vitro development of bovine one-cell embryos: influence of glucose, lactate, pyruvate, amino acids and vitamins. Theriogenology 1992;37:963-978.

[12] Vajta, G, Murphy, C, Macháty, Z, Prather, RS. In straw dilution of *in vitro* produced bovine blastocysts after vitrification with the open pulledstraw (OPS) method. Veterinary Record 1999;144:180–181.

[13] Gordon, I. Controlled Reproduction in Sheep and Goats. University Press, Cambridge, UK; 1997.

[14] Fonseca, JF, Torres, CA, Santos, ADF, Maffili, VV, Amorim, LS, Moraes, EA. Progesterone and behavioral features when estrous is induced in Alpine goats. Animal Reproduction Science 2008;103(3-4):366–373.

[15] Dogan, I, Nur, Z, Gunay, U, Soylu, MK, Sonmez, C. Comparison of fluorgestone and medroxyprogesterone intravaginal sponges for oestrus synchronization in Saanen does during the transition period. South African Journal of Animal Science 2004;34(1):18–22.

[16] Moaeen-ud-Din, M, Yang, LG, Chen, SL, Zhang, ZR, Xiao, JZ, Wen, QY, Dai, M. Reproductive performance of Matou goat under sub-tropical monsoonal

climate of Central China. Tropical Animal Health and Production 2008;40(1):17-23.

[17] Greyling, JPC, Van Der Nest, M. Synchronization of oestrus in goats: dose effect of progestagen. Small Ruminant Research 2000;36(2):201–207.

[18] Fonseca, JF, Bruschi, JH, Santos, ICC, Viana, JHM, Magalhães, ACM. Induction of estrus in non-lactating dairy goats with different estrous synchrony protocols. Animal Reproduction Science 2005;85:117–124.

[19] Souza, JMG, Torres, CA, Maia, ALRS, Brandão, FZ, Bruschi, JH, Viana, JHM, Oba, E, Fonseca, JF. Autoclaved, previously used intravaginal progesterone devices induces estrus and ovulation in anestrous Toggenburg goats. Animal Reproduction Science 2011;129(1-2):50–55.

[20] Freitas, VJF, Baril, G, Saumande, J. Estrus synchronization in dairy goats: use of fluorogestone acetate vaginal sponges or norgestomet ear implants. Animal Reproduction Science 1997;46(3-4):237–244.

[21] Romano, JE. Synchronization of estrus using CIDR, FGA or MAP intravaginal pessaries during the breeding season in Nubian goats. Small Ruminant Research 2004;55:15–19.

[22] Simões, J, Baril, G, Almeida, JC, Azevedo, J, Fontes, P, Mascarenhas, R. Time of ovulation in nulliparous and multiparous goats. Animal 2008;2(5):761-768.

[23] Fonseca, JF, Souza, JMG, Bruschi, JH, Viana, JHM, Brandão, FZ, Silva, WJ, Denadai, R, Maia, ALRS, Facó, O. Induction of estrus in cyclic Alpine goats with short-term progestagen protocols with or without eCG administration. Reproduction, Fertility, and Development 2010;22:169.

[24] Souza, JMG, Torres, CAA, Ribeiro, SDA, Ribeiro, AC, Becalete, L, Rigo, AG, Silva, WJ, Facó, O, Fonseca, JF. Induction of estrus in Anglo-Nubian goats in the transition season with short-term progestagen protocols with or without eCG administration. Reproduction, Fertility and Development 2010;22:380.

[25] Fonseca, JF, Maffili, VV, Santos, ADF, Furst, R, Prosperi, CP, Rovay, H, Souza, JMG, Torres, CA. Effects of prostaglandin administration 10 days apart on reproductive parameters of cyclic dairy nulliparous goats. Arquivo Brasileiro de Medicina Veterinária e Zootecnia 2012;64(2):349–358.

[26] Chavez, R, Cruz, ME, Domhinguez, R. Differences in the ovulation rate of the right or left ovary in unilaterally ovariectomized rats: effect of ipsi- and contralateral vagus nerves on the remaining ovary. The Journal of Endocrinology 1987;113(3):397-401.

[27] Dogan, I, Nur, Z, Gunay, U, Sagyrkaya, H, Soylu, MK, Sonmez, C. Estrous synchronization during the natural breeding season in Anatolian Black does. Veterinay Medicine – Czech 2005;50(1):33–38.

[28] Gaafar, KM, Gabr, MK, Teleb, DF. The hormonal profile during estrous cycle and gestation in Damascus goat. Small Ruminant Research 2005;57(1):85-93.

[29] Talebi, J, Moghaddam, A, Souri, M, Mirmahmoudi, R. Steroid hormone profile of Markhoz does (Iranian Angora) throughout estrous cycle and gestation period. Tropical Animal Health and Production 2012;44:355-360.

[30] Koeman, J, Keefer, CL, Baldassarre, H, Downey, BR. Developmental competence of prepubertal and adult goat oocytes cultured in semi-defined media following laparoscopic recovery. Theriogenology 2003;60(5):879–889.

[31] Pierson, J, Wang, B, Neveu, N, Sneek, L, Coté, F, Karatzas, CN, Baldassarre, H. Effects of repetition, interval between treatments and season on the results from laparoscopic ovum pick-up in goats. Reproduction, Fertility, and Development 2004;16(8):795–799.

[32] Souza, JMG, Duffard N, Bertoldo, MJ, Locatelli, Y, Corbin, E, Fatet, A, Freitas, VJF, Mermillod, P. Influence of heparin or the presence of *cumulus* cells during fertilization on the *in vitro* production of goat embryos. Animal Reproduction Science 2013;138:82-89.

[33] Cox, JF, Alfaro, V. *In vitro* fertilization and development of OPU derived goat and sheep oocytes. Reproduction in Domestic Animals 2007;42(1):83–87.

[34] Rizos, D, Ward, F, Duffy, P, Boland, MP, Lonergan, P. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. Molecular Reproduction and Development 2002;61(2):234–248.

[35] Romaguera, R, Casanovas, A, Morató, R, Izquierdo, D, Catalá, M, Jimenez-Macedo, AR, Mogas, T, Paramio, MT. Effect of follicle diameter on oocyte apoptosis, embryo development and chromosomal ploidy in prepubertal goats. Theriogenology 2010;74(3):364–373.

[36] Dinnyes, A, Carolan, C, Lonergan, P, Massip, A, Mermillod, P. Survival of frozen or vitrified bovine blastocysts produced in vitro in synthetic oviduct fluid. Theriogenology 1996;46:1425-1439.

9 CONCLUSÕES

- Taxas similares de maturação, fecundação e desenvolvimento *in vitro* foram obtidas a partir do uso de meios de maturação indefinido, semidefinido ou definido em oócitos oriundos de ovários de abatedouro;
- Oócitos derivados de ovários de abatedouro ou colhidos por laparoscopia possuem competência ao desenvolvimento similares, contudo a cinética da maturação *in vitro* e suas exigências são diferentes;
- Embriões que desenvolvem-se a partir de oócitos oriundos de ovários de abatedouro ou colhiddos por laparoscopia possuem qualidade similar, demonstrando que a etapa de desenvolvimento *in vitro* é eficiente para oócitos de ambas as fontes;
- A inclusão de heparina no meio de fecundação *in vitro* eleva as taxas de produção de embriões;
- As células do *cumulus* em contato direto ou não aos oócitos durante a maturação e fecundação *in vitro* elevam as taxas de produção de embriões;
- Parte dos oócitos já encontrados desnudos no momento da colheita desenvolvem-se satisfatoriamente até blastocistos quando maturados e fecundados na presença de complexos *cumulus*-oóitos intactos;
- Os parâmetros relativos ao estro, padrão ovulatório e perfil plasmático de progesterona de caprinos da raça Canindé são similares entre nulíparas e pluríparas e ainda a outras raças caprinas descritas na literatura;
- Foi possível obter pela primeira vez embriões *in vitro* a partir de colheita de oócitos por laparoscopia na raça caprina Canindé ameaçada de extinção e iniciar a formação de um banco de embriões para conservação da raça.

10 PERSPECTIVAS

Diante das conclusões deste trabalho, os resultados obtidos poderão contribuir para elaborar um eficiente sistema de PIVE em caprinos. Todavia, ainda é necessário o desenvolvimento de novas pesquisas até que seja estabelecida a condição ideal para que o maior número possível de oócitos maturados *in vitro* possam ser fecundados e sustentem o subsequente desenvolvimento do embrião. Apesar dos resultados serem promissores com relação aos meios utilizados e aos sistemas empregados em oócitos oriundos de ovários de abatedouro, conforme demonstrado neste trabalho, existem fortes evidências de que as exigências metabólicas e fisiológicas dos oócitos de ambas as fontes são diferentes durante as etapas de maturação e fecundação *in vitro*. Consequentemente, existe ainda um grande desafio para alcançar taxas aceitáveis de produção de embrião em oócitos oriundos de colheita por laparoscopia. Torna-se então imprescindível a compreensão exata destas diferenças para que se possa produzir embriões de forma eficiente em ambos os sistemas.

O desenvolvimento de uma técnica de criopreservação adequada para a produção *in vitro* de embriões também deve ser alvo dos pesquisadores com o intuito de possibilitar a utilização maximizada desta tecnologia. Além destes, a biologia molecular deve ser utilizada de modo crescente nos próximos anos de maneira a contribuir com o estudo de genes específicos que possam determinar a qualidade destes embriões em programas de reprodução assistida. Atualmente, o Brasil aparece em posição de destaque com relação a PIVE em bovinos, com quase 50% do total de embriões produzidos mundialmente. Entretanto, a situação é completamente diferente para caprinos, onde tanto a demanda, como a oferta comercial desta biotécnica, são modestas. Espera-se que com o aprimoramento e repetibilidade da técnica, será possível reverter este cenário nacional atual. Além do ponto de vista comercial, o aprimoramento da técnica resultará em grande auxílio para programas de conservação de raças ameaçadas de extinção, como foi demonstrado no presente trabalho.

11 REFERÊNCIAS BIBLIOGRÁFICAS

ABD, E.L.; RAZEK, I.M.; CHARPIGNY, G.; DELALLEAU, N.; MARQUANT-LEGUIENNE, B.; MERMILLOD, P.; GUYADER-JOLY, C.; HUMBLOT, P. Lipid composition of *in vivo* and *in vitro* produced bovine embryos and of *in vitro* embryos showing different osmotic behavior. *Theriogenology*, v. 53, p. 46, 2000.

ABDULLAH, R.B.; LIOW, S.L.; RAHMAN, A.N.M.A.; CHAN, W.K.; WAN-KHADIJAH, W.E.; NG, S.C. Prolonging the interval from ovarian hyperstimulation to laparoscopic ovum pick-up improves oocyte yield, quality, and developmental competence in goats. *Theriogenology*, v. 70, p. 765-771, 2008.

AKIYAMA, K.; KOBAYASHI, J.; SATO, Y.; SATA, R.; OHASHI, M.; SASAKI, E.; ODA, Y.; OGAWA, Y.; UEDA, S.; NABENISHI, H.; MATOBA, S. Calf production from vitrified bovine sexed embryos following in-straw dilution. *Animal Science Journal*, v. 81, n. 4, p. 461-464, 2010.

ALBERIO, R.; OLIVERA, J.; ROCHE, A.; ALABART, J.; FOLCH, J. Performance of a modified ovum pick-up system using three different FSH stimulation protocols in ewes. *Small Ruminant Research*, v. 46, p. 81-87, 2002.

ALMEIDA, K.C.; PEREIRA, A.F.; ALCANTARA NETO, A.S.; AVELAR, S.R.; BERTOLINI, L.R.; BERTOLINI, M.; FREITAS, V.J.; MELO, L.M. Real-time qRT-PCR analysis of EGF receptor in *cumulus*-oocyte complexes recovered by laparoscopy in hormonally treated goats. *Zygote*, v. 19, n. 2, p. 127-136, 2011.

AMSTISLAVSKY, S.Y.; TRUKSHIN, I.S. Cryobanking mammalian embryos: priorities and the optimal choice of reproductive technologies. *Russian Journal of Developmental Biology*, v. 41, n. 1, p. 13–23, 2010.

ANDRABI, S.M.H.; MAXWELL, W.M.C. A review on reproductive biotechnologies for conservation of endangered mammalian species. *Animal Reproduction Science*, v. 99, p. 223–243, 2007.

ARUNAKUMARI, G.; SHANMUGASUNDARAM, N.; RAO, V.H. Development of morulae from the oocytes of cultured sheep preantral follicles. *Theriogenology*, v. 74, p. 884-894, 2010.

ASAB. Guidelines for the treatment of animals in behavioral research and teaching. *Animal Behaviour*, v. 71, p. 245–253, 2006.

ASSIDI, M.; DUFORT, I.; ALI, A.; HAMEL, M.; ALGRIANY, O.; DIELEMANN, S.; SIRARD, M.A. Identification of potential markers of oocyte competence expressed in bovine *cumulus* cells matured with follicle-stimulating hormone and/or phorbol myristate acetate *in vitro*. *Biology of reproduction*, v. 79, n. 2, p. 209-22, 2008.

AVELAR, S.R.G.; MOURA, R.R.; SOUSA, F.C.; PEREIRA, A.F.; ALMEIDA, K.C.; MELO, C.H.S.; TELES-FILHO, A.C.A.; BARIL, G.; MELO, L.M.; TEIXEIRA, D.I.A.; FREITAS, V.J.F. Oocyte production and *in vitro* maturation in Canindé goats following hormonal ovarian stimulation. *Animal Reproduction*, v. 9, p. 1-7, 2012.

BAGUISI, A.; BEHBOODI, E.; MELICAN, D.T.; POLLOCK, J.S.; DESTREMPES, M.M.; CAMMUSO, C.; WILLIAMS, J.L.; NIMS, S.D.; PORTER, C.A.; MIDURA, P.; PALACIOS, M.J.; AYRES, S.L.; DENNISTON, R.S.; HAYES, M.L.; ZIOMEK, C.A.; MEADE, H.M.; GODKE, R.A.; GAVIN, W.G.; OVERSTRÖM, E.W.; ECHELARD, Y. Production of goats by somatic cell nuclear transfer. *Nature Biotechnology*, v. 17, p. 456-461, 1999.

BALDASSARRE, H. Practical aspects for implementing *in vitro* embryo production and cloning programs in sheep and goats. *Animal Reproduction*, v. 9, p. 188-194, 2012.

BALDASSARRE, H.; de MATOS, D.G.; FURNUS, C.C.; CASTRO, T.E.; CABRERA FISCHER, E.I. Technique for efficient recovery of sheep oocytes by laparoscopic folliculocentesis. *Animal Reproduction Science*, v. 35, p. 145-150, 1994.

BALDASSARRE, H.; FURNUS, C.C.; de MATOS, D.G.; PESSI, H. *In vitro* production of sheep embryos using laparoscopic folliculocentesis: alternative gonadotrophin treatments for stimulation of oocyte donors. *Theriogenology*, v. 45, n. 3, p. 707-17, 1996.

BALDASSARRE, H.; KARATZAS, C.N. Advanced assisted reproduction technologies (ART) in goats. *Animal Reproduction Science*, v. 82-83, p. 255-266, 2004.

BALDASSARRE, H.; KEEFER, C.; GAUTHIER, M.; BHATIA, B.; BEGIN, I.; PIERSON, J.; LAURIN, D.; TRIGG, T.; DOWNEY, B.R.; KARATZAS, C.N. Laparoscopic ovum pick up and zygote recovery in goats treated with deslorelin implants before superovulation. *Theriogenology*, v. 55, p. 510, 2001.

BALDASSARRE, H.; KEEFER, C.; WANG, B.; LAZARIS, A.; KARATZAS, C.N.. Nuclear transfer in goats using in vitro matured oocytes recovered by laparoscopic ovum pick-up. *Cloning and stem cells*, v. 5, p. 279-285, 2003.

BALDASSARRE, H.; RAO, K.M.; NEVEU, N.; BROCHU, E.; BEGIN, I.; BEHBOODI, E.; HOCKLEY, D.K. Laparoscopic ovum pick-up followed by *in vitro* embryo production for the reproductive rescue of aged goats of high genetic value. *Reproduction, Fertility and Development*, v. 19, p. 612-616, 2007.

BALDASSARRE, H.; WANG, B.; KAFIDI, N.; GAUTHIER, M.; NEVEU, N.; LAPOINTE, J.; SNEEK, L.; LEDUC, M.; DUGUAY, F.; ZHOU, J.F.; LAZARIS, A.; KARATZAS, C.N. Production of transgenic goats by pronuclear microinjection of in vitro produced zygotes derived from oocytes receovered by laparoscopy. *Theriogenology*, v. 59, p. 831-839, 2003.

BALDASSARRE, H.; WANG, B.; KEEFER, C.L.; LAZARIS, A.; KARATZAS, C.N. State of the art in the production of transgenic goats. *Reproduction, Fertility and Development*, v. 16, n. 4, p. 465-70, 2004.

BALDASSARRE, H.; WANG, B.; PIERSON, J.; NEVEU, N.; SNEEK, L.; LAPOINTE, J.; COTE, F.; KAFIDI, N.; KEEFER, C.L.; LAZARIS, A.; KARATZAS, C.N. Prepubertal propagation of transgenic cloned goats by laparoscopic ovum pick-up and *in vitro* embryo production. *Cloning Stem Cells*, v. 6, n. 1, p. 25-29, 2004.

BARTELS, P.; FRIEDMANN, Y.; LUBBE, K.; MORTIMER, D.; RASMUSSEN, L.A.; GODKE, R.A. The live birth of an eland (*Taurotragus oryx*) calf following estrous synchronization and artificial insemination using frozen-thawed epididymal sperm. *Theriogenology*, v. 55, p. 381, 2001.

BASRUR, P.K.; KING, W.A. Genetics then and now: breeding the best and biotechnology. *Revue scientifique et technique*, v. 24, n. 1, p. 31-49, 2005.

BEDFORD, J.M. Influence of abdominal temperature on epididymal function in the rat and rabbit. *American Journal of Anatomy*, v. 152, p. 509-622, 1978.

BERLINGUER, F.; GONZALEZ-BULNES, A.; SPEZZIGU, A.; CONTRERAS-SOLIS, I.; SUCCU, S.; MCNEILLY, A.S.; NAITANA, S.; LEONI, G.G. Effect of

aging on follicular function may be relieved by exogenous gonadotropin treatment in a sheep model. *Reproduction*, v. 144, n. 2, p. 245-255, 2012.

BERLINGUER, F.; GONZALEZ-BULNES, A.; SUCCU, S.; LEONI, G.; MOSSA, F.; BEBBERE, D.; ARIZNAVARRETA, C.; TRESGUERRES, J.A.; VEIGA-LOPEZ, A.; NAITANA, S. Effects of progestagens on follicular growth and oocyte developmental competence in FSH-treated ewes. *Domestic Animal Endocrinology*, v. 32, p. 303-314, 2007.

BERLINGUER, F.; GONZALEZ-BULNES, A.; SUCCU, S.; LEONI, G.G.; VEIGA-LOPEZ, A.; MOSSA, F.; GARCIA-GARCIA, R.M.; BEBBERE, D.; GALIOTO, M.; COCERO, M.J.; NAITANA, S. GnRH antagonist enhance follicular growth in FSH-treated sheep but affect developmental competence of oocytes collected by ovum pick-up. *Theriogenology*, v. 65, n. 6, p. 1099-109, 2006.

BERLINGUER, F.; LEONI, G.; BOGLIOLO, L.; PINTUS, P.P.; ROSATI, I.; LEDDA, S.; NAITANA, S. FSH different regimes affect the developmental capacity and cryotolerance of embryos derived from oocytes collected by ovum pick-up in donor sheep. *Theriogenology*, v. 61, p. 1477-1486, 2004.

BERLINGUER, F.; LEONI, G.G.; SUCCU, S.; SPEZZIGU, A.; MADEDDU, M.; SATTA, V.; BEBBERE, D.; CONTRERAS-SOLIS, I.; GONZALEZ-BULNES, A.; NAITANA, S. Exogenous melatonin positively influences follicular dynamics, oocyte developmental competence and blastocyst output in a goat model. *Journal of Pineal Research*, v. 46, n. 4, p. 383–391, 2009.

BETTENCOURT, E.M.; BETTENCOURT, C.M.; CHAGAS E SILVA, J.; FERREIRA, P.; MANITO, C.I.; MATOS, C.M.; ROMÃO, R.J.; ROCHA, A. Effect of season and gonadotrophin preparation on superovulatory response and embryo quality in Portuguese Black Merinos. *Small Ruminant Research*, v. 74, p. 134–139, 2008.

BETTENCOURT, E.M.; BETTENCOURT, C.M.; CHAGAS E SILVA, J.; FERREIRA, P.; MATOS, C.P.; ROMÃO, R.J.; ROCHA, A. Fertility rates following the transfer of ovine embryos cryopreserved using three protocols. *Small Ruminant Research*, v. 82, n. 2, p. 112-116, 2009.

BOETTCHER, P.J.; STELLA, A.; PIZZI, F.; GANDINI, G. The combined use of embryos and semen for cryogenic conservation of mammalian livestock genetic resources. *Genetics Selection Evolution*, v. 37, p. 657–675, 2005.

BOLS, P.E. Puncture of immature ovarian follicles in bovine assisted reproduction. *Verhandelingen - Koninklijke Academie voor Geneeskunde van België*, v. 67, p. 177-202, 2005.

BOLS, P.E.; YSEBAERT, M.T.; VAN SOOM, A.; DE KRUIF, A. Effects of needle tip bevel and aspiration procedure on the morphology and developmental capacity of bovine compact *cumulus* oocyte complexes. *Theriogenology*, v. 47, p. 1221-1236, 1997.

BONI, R. Ovum pick-up in cattle: a 25 yr retrospective analysis. *Animal Reproduction* v. 9, p. 362-369, 2012.

BORMANN, C.L.; MOIGE ONGERI, E.; KRISHER, R.L. The effect of vitamins during maturation of caprine oocytes on subsequent developmental potential *in vitro*. *Theriogenology*, v. 59, p. 1373-1380, 2003.

BUHI, W.C.; ALVAREZ, I.M.; KOUBA, A.J. Oviductal regulation of fertilization and early embryonic development. *Journal of reproduction and fertility*, v. 52, p. 285-300, 1997.

CATALÁ, M.G.; IZQUIERDO, D.; UZBEKOVA, S.; MORATÓ, R.; ROURA, M.; ROMAGUERA, R.; PAPILLIER, P.; PARAMIO, M.T. Brilliant Cresyl Blue stain selects largest oocytes with highest mitochondrial activity, maturation-promoting factor activity and embryo developmental competence in prepubertal sheep. *Reproduction*, v. 142, n. 4, p. 517-27, 2011.

CHAGAS E SILVA, J.; LOPES DA COSTA, L.; CIDADÃO, R.; ROBALO SILVA, J. Plasma progesterone profiles, ovulation rate, donor embryo yield and recipient embryo survival in native Saloia sheep in the fall and spring breeding seasons. *Theriogenology*, v. 60, n. 3, p. 521-532, 2003.

CHANG, M.C. Fertilization of rabbit ova *in vitro. Nature*, 184, n. 7, 466-7, 1959.

CHAVEZ, R.; CRUZ, M.E.; DOMHINGUEZ, R. Differences in the ovulation rate of the right or left ovary in unilaterally ovariectomized rats: effect of ipsi- and contralateral vagus nerves on the remaining ovary. *The Journal of Endocrinology*, v. 113, n. 3, p. 397-401, 1987.

CHENG, F.P.; WU, J.T.; CHAN, J.P.W.; WANG, J.S.; FUNG, H.P.; COLENBRANDER, B.; TUNG, K.C. The effect of different extenders on post-thaw sperm survival, acrosomal integrity and longevity in cryopreserved semen of Formosan Sika deer and Formosan Sambar deer. *Theriogenology*, v. 61, p. 1605-1616, 2004.

COCERO, M.J.; ALABART, J.L.; HAMMAMI, S.; MARTÍ, J.I.; LAHOZ, B.; SÁNCHEZ, P.; ECHEGOYEN, E.; BECKERS, J.F.; FOLCH, J. The Efficiency of *In vitro* Ovine Embryo Production Using an Undefined or a Defined Maturation Medium is Determined by the Source of the Oocyte. *Reproduction in domestic animals*, v. 46, n. 3, p. 463-70, 2011.

COGNIÉ, Y. State of the art in sheep-goat embryo transfer. *Theriogenology*, v. 51, n. 1, p. 105-16, 1999.

COGNIÉ, Y.; BARIL, G.; POULIN, N.; MERMILLOD, P. Current status of embryo technologies in sheep and goat. *Theriogenology*, v. 59, p. 171-188, 2003.

COGNIÉ, Y.; POULIN, N.; LOCATELLI, Y.; MERMILLOD, P. State-of-the-art production, conservation and transfer of *in-vitro*-produced embryos in small ruminants. *Reproduction, Fertility and Development*, v. 16, p. 437-445, 2004.

COGNIÉ, Y.; POULIN, N.; PIGNON, P.; SULON, J.; BECKERS, J.F.; GUERIN, Y. Does heparin affect developmental ability of IVP goat oocytes? 11th, AETE, Hannover, Germany, p. 146, 1995.

COLOMA, M.A.; TOLEDANO-DÍAZ, A.; LÓPEZ-SEBASTIÁN, A.; SANTIAGO-MORENO, J. The influence of washing Spanish ibex (*Capra pyrenaica*) sperm on the effects of cryopreservation in dependency of the photoperiod. *Theriogenology*, v. 73, p. 900-908, 2010.

COMISSION ON GENETIC RESOURCES – FAO. *The State of the World's Animal Genetic Resources for Food and Agriculture*. Rome: 2007. 524p.

COMIZZOLI, P.; MERMILLOD, P.; COGNIÉ, Y.; CHAI, N.; LEGENDRE, X.; MAUGET, R. Successful *in vitro* production of embryos in the red deer (*Cervus elaphus*) and the sika deer (*Cervus nippon*). *Theriogenology*, v. 55, p. 649–659, 2001.

COMIZZOLI, P.; MERMILLOD, P.; MAUGET, R. Reproductive biotechnologies for endangered mammalian species. *Reproduction Nutrition Development*, v. 40, p. 493–504, 2000.

COONROD, S.A.; FLORES-FOXWORTH, G.; MORENO, J.F.; WESTHUSIN, M.; BYRD, S.R.; KRAEMER, D.C. Birth of Armenian red sheep (*Ovis orientalis*) lambs to domestic sheep (*Ovis aries*) following interspecific transfer of IVM-IVF derived embryos. *Theriogenology*, v. 41, p. 182, 1994.

COX, J.F.; ALFARO, V. *In vitro* fertilization and development of OPU derived goat and sheep oocytes. *Reproduction in Domestic Animals*, v. 42, p. 83-87, 2007.

COX, J.F.; SARAVIA, F.; BRIONES, M.; SANTA MARÍA, A. Dose-dependent effect of heparin on fertilizing ability of goat spermatozoa. *Theriogenology*, v. 44, p. 451-460, 1995.

COX, J.F.; ZAVALA, A.; SARAVIA, F.; RIVAS, C.; ALFARO, V. Fertilization efficiency of in vitro matured oocytes transferred to oviducts of inseminated goats: a model to assess in vivo fertilization performance of goat spermatozoa. *Theriogenology*, v. 58, p. 1-8, 2002.

COY, P.; AVILES, M. What controls polyspermy in mammals, the oviduct or the oocyte? *Biological reviews of the Cambridge Philosophical Society*, v. 85, n. 3, p. 593-605, 2010.

CROZET, N.; AHMED-ALI, M.; DUBOS, M.P. Developmental competence of goat oocytes from follicles of different size categories following maturation, fertilization and culture *in vitro*. *Journal of reproduction and fertility*, v. 103, p. 293-298, 1995.

CROZET, N.; De SMEDT, V.; AHMED-ALI, M.; SÉVELLEC, C. Normal development following *in vitro* oocyte maturation and fertilization in the goat. *Theriogenology*, v. 39, p. 206, 1993.

de CASTRO, T.; RUBIANES, E.; MENCHACA, A.; RIVERO, A. Ovarian dynamics, serum estradiol and progesterone concentrations during the interovulatory interval in goats. *Theriogenology*, v. 52, p. 399-411, 1999.

de GRAAF, S.P.; BEILBY, K.H.; UNDERWOOD, S.L.; EVANS, G.; MAXWELL, W.M. Sperm sexing in sheep and cattle: the exception and the rule. *Theriogenology*, v. 71, p. 89-97, 2009.

de MATOS, D.G.; FURNUS, C.C.; MOSES, D.F.; BALDASSARRE, H. Effect of cysteamine on glutathione level and developmental capacity of bovine oocyte matured *in vitro*. *Molecular reproduction and development*, v. 42, n. 4, p. 432-6, 1995.

de MATOS, D.G.; GASPARRINI, B.; PASQUALINI, S.R.; THOMPSON, J.G. Effect of glutathione synthesis stimulation during in vitro maturation of ovine oocytes on embryo development and intracellular peroxide content. *Theriogenology*, v. 57, p. 1443-1451, 2002.

DEMEESTERE, I.; DELBAERE, A.; GERVY, C.; VAN DEN BERGH, M.; DEVREKER, F.; ENGLERT, Y. Effect of preantral follicle isolation technique on *in vitro* follicular growth, oocyte maturation and embryo development in mice. *Human Reproduction*, v. 17, p. 2152-2159, 2002.

DEMIRCI, B.; LORNAGE, J.; SALLE, B.; POIREL, M.T.; GUERIN, J.F.; FRANCK, M. The cryopreservation of ovarian tissue: uses and indications in veterinary medicine. *Theriogenology*, v. 60, p. 999–1010, 2003.

DEMMERS, K.J.; JABBOUR, H.N.; DEAKIN, D.W.; FLINT, A.P.F. Production of interferon by red deer (*Cervus elaphus*) conceptuses and the effects of roIFN-tau on the timing of luteolysis and the success of asynchronous embryo transfer. *Journal of Reproduction and Fertility*, v. 118, p. 387–395, 2000.

DI FRANCESCO, S.; BOCCIA, L.; CAMPANILE, G.; DI PALO, R.; VECCHIO, D.; NEGLIA, G.; ZICARELLI, L.; GASPARRINI, B. The effect of season on oocyte quality and developmental competence in Italian Mediterranean buffaloes (*Bubalus bubalis*). *Animal Reproduction Science*, v. 123, p. 48-53, 2011.

DINNYES, A.; CAROLAN, C.; LONERGAN, P.; MASSIP, A.; MERMILLOD, P. Survival of frozen or vitrified bovine blastocysts produced *in vitro* in synthetic oviduct fluid. *Theriogenology*, v. 46, p. 1425-1439, 1996.

DOGAN, I.; NUR, Z.; GUNAY, U.; SAGYRKAYA, H.; SOYLU, M.K.; SONMEZ, C. Estrous synchronization during the natural breeding season in Anatolian Black does. *Veterinay Medicine – Czech*, v. 50, n. 1, p. 33–38, 2005.

DOGAN, I.; NUR, Z.; GUNAY, U.; SOYLU, M.K.; SONMEZ, C. Comparison of fluorgestone and medroxyprogesterone intravaginal sponges for oestrus synchronization in Saanen does during the transition period. *South African Journal of Animal Science*, v. 34, n. 1, p. 18–22, 2004.

DONNAY, I.; PARTRIDGE, R.J.; LEESE, H.J. Can embryo metabolism be used for selecting bovine embryos before transfer? *Reproduction, Nutrition, Development*, v. 39, n. 5-6, p. 523-33, 1999.

DUBY, R.T.; DAMIANI, P.; LOONEY, C.R.; FISSORE, R.A.; ROLB, J.M. Prepubertal calves as oocyte donors: promises and problems. *Theriogenology*, v. 45, p. 121-130, 1996.

DURRANT, B.S. The importance and potential of artificial insemination in CANDES (companion animals, non-domestic, endangered species). *Theriogenology*, v. 71, p. 113-122, 2009.

EPPIG, J.J. The participation of cyclic adenosine monophosphate (cAMP) in the regulation of meiotic maturation of oocytes in the laboratory mouse. *Journal of Reproduction and Fertility*, v. 38, p. 3-8, 1989.

EVANS, A.C.; DUFFY, P.; HYNES, N.; BOLAND, M.P. Waves of follicle development during the estrous cycle in sheep. *Theriogenology*, v. 53, n. 3, p. 699-715, 2000.

EYESTONE, W.H.; FIRST, N.L. Co-culture of early cattle embryos to the blastocyst stage with oviducal tissue or in conditioned medium. *Journal of Reproduction and Fertility*, v. 85, n. 2, p. 715-20, 1989.

FATEHI, A.N.; ROELEN, B.A.J.; COLENBRANDER, B.; SCHOEVERS, E.J.; GADELLA, B.M.; BEVERS, M.M.; VAN DEN HURK, R. Presence of *cumulus* cells during in vitro fertilization protects the bovine oocyte against oxidative stress and improves first cleavage but does not affect further development. *Zygote*, v. 13, p. 177-185, 2005.

FAUSTINO, L.R.; SANTOS, R.R.; SILVA, C.M.; PINTO, L.C.; CELESTINO, J.J.; CAMPELLO, C.C.; FIGUEIREDO, J.R.; RODRIGUES, A.P. Goat and sheep ovarian tissue cryopreservation: effects on the morphology and development of primordial follicles and density of stromal cell. *Animal Reproduction Science*, v. 122, p. 90-97, 2010.

FERNANDEZ-ARIAS, A.; FOLCH, J.; ALABART, J.L.; RAMON, J. Successful interspecific embryo transfer between Spanish ibex (*mpvrenaica*) and domestic goat (*Capra hircus*) using microosmotic pumps for FSH administration. *Theriogenology*, v. 45, p. 247, 1996.

FERRY, L.; MERMILLOD, P.; MASSIP, A.; DESSY, F. Bovine embryos cultured in serum-poor oviduct-conditioned medium need cooperation to reach the blastocyst stage. *Theriogenology*, v. 42, n. 3, p. 445-53, 1994.

FIGUEIREDO, J.R.; CELESTINO, J.J.H.; RODRIGUES, A.P.R.; SILVA, J.R.V. Importância da biotécnica de MOIFOPA para o estudo da foliculogênese e produção *in vitro* de embriões em larga escala. *Revista Brasileira de Reprodução Animal*, v. 31, n. 2, p. 143-152, 2007.

FOLCH, J.; COCERO, M.J.; CHESNÉ, P.; ALABART, J.L.; DOMÍNGUEZ, V.; COGNIÉ, Y.; ROCHE, A.; FERNÁNDEZ-ARIAS, A.; MARTÍ, J.I.; SÁNCHEZ, P.; ECHEGOYEN, E.; BECKERS, J.F.; SÁNCHEZ BONASTRE, A.; VIGNON, X. First birth of an animal from an extinct subspecies (*Capra pyrenaica pyrenaica*) by cloning. *Theriogenology*, v. 71, p. 1026-1034, 2009.

FONSECA, J.F.; BRUSCHI, J.H.; SANTOS, I.C.C.; VIANA, J.H.M.; MAGALHÃES, A.C.M. Induction of estrus in non-lactating dairy goats with different estrous synchrony protocols. *Animal Reproduction Science*, v. 85, p. 117–124, 2005.

FONSECA, J.F.; MAFFILI, V.V.; SANTOS, A.D.F.; FURST, R.; PROSPERI, C.P.; ROVAY, H.; SOUZA, J.M.G.; TORRES, C.A. Effects of prostaglandin administration 10 days apart on reproductive parameters of cyclic dairy nulliparous goats. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, v. 64, n. 2, p. 349–358, 2012.

FONSECA, J.F.; SOUZA, J.M.G. Manejo reprodutivo de caprinos e ovinos. In: SIMPÓSIO INTERNACIONAL SOBRE CAPRINOS E OVINOS DE CORTE, 5., *Anais…*, João Pessoa, PB, Brasil, 2011.

FONSECA, J.F.; SOUZA, J.M.G.; BRUSCHI, J.H.; VIANA, J.H.M.; BRANDÃO, F.Z.; SILVA, W.J.; DENADAI, R.; MAIA, A.L.R.S.; FACÓ, O. Induction of estrus in cyclic Alpine goats with short-term progestagen protocols with or without eCG administration. *Reproduction, Fertility, and Development*, v. 22, p. 169, 2010.

FONSECA, J.F.; SOUZA, J.M.G.; CAMARGO, L.S.A. Produção de Oócitos e Embriões de Pequenos Ruminantes: Passado, Presente e Futuro. *Acta Scientiae Veterinariae*, v. 38, n. 2, p. 337-369, 2010.

FONSECA, J.F.; TORRES, C.A.; SANTOS, A.D.F.; MAFFILI, V.V.; AMORIM, L.S.; MORAES, E.A. Progesterone and behavioral features when estrous is induced in Alpine goats. *Animal Reproduction Science*, v. 103, n. 3-4, p. 366–373, 2008.

FORCADA, F.; AIT AMER-MEZIANE, M.; ABECIA, J.A.; MAUREL, M.C.; CEBRIÁN-PÉREZ, J.A.; MUIÑO-BLANCO, T.; ASENJO, B.; VÁZQUEZ, M.I.; CASAO, A. Repeated superovulation using a simplified FSH/eCG treatment for *in vivo* embryo production in sheep. *Theriogenology*, v. 75, n. 4, p. 769–776, 2011.

FREITAS, V.J.F.; BARIL, G.; SAUMANDE, J. Estrus synchronization in dairy goats: use of fluorogestone acetate vaginal sponges or norgestomet ear implants. *Animal Reproduction Science*, v. 46, n. 3-4, p. 237–244, 1997.

FREITAS, V.J.F.; MELO, L.M. *In vitro* embryo production in small ruminants. *Revista Brasileira de Zootecnia*, v. 39, p. 409-413, 2010.

FREITAS, V.J.F.; SEROVA, I.A.; ANDREEVA, L.E.; DVORYANCHIKOV, G.A.; LOPES Jr, E.S.L., TEIXEIRA, D.I.A.; DIAS, L.P.; AVELAR, S.R.; MOURA, R.R.; MELO, L.M.; PEREIRA, A.F.; CAJAZEIRAS, J.B.; ANDRADE, M.L.; ALMEIDA, K.C.; SOUSA, F.C.; CARVALHO, A.C.; SEROV, O.L. Production of transgenic goat (*Capra hircus*) with human Granulocyte Colony Stimulating Factor (hG-CSF) gene in Brazil. *Anais da Academia Brasileira de Ciências*, v. 79, p. 585-592, 2007.

FUKUI, Y.; MCGOWAN, L.T.; JAMES, R.W.; PUGH, P.A.; TERVIT, H.R. Factors affecting the *in-vitro* development to blastocysts of bovine oocytes matured and fertilized *in vitro*. *Journal of Reproduction and Fertility*, v. 92, n. 1, p. 125-31, 1991.

GAAFAR, K.M.; GABR, M.K.; TELEB, D.F. The hormonal profile during estrous cycle and gestation in Damascus goat. *Small Ruminant Research*, v. 57, n. 1, v. 85-93, 2005.

GALAL, S. Biodiversity in goats. *Small Ruminant Research*, v. 60, p. 75-81, 2005.

GALLI, C.; CROTTI, G.; NOTARI, C.; TURINI, P.; DUCHI, R.; LAZZARI, G. Embryo production by ovum pick up from live donors. *Theriogenology*, v. 55, p. 1341-1357, 2001.

GANDOLFI, F.; BREVINI, T.A.L. *In vitro* maturation of farm animal oocytes: a useful tool for investigating the mechanisms leading to full-term development. *Reproduction, Fertility and Development*, v. 22, p. 495-507, 2010.

GANDOLFI, F.; MOOR, R.M. Stimulation of early embryonic development in the sheep by co-culture with oviduct epithelial cells. *Journal of Reproduction and Fertility*, v. 81, n. 1, p. 23-8, 1987.

GARCIA-GARCIA, R.M.; WARD, F.; FAIR, S.; O'MEARA, C.M.; WADE, M.; DUFFY, P.; LONERGAN, P. Development and quality of sheep embryos cultured in commercial G1.3/G2.3 sequential media. *Animal Reproduction Science*, v. 98, n. 3-4, p. 233-40, 2007.

GIBBONS, A.; PEREYRA BONNET, F.; CUETO, M.; CATALA, M.; SALAMONE, D.; GONZÁLEZ-BULNES, A. Procedure for maximizing oocyte harvest for *in vitro* embryo production in small ruminants. *Reproduction in Domestic Animals*, v. 42, p. 423-426, 2007.

GIBBONS, A.; PEREYRA BONNET, F.; CUETO, M.I.; SALAMONE, D.; CATALA, M. Recovery of sheep and goat oocytes by laparoscopy. *Acta Scientiae Veterinariae*, v. 36, p. 223-230, 2008.

GIL, M.A.; CUELLO, C.; PARRILLA, I.; VAZQUEZ, J.M.; ROCA, J.; MARTINEZ, E.A. Advances in swine *in vitro* embryo production technologies. *Reproduction in domestic animals*, v. 45, n. 2, p. 40-8, 2010.

GILCHRIST, R.B.; LANE, M.; THOMPSON, J.G. Oocyte-secreted factors: regulators of *cumulus* cell function and oocyte quality. *Human Reproduction Update*, v. 14, n. 2, p. 159-77, 2008.

GILCHRIST, R.B.; THOMPSON, J.G. Oocyte maturation: Emerging concepts and tecnhnologies to improve developmental potential in vitro. *Theriogenology*, v. 67, p. 6-15, 2007.

GINTHER, O.J.; KOT, K. Follicular dynamics during the ovulatory season in goats. *Theriogenology*, v. 42, n. 6, p. 987–1001, 1994.

GLOWKA, L.; BURHENNE-GUILMIN, F.; SYNGE, H. *A guide to the convention on biological diversity, IUCN*. IUCN – the World Conservation Union (Gland, Switzerland), 1994.

GONCALVES, R.F.; STAROS, A.L.; KILLIAN, G.J. Oviductal fluid proteins associated with the bovine zona pellucida and the effect on *in vitro* sperm-egg binding, fertilization and embryo development. *Reproduction in Domestic Animals*, v. 43, n. 6, p. 720-9, 2008.

GONZALEZ-BULNES, A.; BAIRD, D.T.; CAMPBELL, B.K.; COCERO, M.J.; GARCÍA-GARCÍA, R.M.; INSKEEP, E.K.; LÓPEZ-SEBASTIÁN, A.; MCNEILLY, A.S.; SANTIAGO-MORENO, J.; SOUZA, C.J.; VEIGA-LÓPEZ, A. Multiple factors affecting the efficiency of MOET in sheep and goats. *Reproduction, Fertility and Development*, v. 16, p. 421-435, 2004.

GONZALEZ-BULNES, A.; VEIGA-LOPEZ, A.; GARCIA, P.; GARCIA-GARCIA, R.M.; ARIZNAVARRETA, C.; SANCHEZ, M.A.; TRESGUERRES, J.A.; COCERO, M.J.; FLORES, J.M. Effects of progestagens and prostaglandin analogues on ovarian function and embryo viability in sheep. *Theriogenology*, v. 63, p. 2523-2534, 2005.

GORDON, I. *Controlled Reproduction in Sheep and Goats.* University Press, Cambridge, UK; 1997.

GORDON, J.W.; SCANGOS, G.A.; PLOTKIN, D.J.; BARBOSA, J.A.; RUDDLE, F.H. Genetic transformation of mouse embryos by microinjection of purified DNA. *Proceedings of the National Academy of Sciences of America*, v. 77, p. 7380-7384, 1980.

GRAFF, K.J.; MEINTJES, M.; DYER, V.W.; PAUL, J.B.; DENNISTON, R.S.; ZIOMEK, C.; GODKE, R.A. Transvaginal ultrasound-guided oocyte retrieval following FSH stimulation of domestic goats. *Theriogenology*, v. 51, p. 1099-1119, 1999.

GRAFF, K.J.; MEINTJES, M.; HAN, Y.; REGGIO, B.C.; DENNISTON, R.S.; GAVIN, W.G.; ZIOMEK, C.; GODKE, R.A. Comparing follicle stimulating hormone from two commercial sources for oocyte production from out-of-season dairy goats. *Journal of Dairy Science*, v. 83, p. 484-487, 2000.

GREYLING, J.P.C.; VAN DER NEST, M. Synchronization of oestrus in goats: dose effect of progestagen. *Small Ruminant Research*, v. 36, n. 2, p. 201–207, 2000.

GUIGNOT, F.; BOUTTIER, A.; BARIL, G.; SALVETTI, P.; PIGNON, P.; BECKERS, J.F.; TOUZÉ, J.L.; COGNIÉ, J.; TRALDI, A.S.; COGNIÉ, Y.; MERMILLOD, P. Improved vitrification method allowing direct transfer of goat embryos. *Theriogenology*, v. 66, p. 1004-1011, 2006.

GULER, A.; POULIN, N.; MERMILLOD, P.; TERQUI, M.; COGNIÉ, Y. Effect of growth factors, EGF and IGF-I, and estradiol on in vitro maturation of sheep oocytes. *Theriogenology*, v. 54, p. 209-218, 2000.

GUTIÉRREZ-ADÁN, A.; LONERGAN, P.; RIZOS, D.; WARD, F.A.; BOLAND, M.P.; PINTADO, B.; DE LA FUENTE, J. Effect of the *in vitro* culture system on the kinetics of blastocyst development and sex ratio of bovine embryos. *Theriogenology*, v. 55, n. 5, p. 1117-26, 2001.

HAMMAMI, S.; MORATO, R.; ROMAGUERA, R.; ROURA, M.; CATALA, M.G.; PARAMIO, M.T.; MOGAS, T.; IZQUIERDO, D. Developmental Competence and Embryo Quality of Small Oocytes from Pre-pubertal Goats Cultured in IVM Medium Supplemented with Low Level of Hormones, Insulin–Transferrin–Selenium and Ascorbic Acid. *Reproduction in Domestic Animals*, v. 48, p. 339-344, 2013.

HAMMER, R.E.; PURSEL, V.G.; REXROAD Jr, C.E.; WALL, R.J.; BOLT, D.J.; EBERT, K.M.; PALMITER, R.D.; BRINSTER, R.L. Production of transgenic rabbits, sheep and pigs by microinjection. *Nature*, v. 315, p. 680-683, 1985.

HAN, Z.B.; LAN, G.C.; WU, Y.G.; HAN, D.; FENG, W.G.; WANG, J.Z.; TAN, J.H. Interactive effects of granulosa cell apoptosis, follicle size, *cumulus*-oocyte complex morphology, and *cumulus* expansion on the developmental competence of goat oocytes: a study using the well-in-drop culture system. *Reproduction*, v. 132, n. 5, p. 749-58, 2006.

HANADA, A. *In vitro* fertilization in goats. *Journal of Animal Reproduction*, v. 31, p. 21-26, 1985.

HANSEN, P.J.; BLOCK, J.; LOUREIRO, B.; BONILLA, L.; HENDRICKS, K.E. Effects of gamete source and culture conditions on the competence of *in vitro*-produced embryos for post-transfer survival in cattle. *Reproduction, fertility, and development*, v. 22, n. 1, p. 59-66, 2010.

HARPER, K.M.; BRACKETT, B.G. Bovine blastocyst development after follicle-stimulating hormone and platelet-derived growth factor treatment for oocyte maturation *in vitro*. *Zygote*, v. 1, n. 1, p. 27-34, 1993.

HINRICHS, K. *In vitro* production of equine embryos: state of the art. *Reproduction in domestic animals*, v. 45, v. 2, p. 3-8, 2010.

HOLT, W.V.; ABAIGAR, T.; JABBOUR, H.N. Oestrous synchronization, semen preservation and artificial insemination in the Mohor gazelle (*Gazella dama mhorr*) for the establishment of a genome resource bank programme. *Reproduction, Fertility and Development*, v. 8, p. 1215–1222, 1996a.

HOLT, W.V.; BENNETT, P.M.; VOLOBOUEV, V. Genetic resource banks in wildlife conservation. *Journal of Zoology*, v. 238, p. 531-544, 1996b.

HOLT, W.V.; MOORE, H.D.M.; NORTH, R.D.; HARTMAN, T.D.; HODGES, J.K. Hormonal and behavioural detection of oestrus in blackbuck, *Antilopre cervicapra*, and successful artificial insemination with fresh and frozen semen. *Journal of Reproduction and Fertility*, v. 82, p. 717–725, 1988.

HOLT, W.V.; PICKARD, A.R. Role of reproductive technologies and genetic resource banks in animal conservation. *Reviews of Reproduction*, v. 4, p. 143–150, 1999.

ISACHENKO, V.; ALABART, J.L.; DATTENA, M.; NAWROTH, F.; CAPPAI, P.; ISACHENKO, E.; COCERO, M.J.; OLIVEIRA, J.; ROCHE, A.; ACCARDO, C.; KRIVOKHARCHENKO, A.; FOLCH, J. New technology for vitrification and field (microscope-free) warming and transfer of small ruminant embryos. *Theriogenology*, v. 59, p. 1209-1218, 2003.

ITOH, T.; HOSHI, H. Efficient isolation and long-term viability of bovine small preantral follicles *in vitro. In Vitro Cellular and. Developmental Biology. Animal*., v. 36, n. 4, p. 235-240, 2000.

JABLONKA-SHARIFF, A.; REYNOLDS, L.P.; REDMER, D.A. Effects of gonadotropin treatment and withdrawal on follicular growth, cell proliferation, and atresia in ewes. *Biology of Reproduction*, v. 55, n. 3, p. 693-702, 1996.

JOHNSTON, L.A.; PARRISH, J.J.; MONSON, R.; LEIBFRIED-RUTLEDGE, L.; SUSKO-PARRISH, J.L.; NORTHEY, D.L.; RUTLEDGE, J.J.; SIMMONS, L.G. Oocyte maturation, fertilization and embryo development in vitro and in vivo in the gaur (*Bos gaurus*). *Journal of Reproduction and Fertility*, v. 100, p. 131–136, 1994.

KARAMI SHABANKAREH, H.; SARSAIFI, K.; MEHRANNIA, T. *In vitro* maturation of ovine oocytes using different maturation media: effect of human menopausal serum. *Journal of Assisted Reproduction and Genetics*, v. 28, n. 6, p. 531-537, 2011.

KATSKA-KSIAZKIEWICZ, L.; OPIELA, J.; RYNSKA, B. Effects of oocyte quality, semen donor and embryo co-culture system on the efficiency of blastocyst production in goats. *Theriogenology*, v. 68, p. 736-744, 2007.

KATSKA-KSIAZKIEWICZ, L.; RYNSKA, B.; GAJDA, B.; SMORAG, Z. Effect of donor stimulation, frozen semen and heparin treatment on the efficiency of *in vitro* embryo production in goats. *Theriogenology*, v. 62, p. 576-586, 2004.

KEEFER, C.L.; KEYSTON, R.; LAZARIS, A.; BHATIA, B.; BEGIN, I.; BILODEAU, A.S.; ZHOU, F.J.; KAFIDI, N.; WANG, B.; BALDASSARRE, H.; KARATZAS, C.N. Production of cloned goats after nuclear transfer using adult somatic cells. *Biology of reproduction*, v. 66, n. 1, p. 199-203, 2002.

KESKINTEPE, L.; DARWISH, G.M.; YOUNIS, A.I.; BRACKETT, B.G. *In vitro* development of morulae from immature caprine oocytes. *Zygote*, v. 2, p. 97-102, 1994.

KHATUN, M.; BHUIYAN, M.M.; AHMED, J.U.; HAQUE, A.; RAHMAN, M.B; SHAMSUDDIN, M. *In vitro* maturation and fertilization of prepubertal and pubertal black Bengal goat oocytes. *Journal of Veterinary Science*, v. 12, p. 75-82, 2011.

KISHIDA, R.; LEE, E.S.; FUKUI, Y. *In vitro* maturation of porcine oocytes using a defined medum and developmental capacity after intracytoplasmic sperm injection. *Theriogenology*, v. 62, n. 9, p. 1663-76, 2004.

KLING, J. First US approval for a transgenic animal drug. *Nature Biotechnology*, v. 27, n. 4, 302-304, 2009.

KO, J.H.; LEE, C.S.; KIM, K.H.; PANG, M.G.; KOO, J.S.; FANG, N.; KOO, D.B.; OH, K.B.; YOUN, W.S.; ZHENG, G.D.; PARK, J.S.; KIM, S.J.; HAN, Y.M.; CHOI, I.Y.; LIM, J.; SHIN, S.T.; JIN, S.W.; LEE, K.K.; YOO, O.J. Production of biologically active human granulocyte colony stimulating factor in the milk of transgenic goat. *Transgenic Research*, v. 9, p. 215-222, 2000.

KOEMAN, J.; KEEFER, C.L.; BALDASSARRE, H.; DOWNEY, B.R. Developmental competence of prepubertal and adult goat oocytes cultured in semi-defined media following laparoscopic recovery. *Theriogenology*, v. 60, p. 879-889, 2003.

KORHONEN, K.K.K.; KETOJA, E.; MATOMÄKI, J.; HALMEKYTÖ, M.; PEIPPO, J. Effects of serum-free *in vitro* maturation of bovine oocytes on subsequent embryo development and cell allocation in two developmental stages of day 7 blastocysts. *Reproduction in Domestic Animals*, v. 45, p. 42-49, 2010.

LEDDA, S.; LEONI, G.; BOGLIOLO, L.; NAITANA, S. Oocyte cryopreservation and ovarian tissue banking. *Theriogenology*, v. 55, p. 1359-1371, 2001.

LEESE, H.J.; HUGENTOBLER, S.A.; GRAY, S.M.; MORRIS, D.G.; STURMEY, R.G.; WHITEAR, S.L.; SREENAN, J.M. Female reproductive tract fluids: composition, mechanism of formation and potential role in the developmental origins of health and disease. *Reproduction, Fertility and Development*, v. 20, n. 1, p. 1-8, 2008.

LEONI, G.G.; SUCCU, S.; SATTA, V.; PAOLO, M.; BOGLIOLO, L.; BEBBERE, D.; SPEZZIGU, A.; MARDEDDU, M.; BERLINGUER, F.; LEDDA, S.; NAITANA, S. *In vitro* production and cryotolerance of prepubertal and adult goat blastocysts obtained

from oocytes collected by laparoscopic oocyte-pick-up (LOPU) after FSH treatment. *Reproduction, Fertility and Development*, v. 21, p. 901-908, 2009.

LI, X.C.; YUE, H.; LI, C.Y.; HE, X.H.; ZHAO, Q.J.; MA, Y.H.; GUAN, W.J.; MA, J.Z. Establishment and characterization of a fibroblast cell line derived from Jining Black Grey goat for genetic conservation. *Small Ruminant Research*, v. 87, p. 17-26, 2009.

LOCATELLI, Y.; COGNIÉ, Y.; VALLET, J.C.; BARIL, G.; VERDIER, M.; POULIN, N.; LEGENDRE, X.; MERMILLOD, P. Successful use of oviduct epithelial cell coculture for *in vitro* production of viable red deer (Cervus elaphus) embryos. *Theriogenology*, v. 64, p. 1729-1739, 2005.

LOCATELLI, Y.; POULIN, N.; BARIL, G.; TOUZÉ, J.L.; BOUTTIER, A.; COGNIÉ, Y.; MERMILLOD, P. FSH treatment before laparoscopic ovum pick-up in goat influences quantity and quality of recovered oocytes. *Reproduction, Fertility and Development*, v. 16, p. 513, 2004.

LOCATELLI, Y.; POULIN, N.; BARIL, G.; TOUZÉ, J.L.; FATET, A.; BECKERS, J.F.; MERMILLOD, P. *In vitro* maturation treatment affects developmental competence of laparoscopic ovum pickup-derived oocytes in follicle-stimulating hormone-stimulated goats. *Reproduction Fertility and Development*, v. 20, p. 182-183, 2008.

LOCATELLI, Y.; VALLET, J.C.; HUYGHE, F.P.; COGNIÉ, Y.; LEGENDRE, X.; MERMILLOD, P. Laparoscopic ovum pick-up and *in vitro* production of sika deer embryos: Effect of season and culture conditions. *Theriogenology*, v. 66, p. 1334-1342, 2006.

LONERGAN, P.; CAROLAN, C.; VAN LANGENDONCKT, A.; DONNAY, I.; KHATIR, H.; MERMILLOD, P. Role of epidermal growth factor in bovine oocyte maturation and preimplantation embryo development *in vitro. Biology of reproduction*, v. 54, n. 6, p. 1420-9, 1996.

LONERGAN, P.; FAIR, T. *In vitro*-produced bovine embryos: dealing with the warts. *Theriogenology*, v. 69, n. 1, p. 17-22, 2008.

LONERGAN, P.; FAIR, T.; CORCORAN, D.; EVANS, A.C. Effect of culture environment on gene expression and developmental characteristics in IVF-derived embryos. *Theriogenology*, v. 65, n. 1, p. 137-52, 2006.

LONERGAN, P.; KHATIR, H.; CAROLAN, C.; MERMILLOD, P. Bovine blastocyst production *in vitro* after inhibition of oocyte meiotic resumption for 24 h. *Journal of Reproduction and Fertility*, v. 109, n. 2, p. 355-365, 1997.

LONERGAN, P.; RIZOS, D.; KANKA, J.; NEMCOVA, L., MBAYE, A.M.; KINGSTON, M.; WADE, M.; DUFFY, P.; BOLAND, M.P. Temporal sensitivity of bovine embryos to culture environment after fertilization and the implications for blastocyst quality. *Reproduction*, v. 126, p. 337-346, 2003.

LOPES JR, E.S.; MAIA, E.L.M.M.; PAULA, N.R.O.; TEIXEIRA, D.I.A.; VILLAROEL, A.B.S.; RONDINA, D.; FREITAS, V.J.F. Effect of age of donor on embryo production in Morada Nova (white variety) ewes participating in a conservation programme in Brazil. *Tropical Animal Health and Production*, v. 38, n. 7-8, p. 555-561, 2006.

LOSKUTOFF, N.M.; BARTELS, P.; MEINTJES, M.; GODKE, R.A.; SCHIEWE, M.C. Assisted reproductive technology in nondomestic ungulates: a model approach to preserving and managing genetic diversity. *Theriogenology*, v. 43, p. 3–12, 1995.

LUCCI, C.M.; AMORIM, C.A.; BÁO, S.N.; FIGUEIREDO, J.R.; RODRIGUES, A.P.; SILVA, J.R.; GONÇALVES, P.B. Effect of the interval of serial sections of ovarian in the tissue chopper on the number of isolated caprine preantral follicles. *Animal Reproduction Science*, v. 56, p. 39-49, 1999.

LUCCI, C.M.; SILVA, R.V.; CARVALHO, C.A.; FIGUEIREDO, R.; BÁO, N. Light microscopical and ultrastructural characterization of goat preantral follicles. *Small Ruminant Research*, v. 41, p. 61-69, 2001.

MAGALHÃES, D.M.; DUARTE, A.B.; ARAÚJO, V.R.; BRITO, I.R.; SOARES, T.G.; LIMA, I.M.; LOPES, C.A.; CAMPELLO, C.C.; RODRIGUES, A.P.; FIGUEIREDO, J.R. *In vitro* production of a caprine embryo from a preantral follicle cultured in media supplemented with growth hormone. *Theriogenology*, v. 75, p. 182-188, 2011.

MALIK, R.K.; LOHAN, I.S.; DHANDA, O.P.; TULI, R.K. Test for the acrosomal reaction of goat spermatozoa treated with heparin. *Small Ruminant Research*, v. 26, p. 163-166, 1997.

MARA, L.; PILICHI, S.; SANNA, A.; ACCARDO, C.; CHESSA, B.; CHESSA, F.; DATTENA, M.; BOMBOI, G.; CAPPAL, P. Sexing of *in vitro* produced ovine embryos by duplex PCR. *Molecular Reproduction and Development*, v. 69, p. 35–42, 2004.

MARCHAL, R.; FEUGANG, J.M.; PERREAU, C.; VENTURI, E.; TERQUI, M.; MERMILLOD, P. Meiotic and developmental competence of prepubertal and adult swine oocytes. *Theriogenology*, v. 56, n. 1, p. 17-29, 2001.

MARIANTE, A.S.; ALBUQUERQUE, M.S.M.; EGITO, A.A.; MCMANUS, C.; LOPES, M.A.; PAIVA, S.R. Present status of the conservation of livestock genetic resources in Brazil. *Livestock Science*, v. 120, p. 204–212, 2009.

MARIANTE, A.S.; CAVALCANTE, N. *Animals of the Discovery. Domestic breeds in the history of Brazil*. Clécia M. 2nd Ed. Brasília: Embrapa Recursos Genéticos e Biotecnologia, 2006.

MARQUES, M.G.; NICACIO, A.C.; de OLIVEIRA, V.P.; NASCIMENTO, A.B.; CAETANO, H.V.; MENDES, C.M.; MELLO, M.R.; MILAZZOTTO, M.P.; ASSUMPÇÃO, M.E.; VISINTIN, J.A. *In vitro* maturation of pig oocytes with different media, hormone and meiosis inhibitors. *Animal Reproduction Science*, v. 97, n. 3-4, p. 375-81, 2007.

MARTÍNEZ-PASTOR, F.; MARTÍNEZ, F.; ÁLVAREZ, M.; MAROTO-MORALES, A.; GARCÍA-ALVAREZ, O.; SOLER, A.J.; GARDE, J.J.; PAZ, P.; ANEL, L. Cryopreservation of Iberian red deer (*Cervus elaphus hispanicus*) spermatozoa obtained by electroejaculation. *Theriogenology*, v. 71, p. 628–638, 2009.

MARTINO, A.; PALOMO, M.J.; MOGAS, T.; PAMMIO, M.T. Influence of the collection technique of prepubertal goat oocytes on *in vitro* maturation and fertilization. *Theriogenology*, v. 42, p. 859-873, 1994.

MARTINS, F.S.; CELESTINO, J.J.; SARAIVA, M.V.; MATOS, M.H.; BRUNO, J.B.; ROCHA-JUNIOR, C.M.; LIMA-VERDE, I.B.; LUCCI, C.M.; BÁO, S.N.; FIGUEIREDO, J.R. Growth and differentiation factor-9 stimulates activation of goat primordial follicles *in vitro* and their progression to secondary follicles. *Reproduction, Fertility and Development*, v. 20, p. 916-924, 2008.

MASSIP, A.; MERMILLOD, P.; DINNYES, A. Morphology and biochemistry of *in-vitro* produced bovine embryos: Implications for their cryopreservation. *Human Reproduction*, v. 10, n. 11, p. 3004-3011, 1995.

MATTIOLI, M.; BARBONI, B. Signal transduction mechanism for LH in the *cumulus*-oocyte complex. *Molecular and Cellular Endocrinology*, v. 161, p. 19-23, 2000.

MERMILLOD, P.; DALBIES-TRAN, R.; UZBEKOVA, S.; THELIE, A.; TRAVERSO, J.M.; PERREAU, C.; PAPILLIER, P.; MONGET, P. Factors affecting oocyte quality: who is driving the follicle? *Reproduction in domestic animals*, v. 43, Suppl 2, p. 393-400, 2008.

MERMILLOD, P.; MARCHAL. R. *In-vitro* maturation of oocytes from domestic species. *Human Reproduction*, v. 14, p. 384-385, 1999.

MERMILLOD, P.; MARCHAL, R. Mammalian oocyte maturation. *M S-Medecine Sciences*, v. 15, n. 2, p. 148-156, 1999.

MERMILLOD, P.; OUSSAID, B.; COGNIÉ, Y. Aspects of follicular and oocyte maturation that affect the developmental potential of embryos. *Journal of Reproduction and Fertility*, v. 54, p. 449-460, 1999.

MERMILLOD, P.; TOMANEK, M.; MARCHAL, R.; MEIJER, L. High developmental competence of cattle oocytes maintained at the germinal vesicle stage for 24 hours in culture by specific inhibition of MPF kinase activity. *Molecular reproduction and development*, v. 55, n. 1, p. 89-95, 2000.

MOAEEN-UD-DIN, M.; YANG, L.G.; CHEN, S.L.; ZHANG, Z.R.; XIAO, J.Z.; WEN, Q.Y.; DAI, M. Reproductive performance of Matou goat under sub-tropical monsoonal climate of Central China. *Tropical Animal Health and Production*, v. 40, n. 1, p. 17-23, 2008.

MOGAS, T.; PALOMO, M.J.; IZQUIERDO, M.D.; PARAMIO, M.T. Developmental capacity of *in vitro* matured and fertilized oocytes from prepubertal and adult goats. *Theriogenology*, v. 47, n. 6, p. 1189-1203, 1997.

MORATO, R.; ROMANGUERA, R.; IZQUIERDO, D.; PARAMIO, M.T.; MOGAS, T. Vitrification of *in vitro* produced goat blastocysts: Effects of oocyte donor age and development stage. *Cryobiology*, v. 63, p. 240-244, 2011.

MORI, T.; AMANO, T.; SHIMIZU, H. Roles of Gap Junctional Communication of *Cumulus* Cells in Cytoplasmic Maturation of Porcine Oocytes Cultured *In Vitro*. *Biology of Reproduction*, v. 62, p. 913-919, 2000.

MORROW, C.J.; WOLFE, B.A.; ROTH, T.L.; WILDT, D.E.; BUSH, M.; BLUMER, E.S.; ATKINSON, M.W.; MONFORT, S.L. Comparing ovulation synchronization protocols for artificial insemination in the scimitar-horned oryx (*Oryx dammah*). *Animal Reproduction Science*, v. 59, p. 71–86, 2000.

MORTON, K.M. Developmental capabilities of embryos produced in vitro from prepubertal lamb oocytes. *Reproduction in Domestic Animals*, v. 43, p. 137-143, 2008.

MORTON, K.M.; De GRAAF, S.P.; CAMPBELL, A.; TOMKINS, L.M.; MAXWELL, W.M.; EVANS, G. Repeat ovum pick-up and in vitro embryo production from adult ewes with and without FSH treatment. *Reproduction in Domestic Animals*, v. 40, p. 422-428, 2005.

MORTON, K.M.; MAXWELL, W.M.C.; EVANS, G. Effect of Aspiration Pressure during Oocyte Harvesting on Oocyte Recovery and *in vitro* Development of Ovine Oocytes. *Reproduction in Domestic Animals*, v. 43, p. 106-110, 2008.

MOURA, R.R.; MELO, L.M.; FREITAS, V.J.F. Production of recombinant proteins in milk of transgenic and non-transgenic goats. *Brazilian Archives of Biology and Technology*, v. 54, p. 927-938, 2011.

MU, X.F.; JIN, X.L.; FARNHAM, M.M.; LI, Y.; O'NEILL, C. DNA damage-sensing kinases mediate the mouse 2-cell embryo's response to genotoxic stress. *Biology of reproduction*, v. 85, n. 3, p. 524-35, 2011.

MURUVI, W.; PICTON, H.M.; RODWAY, R.G.; JOYCE, I.M. In vitro growth and differentiation of primary follicles isolated from cryopreserved sheep ovarian tissue. *Animal Reproduction Science*, v. 112, n. 1, p. 36–50, 2009.

NIASARI-NASLAJI, A.; MOSAFERI, S.; BAHMANI, N.; GERAMI, A.; GHARAHDAGHI, A.A.; ABARGHANI, A.; GHANBARI, A. Semen cryopreservation in Bactrian camel (*Camelus bactrianus*) using SHOTOR diluent: Effects of cooling rates and glycerol concentrations. *Theriogenology*, v. 68, p. 618-625, 2007.

ONGER, E.M.; BORMANN, C.L.; BULTER, R.E.; MELICAN, D.; GAVIN, W.G.; ECHLELARD, Y.; KRISHER, R.L.; BEHBOODI, E. Development of goat embryos after *in vitro* fertilization and parthenogenetic activation by different methods. *Theriogenology*, v. 55, p. 1933–1945, 2001.

OPIELA, J.; KATSKA-KSIAZKIEWICZ, L.; LIPIŃSKI, D.; SŁOMSKI, R.; BZOWSKA, M.; RYŃSKA, B. Interactions among activity of glucose-6-phosphate dehydrogenase in immature oocytes, expression of apoptosis-related genes Bcl-2 and Bax, and developmental competence following IVP in cattle. *Theriogenology*, v. 69, n. 5, p. 546-55, 2008.

OTHEN, L.S.; BELLEM, A.C.; GARTLEY, C.J.; AUCKLAND, K.; KING, W.A.; LIPTRAP, R.M.; GOODROWE, K.L. Hormonal control of estrous cyclicity and attempted superovulation in wood bison (*Bison bison athabascae*). *Theriogenology*, v. 52, p. 313-323, 1999.

PAFFONI, A.; BREVINI, T.A.L.; GANDOLFI, F.; RAGNI, G. Parthenogenetic Activation: Biology and Applications in the ART Laboratory. *Placenta*, v. 121-125, 2008.

PALOMO, M.J.; MOGAS, T.; IZQUIERDO, D.; PARAMIO, M.T. The influence of sperm concentration, length of the gamete co-culture and the evolution of different sperm parameters on the *in vitro* fertilization of prepubertal goat oocytes. *Zygote*, v. 18, n. 4, p. 345-55, 2010.

PARAMIO, M.T. *In vivo* and *in vitro* embryo production in goats. *Small Ruminant Research*, v. 89, p. 144-114, 2010.

PARK, J.Y.; SU, Y.Q.; ARIGA, M.; LAW, E.; JIN, S.L.; CONTI, M. EGF-like growth factors as mediators of LH action in the ovulatory follicle. *Science*, v. 303, n. 5658, p. 682-684, 2004.

PARRISH, J.J.; SUSKO-PARRISH, J.; WINER, M.A.; FIRST, N.L. Capacitation of bovine sperm by heparin. *Biology of Reproduction*, v. 38, p. 1171-1180, 1988.

PAVASUTHIPAISIT, K.; LHUANGMAHAMONGKOL, S.; TOCHARUS, C.; KITIYANANT, Y.; PREMPREE, P. Porcine oviductal cells support *in vitro* bovine embryo development. *Theriogenology*, v. 41, n. 5, p. 1127-1138, 1994.

PENFOLD, L.M.; MONFORT, S.L.; WOLFE, B.A.; CITINO, S.B.; WIDLT, D.E. Reproductive physiology and artificial insemination studies in wild and captive gerenuk (*Litocranius walleri walleri*). *Reproduction, Fertility and Development*, v. 17, p. 707–714, 2005.

PEREYRA-BONNET, F.; GIBBONS, A.; CUETO, M.; SIPOWICZ, P.; FERNÁNDEZ-MARTÍN, R.; SALAMONE, D. Efficiency of sperm-mediated gene transfer in the ovine by laparoscopic insemination, *in vitro* fertilization and ICSI. *The Journal of Reproduction and Development*, v. 57, p. 188-196, 2011.

PIERSON, J.; WANG, B.; NEVEU, N.; SNEEK, L.; COTE, F.; KARATZAS, C.N.; BALDASSARRE, H. Effects of repetition, interval between treatments and season on the results from laparoscopic ovum pick-up in goats. *Reproduction, fertility, and development*, v. 16, p. 795-799, 2004.

PONTES, J.H.F.; MELO STERZA, F.A.; BASSO, A.C.; FERREIRA, C.R.; SANCHES, B.V.; RUBIN, K.C.P.; SENEDA, M.M. Ovum pick up, *in vitro* embryo production, and pregnancy rates from a large-scale commercial program using Nelore cattle (Bos indicus) donors. *Theriogenology*, v. 75, p. 1640-1646, 2011.

PONTES, J.H.F.; SILVA, K.C.F.; BASSO, A.C.; RIGO, A.G.; FERREIRA, C.R.; G.M.G., S.; SANCHES, B.V.; PORCIONATO, J.P.F.; VIEIRA, P.H.S.; FAIFER, F.S.; STERZA, F.A.M.; SCHENK, J.L.; SENEDA, M.M. Large-scale *in vitro* embryo production and pregnancy rates from *Bos taurus*, *Bos indicus*, and *indicus-taurus* dairy cows using sexed sperm. *Theriogenology*, v. 74, p. 1349-1355, 2010.

POULIN, N.; GULER, A.; PIGNON, P.; COGNIÉ, Y. *In vitro* production of goat embryos: heparin in IVF medium affect developmental ability. In: INTERNATIONAL CONFERENCE ON GOATS, 6. Pekin, 2: p. 838–40, 1996.

PTAK, G.; CLINTON, M.; BARBONI, B.; MUZZEDDU, M.; CAPPAI, P.; TISCHNER, M.; LOI, P. Preservation of the wild european mouflon: the first example of genetic management using a complete program of reproductive biotechnologies. *Biology of Reproduction*, v. 66, p. 796–801, 2002.

PTAK, G.; DATTENA, M.; LOI, P.; TISCHNER, M.; CAPPAI, P. Ovum pick-up in sheep: efficiency of *in vitro* embryo production, vitrification and birth of offspring. *Theriogenology*, v. 52, p. 1105-1114, 1999.

PTAK, G.; LOI, P.; DATTENA, M.; TISCHNER, M.; CAPPAI, P. Offspring from one-month-old lambs: studies on the developmental capability of prepubertal oocytes. *Biology of reproduction*, v. 61, p. 1568-1574, 1999.

PUKAZHENTHI, B.S.; WILDT, D.E. Which reproductive technologies are most relevant to studying, managing and conserving wildlife? *Reproduction, Fertility and Development*, v. 16, p. 33–46, 2004.

RAHMAN, A.N.M.A.; ABDULLAH, R.B.; EMBONG, W.K.W. Goat Embryo Development from *in vitro* Matured Oocytes of Heterogeneous Quality Through Intracytoplasmic Sperm Injection Technique. *Biotechnology*, v. 6, p. 373-382, 2007.

REBY, D.; CHARLTON, B.D.; LOCATELLI, Y.; McCOMB, K. Oestrous red deer hinds prefer male roars with higher fundamental frequencies. *Proceeding of the Royal Society B*, v. 277, p. 2747-2753, 2010.

REVEL, F.; MERMILLOD, P.; PEYNOT, N.; RENARD, J.P.; HEYMAN, Y. Low developmental capacity of *in vitro* matured and fertilized oocytes from calves compared with that of cows. *Journal of Reproduction and Fertility*, v. 103, n. 1, v. 115-20, 1995.

RHO, G.J.; HAHNEL, A.; BETTERIDGE, K.J. Comparisons of oocyte maturation times and of three methods of sperm penetration for their effects on the production of goat embryos *in vitro*. *Theriogenology*, v. 56, p. 503-516, 2001.

RIBEIRO, M.N.; BRUNO-DE-SOUSA, C.; MARTINEZ-MARTINEZ, A.; GINJA, C.; MENEZES, M.P.C.; PIMENTA-FILHO, E.C.; DELGADO, J.V.; GAMA, L.T. Drift across the Atlantic: genetic differentiation and population structure in Brazilian and Portuguese native goat breeds. *Journal of Animal Breeding and Genetics*, v. 129, p. 79-87, 2012.

RIZOS, D.; CLEMENTE, M.; BERMEJO-ALVAREZ, P.; DE LA FUENTE, J.; LONERGAN, P.; GUTIÉRREZ-ADÁN, A. Consequences of *in vitro* culture conditions

on embryo development and quality. *Reproduction in Domestic Animals*, v. 43, n. 4, p. 44-50, 2008.

RIZOS, D.; RAMIREZ, M.A.; PINTADO, B.; LONERGAN, P.; GUTIERREZ-ADAN, A. Culture of bovine embryos in intermediate host oviducts with emphasis on the isolated mouse oviduct. *Theriogenology*, v. 73, n. 6, p. 777-785, 2010.

RIZOS, D.; WARD, F.; BOLAND, M.P.; LONERGAN, P. Effect of culture system on the yield and quality of bovine blastocysts as assessed by survival after vitrification. *Theriogenology*, v. 56, n. 1, p. 1-16, 2001.

RIZOS, D.; WARD, F.; DUFFY, P.; BOLAND, M.P.; LONERGAN, P. Consequences of bovine oocyte maturation, fertilization or early embryo development *in vitro* versus *in vivo*: implications for blastocyst yield and blastocyst quality. *Molecular Reproduction and Development*, v. 61, p. 234-248, 2002.

RODRIGUEZ, C.; ANEL, L.; ALVAREZ, M.; ANEL, E.; BOIXO, J.C.; CHAMORRO, C.A.; DE PAZ, P. Ovum Pick-up in Sheep: a Comparison between Different Aspiration Devices for Optimal Oocyte Retrieval. *Reproduction in Domestic Animals*, v. 41, p. 106-113, 2006.

RODRIGUEZ-DORTA, N.; COGNIÉ, Y.; GONZALEZ, F.; POULIN, N.; GUIGNOT, F.; TOUZE, J.L.; BARIL, G.; CABRERA, F.; ALAMO, D.; BATISTA, M.; GRACIA, A.; MERMILLOD, P. Effect of coculture with oviduct epithelial cells on viability after transfer of vitrified *in vitro* produced goat embryos. *Theriogenology*, v. 68, p. 908-913, 2007.

RODRIGUEZ-GONZALEZ, E.; LOPEZ-BEJAR, M.; VELILLA, E.; PARAMIO, M.T. Selection of prepubertal goat oocytes using the brilliant cresyl blue test. *Theriogenology*, v. 57, p. 1397-1409, 2002.

ROMAGUERA, R.; CASANOVAS, A.; MORATÓ, R.; IZQUIERDO, D.; CATALÁ, M.; JIMENEZ-MACEDO, A.R.; MOGAS, T.; PARAMIO, M.T. Effect of follicle diameter on oocyte apoptosis, embryo development and chromosomal ploidy in prepubertal goats. *Theriogenology*, v. 74, n. 3, p. 364-373, 2010.

ROMAGUERA, R.; MOLL, X.; MORATO, R.; ROURA, M.; PALOMO, M.J.; CATALA, M.G.; JIMENEZ-MACEDO, A.R.; HAMMAMI, S.; IZQUIERDO, D.; MOGAS, T.; PARAMIO, M.T. Prepubertal goat oocytes from large follicles result in

similar blastocyst production and embryo ploidy than those from adult goats. *Theriogenology*, v. 76, p. 1-11, 2011.

ROMAGUERA, R.; MORATÓ, R.; JIMÉNEZ-MACEDO, A.R.; CATALÁ, M.; ROURA, M.; PARAMIO, M.T.; PALOMO, M.J.; MOGAS, T.; IZQUIERDO, D. Oocyte secreted factors improve embryo developmental competence of COCs from small follicles in prepubertal goats. *Theriogenology*, v. 74, n. 6, p. 1050-9, 2010.

ROMANO, J.E. Synchronization of estrus using CIDR, FGA or MAP intravaginal pessaries during the breeding season in Nubian goats. *Small Ruminant Research*, v. 55, p. 15–19, 2004.

SALHAB, M.; PAPILLIER, P.; PERREAU, C.; GUYADER-JOLY, C.; DUPONT, J.; MERMILLOD, P.; UZBEKOVA, S. Thymosins beta-4 and beta-10 are expressed in bovine ovarian follicles and upregulated in *cumulus* cells during meiotic maturation. *Reproduction, fertility, and development*, v. 22, n. 8, p. 1206-21, 2010.

SALHAB, M.; TOSCA, L.; CABAU, C.; PAPILLIER, P.; PERREAU, C.; DUPONT, J.; MERMILLOD, P.; UZBEKOVA, S. Kinetics of gene expression and signaling in bovine *cumulus* cells throughout IVM in different mediums in relation to oocyte developmental competence, *cumulus* apoptosis and progesterone secretion. *Theriogenology*, v. 75, n. 1, p. 90-104, 2011.

SALLE, B.; DEMIRCI, B.; FRANCK, M.; RUDIGOZ, R.C.; GUERIN, J.F.; LORNAGE, J. Normal pregnancies and live births after autograft of frozen-thawed hemi-ovaries into ewes. *Fertility and Sterility*, v. 77, p. 403-408, 2002.

SANTIAGO-MORENO, J.; TOLEDANO-DÍAZ, A.; PULIDO-PASTOR, A.; GÓMEZ-BRUNET, A.; LÓPEZ-SEBASTIÁN, A. Birth of live Spanish ibex (*Capra pyrenaica hispanica*) derived from artificial insemination with epididymal spermatozoa retrieved after death. *Theriogenology*, v. 66, p. 283-291, 2006.

SANTOS, R.R.; KNIJN, H.M.; VOS, P.L.; OEI, C.H.; VAN LOON, T.; COLENBRANDER, B.; GADELLA, B.M.; VAN DEN HURK, R.; ROELEN, B.A. Complete follicular development and recovery of ovarian function of frozen–thawed, autotransplanted caprine ovarian cortex. *Fertility and Sterility*, v. 91, p. 1455-1458, 2009.

SARAGUSTY, J.; GACITUA, H.; KING, R.; ARAV, A. Post-mortem semen cryopreservation and characterization in two different endangered gazelle species (*Gazella gazella* and *Gazella dorcas*) and one subspecies (*Gazella gazelle acaiae*). *Theriogenology*, v. 66, p. 775-784, 2006.

SARAIVA, M.V.; ROSSETTO, R.; BRITO, I.R.; CELESTINO, J.J.; SILVA, C.M.; FAUSTINO, L.R.; ALMEIDA, A.P.; BRUNO, J.B.; MAGALHÃES, D.M.; MATOS, M.H.; CAMPELLO, C.C.; FIGUEIREDO, J.R. Dynamic medium produces caprine embryo from preantral follicles grown *in vitro*. *Reproductive Sciences*, v. 17, n. 12, p. 1135-1143, 2010.

SASAMOTO, Y.; SAKAGUCHI, M.; KATAGIRI, S.; YAMADA, Y.; TAKAHASHI, Y. The effects of twisting and type of aspiration needle on the efficiency of transvaginal ultrasound-guided ovum pick-up in cattle. *Journal of Veterinary Medical Science*, v. 65, p. 1083-1086, 2003.

SCHMIDT, C. Belated approval of first recombinant protein from animal. *Nature Biotechnology*, v. 24, n. 8, p. 877, 2006.

SHABANKAREH, H.K.; SARSAIFI, K.; MEHRANNIA, T. *In vitro* maturation of ovine oocytes using different maturation media: effect of human menopausal serum. *Journal of Assisted Reproduction and Genetics*, v. 28, n. 6, p. 531-537, 2011.

SHABANKAREH, H.K.; ZANDI, M. Developmental potential of sheep oocytes cultured in different maturation media: effects of epidermal growth factor, insulin-like growth factor I, and cysteamine. *Fertility and Sterility*, v. 94, n. 1, p. 335-340, 2010.

SHIRAZI, A.; ARDALI, M.A.; AHMADI, E.; NAZARI, H.; MAMUEE, M.; HEIDARI, B. The Effect of Macromolecule Source and Type of Media During *in vitro* Maturation of Sheep Oocytes on Subsequent Embryo Development. *Journal of Reproduction and Infertility*, v. 13, p. 13-19, 2012.

SHIRAZI, A.; SHAMS-ESFANDABADI, N.; AHMADI, E.; HEIDARI, B. Effects of Growth Hormone on Nuclear Maturation of Ovine Oocytes and Subsequent Embryo Development. *Reproduction in Domestic Animals*, v. 45, p. 530-536, 2010.

SILVA, J.C.B.; OKABE, W.K.; TRALDI, A.S. From cattle to sheep: a view of the difficulties and success of commercial *in vitro* production of sheep embryos. *Animal Reproduction*, v. 9, p. 195-200, 2012.

SIMÕES, J.; BARIL, G.; ALMEIDA, J.C.; AZEVEDO, J.; FONTES, P.; MASCARENHAS, R. Time of ovulation in nulliparous and multiparous goats. *Animal*, v. 2, n. 5, p. 761-768, 2008.

SINCLAIR, K.D.; MCEVOY, T.G.; MAXFIELD, E.K.; MALTIN, C.A.; YOUNG, L.E.; WILMUT, I.; BROADBENT, P.J.; ROBINSON, J.J. Aberrant fetal growth and development after *in vitro* culture of sheep zygotes. *Journal of reproduction and fertility*, v. 116, n. 1, 177-86, 1999.

SIRARD, M.A.; FIRST, N.L. *In vitro* inhibition of oocyte nuclear maturation in the bovine. *Biology of Reproduction*, v. 39, n. 2, p. 229-34, 1988.

SIRARD, M.A.; RICHARD, F.; BLONDIN, P.; ROBERT, C. Contribution of the oocyte to embryo quality. *Theriogenology*, v. 65, n. 1, p. 126-36, 2006.

SMORAG, Z.; KATSKA-KSIAZKIEWICZ, L.; SKRZYSZOWSKA, M.; JURA, J.; GAJDA, B.; BOCHENEK, M. Animal reproduction biotechnology in Poland. *The International Journal of Developmental Biology*, v. 52, n. 2-3, p. 151-5, 2008.

SNOW, M.; CLEARY, M.; COX, S.L.; SHAW, J.M.; PARIS, M.C.J.; JENKIN, G. Comparison of the effects of in vitro and in situ storage on the viability of mouse ovarian tissue collected after death. *Reproduction, Fertility and Development*, v. 13, p. 389–394, 2001.

SOLER, A.J.; ASTORE, V.; SESTELO, A.; RIVOLTA, M.; JÁCOME, L.N.; GARDE, J.J. Effect of thawing procedure on cryosurvival of deer spermatozoa: work in progress. *Theriogenology*, v. 60, p. 511-520, 2003.

SOLTI, L.; CRICHTON, E.G.; LOSKUTOFF, N.M.; CSEH, S. Economical and ecological importance of indigenous livestock and the application of assisted reproduction to their preservation. *Theriogenology*, v. 53, p. 149-162, 2000.

SOUZA, J.M.G.; BATISTA, R.I.T.P.; MELO, L.M.; FREITAS, V.J.F. Reproductive biotechnologies applied to the conservation of endangered ruminant - Past, Present and Future. *Revista Portuguesa em Ciências Veterinárias*, v. 110, n. 577-580, p. 31–38, 2011.

SOUZA, J.M.G.; DUFFARD, N.; BERTOLDO, M.J.; LOCATELLI, Y.; CORBIN, E.; FATET, A.; FREITAS, V.J.F.; MERMILLOD, P. Influence of heparin or the presence of *cumulus* cells during fertilization on the *in vitro* production of goat embryos. *Animal Reproduction Science*, v. 138, p. 82-89, 2013.

SOUZA, J.M.G.; TORRES, C.A.A.; MAIA, A.L.R.S.; BRANDÃO, F.Z.; BRUSCHI, J.H.; VIANA, J.H.M.; OBA, E.; FONSECA, J.F. Autoclaved, previously used intravaginal progesterone devices induces estrus and ovulation in anestrous Toggenburg goats. *Animal Reproduction Science*, v. 129, n. 1-2, p. 50–55, 2011.

SOUZA, J.M.G.; TORRES, C.A.A.; RIBEIRO, S.D.A.; RIBEIRO, A.C.; BECALETE, L.; RIGO, A.G.; SILVA, W.J.; FACÓ, O.; FONSECA, J.F. Induction of estrus in Anglo-Nubian goats in the transition season with short-term progestagen protocols with or without eCG administration. *Reproduction, Fertility and Development*, v. 22, p. 380, 2010.

STANGL, M.; KUHHOLZER, B.; BESENFELDER, U.; BREM, G. Repeated endoscopic ovum pick-up in sheep. *Theriogenology*, v. 52, p. 709-716, 1999.

STOVER, J.; EVANS, J.; DOLENSEK, E.P. Inter species embryo transfer from the gaur to domestic Holestein. In: ANNUAL MEETING OF AMERICAN ASSOCIATION OF ZOO VETERINARIANS, Woodland Park Zoo, USA. *Proceedings…* p. 122–124, 1981.

SUCCU, S.; LEONI, G.G.; BERLINGUER, F.; MADEDDU, M.; BEBBERE, D.; MOSSA, F.; BOGLIOLO, L.; LEDDA, S.; NAITANA, S. Effect of vitrification solutions and cooling upon *in vitro* matured prepubertal ovine oocytes. *Theriogenology*, v. 68, p. 107-114, 2007.

SUITER, J. Body condition scoring in sheep and goats. Farmnonte 69/94. Available at: <*http://www.agric.wa.gov.au/content/aap/sl/m/fn069_1994.htm*>, 1994. Acesso em 23 de Setember, 2010.

SUTTON, M.L.; GILCHRIST, R.B.; THOMPSON, J.G. Effects of *in-vivo* and *in-vitro* environments on the metabolism of the *cumulus*-oocyte complex and its influence on oocyte developmental capacity. *Human Reproduction Update*, v. 9, n. 1, p. 35-48, 2003.

SUZUKI, K.; ERIKSSON, B.; SHIMIZU, H.; NAGAI, T.; RODRIGUEZ-MARTINEZ, H. Effect of hyaluronan on monospermic penetration of porcine oocytes fertilized *in vitro*. *International Journal of Andrology*, v. 23, p. 13-21; 2000.

TAKAHASHI, Y.; FIRST, N.L. *In vitro* development of bovine one-cell embryos: influence of glucose, lactate, pyruvate, amino acids and vitamins. *Theriogenology*, v. 37, p. 963-978, 1992.

TAKUMA, T.; SAKAI, S.; EZOE, D.; ICHIMARU, H.; JINNOUCHI, T.; KAEDEI, Y.; NAGAI, T.; OTOI, T. Effects of season and reproductive phase on the quality, quantity and developmental competence of oocytes aspirated from Japanese black cows. *Journal of Reproduction and Development*, v. 56, p. 55-59, 2010.

TALEBI, J.; MOGHADDAM, A.; SOURI, M.; MIRMAHMOUDI, R. Steroid hormone profile of Markhoz does (Iranian Angora) throughout estrous cycle and gestation period. *Tropical Animal Health and Production*, v. 44, p. 355-360, 2012.

TAN, W.L.; WAN KHADIJAH, W.E.; ABDULLAH, R.B. Effect of sperm insemination duration on *in vitro* fertilisation (IVF) performance on goats. *Malaysian Journal of Science*, v. 30, p. 105-111, 2011.

TAO TAO, A.R.; NADEAU, S.; SAVOIE, R.; GALLANT, B.; OUELLETTE, R. Effect of *cumulus* cell removal on the fertilization and the day 3 embryo quality in human IVF. *International Congress Series*, v. 1271, p. 135-138, 2004.

TARU SHARMA, G.; MAJUMDAR, A.C.; BONDE, S.W. Chronology of maturational events in goat oocytes cultured *in vitro*. *Small Ruminant Research*, v. 22, p. 25-30, 1996.

TATEMOTO, H.; SAKURAI, N.; MUTO, N. Protection of porcine oocytes against apoptotic cell death caused by oxidative stress during *in vitro* maturation: role of *cumulus* cells. *Biology of Reproduction*, v. 63, p. 805-810, 2000.

TEIXEIRA, P.P.; PADILHA, L.C.; OLIVEIRA, M.E.; MOTHEO, T.F.; DA SILVA, A.S.; BARROS, F.F.; COUTINHO, L.N.; FLÔRES, F.N.; LOPES, M.C.; BANDARRA, M.B.; SILVA, M.A.; VASCONCELOS, R.O.; RODRIGUES, L.F.; VICENTE, W.R. Laparoscopic ovum collection in sheep: Gross and microscopic evaluation of the ovary and influence on ooctye production. *Animal Reproduction Science*, v. 127, n. 3-4, p. 169-175, 2011.

TERVIT, H.R. Laparoscopy/laparotomy oocyte recovery and juvenile breeding. *Animal Reproduction Science*, v. 42, p. 227-238, 1996.

TERVIT, H.R.; WHITTINGHAM, D.G.; ROWSON, L.E. Successful culture *in vitro* of sheep and cattle ova. *Journal of reproduction and fertility*, v. 30, n. 3, p. 493-7, 1972.

THÉLIE, A.; PAPILLIER, P.; PENNETIER, S.; PERREAU, C.; TRAVERSO, J.M.; UZBEKOVA, S.; MERMILLOD, P.; JOLY, C.; HUMBLOT, P.; DALBIÈS-TRAN, R. Differential regulation of abundance and deadenylation of maternal transcripts during bovine oocyte maturation *in vitro* and *in vivo*. *BMC developmental biology*, v. 7, p. 125, 2007.

THUNDATHIL, J.; WHITESIDE, D.; SHEA, B.; LUDBROOK, D.; ELKIN, B.; NISHI, J. Preliminary assessment of reproductive technologies in wood bison (*Bison bison athabascae*): Implications for preserving genetic diversity. *Theriogenology*, v. 68, p. 93-99, 2007.

TIBARY, A.; ANOUASSI, A.; KHATIR, H. Update on reproductive biotechnologies in small ruminants and camelids. *Theriogenology*, v. 64, n. 3, p. 618-38, 2005.

TSAI, T.C.; WU, S.H.; CHEN, H.L.; TUNG, Y.T.; CHENG, W.T.; HUANG, J.C.; CHEN, C.M. Identification of Sex-Specific Polymorphic Sequences in Goat Amelogenin Gene for Embryo Sexing. *Journal of Animal Science*, v. 89, n. 8, p. 2407-14, 2011.

VAJTA, G. Vitrification of the oocytes and embryos of domestic animals. *Animal Reproduction Science*, v. 60-61, p. 357-364, 2000.

VAJTA, G.; MURPHY, C.; MACHÁTY, Z.; PRATHER, R.S. In straw dilution of *in vitro* produced bovine blastocysts after vitrification with the open pulledstraw (OPS) method. *Veterinary Record*, v. 144, p. 180–181, 1999.

VAZQUEZ, M.I.; ABECIA, J.A.; FORCADA, F.; CASAO, A. Effects of exogenous melatonin on *in vivo* embryo viability and oocyte competence of undernourished ewes after weaning during the seasonal anestrus. *Theriogenology*, v. 74, p. 618-626, 2010.

VAZQUEZ, M.I.; FORCADA, F.; CASAO, A.; ABECIA, J.A.; SOSA, C.; PALACIN, I. Undernutrition and exogenous melatonin can affect the in vitro developmental competence of ovine oocytes on a seasonal basis. *Reproduction in Domestic Animals*, v. 45, p. 677-684, 2010.

VERGARA, G.J.; IRWIN, M.H.; MOFFATT, R.J.; PINKERT, C.A. *In vitro* fertilization in mice: Strain differences in response to superovulation protocols and effect of *cumulus* cell removal. *Theriogenology*, v. 47, p. 1245-1252, 1997.

VIANA, J.H.; DE ALMEIDA CAMARGO, L.S.; DE MORAES FERREIRA, A.; DE SA, W.F.; DE CARVALHO FERNANDES, C.A.; DE PINHO MARQUES JUNIOR, A. Short intervals between ultrasonographically guided follicle aspiration improve oocyte quality but do not prevent establishment of dominant follicles in the Gir breed (*Bos indicus*) of cattle. *Animal Reproduction Science*, v. 84, p. 1-12, 2004.

VIGNERON, C.; PERREAU, C.; DUPONT, J.; UZBEKOVA, S.; PRIGENT, C.; MERMILLOD, P. Several signaling pathways are involved in the control of cattle oocyte maturation. *Molecular Reproduction and Development*, v. 69, n. 4, p. 466-74, 2004.

WANG, B.; BALDASSARRE, H.; PIERSON, J.; COTE, F.; RAO, K.M.; KARATZAS, C.N. The *in vitro* and *in vivo* development of goat embryos produced by intracytoplasmic sperm injection using tail-cut spermatozoa. *Zygote*, v. 11, n. 3, p. 219-27, 2003.

WANG, B.; BALDASSARRE, H.; TAO, T.; GAUTHIER, M.; NEVEU, N.; ZHOU, J.F.; LEDUC, M.; DUGUAY, F.; BILODEAU, A.S.; LAZARIS, A.; KEEFER, C.; KARATZAS, C.N. Transgenic goats produced by DNA pronuclear microinjection of *in vitro* derived zygotes. *Molecular, Reproduction and Development*, v. 63, n. 4, p. 437-43, 2002.

WANI, N.A.; WANI, G.M.; KHAN, M.Z.; SIDIQI, M.A. Effect of different factors on the recovery rate of oocytes for *in vitro* maturation and *in vitro* fertilization procedures in sheep. *Small Ruminant Research*, v. 34, p. 71-76, 1999.

WILMUT, I.; SCHNIEKE, A.E.; MCWHIR, J.; KIND, A.J.; CAMPBELL, K.H.S. Viable offspring derived from fetal and adult mammalian cells. *Nature*, v. 385, p. 810–813, 1997.

WIRTU, G.; POPE, C.E.; PACCAMONTI, D.L.; GODKE, R.A.; DRESSER, B.L. Ultrasonographic-guided retrieval and *in vitro* maturation of eland (*Taurotragus oryx*) and bongo (*Tragelaphus eurycerus isaaci*) antelope oocytes. *Animal Reproduction Science*, v. 111, n. 2-4, p. 160–172, 2009.

WOLVEKAMP, M.C.; CLEARY, M.L.; COX, S.L.; SHAW, J.M.; JENKIN, G.; TROUNSON, A.O. Follicular development in cryopreserved Common Wombat ovarian tissue xenografted to Nude rats. *Animal Reproduction Science*, v. 65, n. 1-2, p. 135–147, 2001.

WRENZYCKI, C.; HERRMANN, D.; NIEMANN, H. Messenger RNA in oocytes and embryos in relation to embryo viability. *Theriogenology*, v. 68, n. 1, p. 77-83, 2007.

YADAV, P.S.; SAINI, A.; KUMAR, A.; JAIN, G.C. Effect of oviductal cell co-culture on cleavage and development of goat IVF embryos. *Animal Reproduction Science*, v. 51, n. 4, p. 301-6, 1998.

YOUNIS, A.I.; ZUELKE, K.A.; HARPER, K.M.; OLIVEIRA, M.A.; BRACKETT, B.G. *In vitro* fertilization of goat oocytes. *Biology of Reproduction*, v. 44, p. 1177-1182, 1991.

ZHANG, L.; JIANG, S.; WOZNIAK, P.J.; YANG, X.; GODKE, R.A. *Cumulus* cell function during bovine oocyte maturation, fertilization, and embryo development *in vitro*. *Molecular, Reproduction and Development*, v. 40, p. 338-344, 1995.

ZOHEIR, K.M.; ALLAM, A.A. A rapid method for sexing the bovine embryo. *Animal Reproduction Science*, v. 119, n. 1-2, p. 92-96, 2010.

12 APÊNDICES

12.1 APÊNDICE A

RESUMO PUBLICADO: Anais do Congresso Mundial de Caprinos, IGA, 2012*.

INFLUENCE OF *CUMULUS* CELLS ATTACHMENT DURING *IN VITRO* FERTILIZATION OF *IN VITRO* MATURED GOAT OOCYTES

Souza, J.M.G.[1,2*], N. Duffard[1,3], M.J. Bertoldo[1], Y. Locatelli[1,3], A. Fatet[1], E. Corbin, V.J.F. Freitas[2], P. Mermillod[1]

[1]INRA, UMR 7247, Physiologie de la Reproduction et des Comportements, INRA, CNRS, Université de Tours, IFCE. 37380 Nouzilly, France; [2]Laboratory of Physiology and Control of Reproduction, Veterinary School, State University of Ceará, Av. Dedé Brasil, 1700, 60740-90, Fortaleza, CE, Brazil; [3]Museum National d'Histoire Naturelle, Parc Animalier de la Haute Touche, 36290, Obterre, France. *Bolsista CAPES E-mail: joannavet@gmail.com

Considerable research into IVP technology has been undertaken to enhance embryo production. However, specific questions are yet to be answered. The objective of the present study was to assess the influence of *cumulus* cell presence when not attached to oocyte during IVF on embryo development and to evaluate the possibility of using oocytes found denuded at collection to produce embryos. Throughout five collections of slaughterhouse goat ovaries, all oocytes presenting a homogenous ooplasm were selected. Most (1316) were *cumulus* oocyte complexes (COC) while some (*n*=381) were already denuded at collection (DOC). COC and DOC were washed and placed in wells containing 50 oocytes in 500 µL of maturation medium (TCM 199 supplemented with 10 ng/mL EGF and 100 µM/mL cysteamine). Matured oocytes were incubated with frozen-thawed semen in SOF, containing 10% heat-inactivated sheep serum, 40 µg/mL gentamicin and 5 µg/mL heparin. Some oocytes were denuded on purpose (DOP) by vortex before IVF. After fertilization, all presumptive zygotes were denuded by vortex and cultured for 8 days in groups of 25 in oil overlaid droplets of 25 µL of SOF medium supplemented with 10% FCS at 48 hpi. The proportions of oocytes that cleaved and

developed to the blastocyst stage were assessed at Days 2 and 8 post-insemination, respectively. Cleavage rate and blastocyst yield were compared using ANOVA followed by Tukey's multiple comparisons. The cleavage and blastocyst rates calculated from total oocytes were lower for DOC (33%±1.6; 11%±2.1, respectively, mean±SEM) as compared to COC (76%±3.5; 59%±4.4). However, when equal numbers of COC and DOC (25/25) were mixed at the start of IVM (COC DOC IVM), the overall cleavage and blastocysts rates were similar to COC (67%±1.4; 44%±3.6), indicating that the presence of DOC is not detrimental to COC and that COC could rescue some DOC. When DOC and COC were mixed equally for IVF (COC DOC IVF), the rates were significantly reduced (57%±2.3; 39%±1.2) compared to COC alone. Removal of *cumulus* before IVF (DOP) resulted in a tendency to reduce cleavage and development rates (69%±4.3; 46%±3.9) as compared to COC. This tendency was not observed when unattached *cumulus* cells (DOPCC: 72%±4.7; 52%±4.3) or equal number of COC were added to DOP during IVF (DOPCOC: 70%±2.8; 52%±5.0). Collectively, these data suggest the possibility to use denuded oocytes to increase *in vitro* production of goat embryos and that unattached *cumulus* cells added during IVF may help denuded oocytes to be fertilized.

This work was supported by a grant from Région Centre, France (PIVER program, #200800030493, 2008-2011) and CAPES, Brazil.

***Resumo selecionado e apresentado oralmente durante a XI International Conference on Goats (IGA), Gran Canaria, Espanha, 2012.**

12.2 APÊNDICE B

RESUMO PUBLICADO: *Reproduction in Domestic Animals*, **47 (Supl. 4)**: 511, 2012.

HEPARIN AND *CUMULUS* CELLS AFFECT *IN VITRO* FERTILIZATION AND DEVELOPMENTAL COMPETENCE OF GOAT OOCYTES

Joanna M G Souza[1,2*], Nicolas Duffard[1,3], Michael J Bertoldo[1], Christine Perreau[1], Yann Locatelli[1,3], Alice Fatet[1], Vicente J F Freitas[2], Pascal Mermillod[1]

1Unité de Physiologie de la Reproduction et des Comportements, INRA, Nouzilly, France; 2Laboratory of Physiology and Control of Reproduction, Veterinary School, State University of Ceará, Fortaleza, CE, Brazil; 3Laboratoire de l'espace animalier de la Haute Touche, MNHN, Obterre, France. *Bolsista CAPES E-mail: joannavet@gmail.com

Considerable research has been focused on *in vitro* production (IVP) of goat embryos to improve its efficiency. However, simplified methods providing high and reproducible results would allow a wider use of this promising technology. The aim of this study was to observe the effects of FSH in maturation medium, the use of heparin in fertilization medium and the time of *cumulus* cell removal on embryo development in goats. During six replicates, slaughterhouse-derived oocytes were randomly allocated to two IVM groups: TCM 199 supplemented with 10 ng/mL EGF and 100 µM/mL cysteamine with or without 50 ng/mL oFSH. Matured oocytes were incubated with frozen-thawed semen in synthetic oviduct fluid (SOF), containing 10% heat-inactivated sheep serum, 40 µg/mL gentamicin and the presence of 5 µg/mL heparin or not. The *cumulus* oophorus was removed by vortex before (denuded oocyte group; DO) or after (*cumulus* oocyte complex group; COC) fertilization (total of 8 different treatments). After fertilization, presumptive zygotes were cultured for 8 days in groups of 25 in oil overlaid droplets of 25 µL of SOF medium supplemented with 10% FCS at 48 hpi. The proportions of oocytes that cleaved and developed to the blastocyst stage were assessed at Days 2 and 8 post-insemination, respectively. Cleavage rate and blastocyst yield were compared using chi-square analysis with $P<0.05$ considered significant. No differences were observed in cleavage rate among all conditions. Similarly, FSH did not influence

embryo development (not shown). However in COC, the presence of heparin tended to increase cleavage rate, as well as development of cleaved embryos to the blastocyst stage. This resulted in a significantly increased yield of blastocysts from oocytes entering into IVM ($P<0.05$; Table 1). This effect was not observed in DO. In conclusion, these data indicate that when EGF and cysteamine are added to maturation media, FSH offers no further benefit to developmental competence. However, embryo development can significantly be affected by the moment oocytes are denuded and the presence or absence of heparin during fertilization. These results suggest that *cumulus* cells have an important role in the regulation of *in vitro* fertilization of goat oocytes and heparin enhances these interactions.

Table 1. Effect of heparin on intact *cumulus*-oocyte complexes (COC) or denuded oocytes (DO) during IVF on *in vitro* production of goat embryos (6 replicates).

Conditions	Heparin	Oocytes (n)	Cleavage (%)	Day 8 pi (%)	
				from cleaved	oocytes
COC	-	365	66[a]	63[a]	42[a]
	+	364	72[a]	75[a]	54[b]
DO	-	360	64[a]	59[a]	38[a]
	+	358	68[a]	61[a]	41[a]

Within a column, values with different superscripts differ significantly ($P<0.05$).
pi: post-insemination

Resumo apresentado no International Conference on Animal Reproduction (ICAR), Vancouver, Canadá, 2012.

12.3 APÊNDICE C

RESUMO PUBLICADO: *Acta Scientiae Veterinariae*, **39 (Supl. 1):** 411, 2011.

IN VITRO EMBRYO PRODUCTION (IVEP) AFTER LAPAROSCOPIC OOCYTE RECOVERY (LOR) IN CANINDÉ GOATS

Pereira, A.F.; Moura, R.R.; Batista, R.I.T.P.; **Souza, J.M.G.;** Alcantara Neto, A.S.; Melo, C.H.S.; Campelo I.S.; Vieira M.P.; Teixeira, D.I.A.; Melo, L.M.; Freitas, V.J.F.

Laboratório de Fisiologia e Controle da Reprodução, Faculdade de Veterinária, Universidade Estadual do Ceará – Fortaleza-CE, Brasil. E-mail: vjff@pq.cnpq.br

Currently, a major concern of livestock is the biodiversity preservation. In Northeast Brazil, there are several naturalized goat breeds at risk of extinction, including the Canindé. Reproductive biotechnologies could participate in this process. From these, IVEP after LOR may accelerate the genetic material recovery. Nevertheless, few studies demonstrate the real efficiency of this system in goats. Therefore, the aim of this study was to evaluate the IVEP coupled with LOR as biotechnique to create an embryo bank for the preservation of Canindé goats. Thus, 20 adult and cyclic goats (five females per session) received intravaginal sponges with 60 mg medroxyprogesterone acetate (Progespon, Buenos Aires, Argentina) for 11 days associated with 70μg cloprostenol (Prolise, Buenos Aires, Brazil) in the eighth day. Thirty-six hours before sponge removal, animals received 70 mg pFSH (Folltropin, Ontario, Canadá) and 200 IU eCG (Novormon, Buenos Aires, Argentina). The follicles, visualized by laparoscopy, were classified as small (< 3mm), medium (3 to 4 mm) and large (> 4mm) and aspirated just after the sponge removal using an aspiration system for small ruminants (Watanabe, Cravinhos, Brazil). *Cumulus* -oocyte complexes (COCs) were recovered and classified (grade I to IV) based in the presence of *cumulus* cells and cytoplasm homogeneity. Grade I and II structures were matured in modified TCM199, for 24 h at 38.5°C and 5% CO_2. After this period, COCs were fertilized with fresh spermatozoa ($2x10^6$ sperm/mL) in SOF-FIV medium supplemented with heparin for 16 h in the same maturation conditions. The presumptive zygotes were cultured in SOF-CIV medium, in the same fertilization conditions, for seven days. A total of 245 follicles were punctured and distributed in small (31.5%), medium (35.9%) and large (32.6%). The oocyte recovery

rate was 74.3% (182/245) with an average of 9.1 oocytes per goat. Regarding to oocyte quality, 13.2% (24/182), 68.1% (124/182), 5.5% (10/182) and 13.2% (24/182) were classified as grade I, II, III and IV, respectively. The average of COCs submitted to maturation (grade I and II) was 7.5 per goat. From the presumptive zygotes in vitro incubated, 58.3% (84/144) cleaved after 48 h of culture. The blastocyst rate was 52.1% (75/144) regarding the total number of structures in culture. The total percentage of blastocyst in relation to cleaved embryos was 89.3% (75/84). In conclusion, the IVEP-LOR system was efficient to produce Canindé goat blastocysts and may be used in the creation of an embryo bank in order to preserve the breed.

Keywords: goat, Canindé, embryo

12.4 APÊNDICE D

RESUMO PUBLICADO: *Acta Scientiae Veterinariae*, **38 (Supl. 2):** 753, 2010.

THE EFFECT OF DIFFERENT HORMONAL TREATMENTS FOR OVARIAN ESTIMULATION ON OOCYTE PRODUCTION AND *IN VITRO* MATURATION IN CANINDÉ GOATS

Freitas, V.J.F.; Pereira, A.F.; Alcântara Neto, A.S.; Sousa, F.C.; Moura, R.R.; Albuquerque, E.S.; Luciano, C.M.S.; **Souza, J.M.G.;** Teles Filho, A.C.; Teixeira, D.I.A.; Melo, L.M.

Laboratório de Fisiologia e Controle da Reprodução, Faculdade de Veterinária, Universidade Estadual do Ceará – Fortaleza-CE, Brasil. E-mail: vjff@pq.cnpq.br

The Canindé breed was naturalized in Northeastern Braziland is characterized by its high rusticity. Due to the risk of being extinct, the use of reproductive biotechnologies may greatly increase the number of individuals of this breed. The aim of this study was to evaluate the effect of different hormonal treatments for ovarian stimulation on the quantity and quality of *cumulus*-oocyte complexes (COCs) recovered by laparoscopy and their subsequent in vitro maturation rate (IVM). Eighteen Canindé goats received intravaginal sponge impregnated with 60 mg of medroxyprogesterone acetate (Progespon, Syntex, Buenos Aires, Argentina) for 11 days and 50 µg d-cloprostenol (Ciosin, Schering Plough, São Paulo, Brazil) i.m. at eighth day of progestagen treatment. Goats were allocated into three groups and received: i) five doses (FD) of pFSH (120 mg, Folltropin-V, Bioniche, Ontario, Canada) i.m. in 12 h intervals from the eight day; ii) three doses (TD) of pFSH (120 mg) also from day eight, in 24 h intervals and iii) single dose (SD) of pFSH (70 mg) associated to eCG (200 IU; Novormon, Syntex, Buenos Aires, Argentina) i.m., administrated 36 h before sponge removal. It was performed three hormonal treatment/oocyte recovery sessions and for each of them, six goats were allocated to a different hormonal treatment. Oocyte recovery was performed by laparoscopy at the same moment of sponge removal and submitted to IVM in 5% CO_2 and 38.5 °C for 24 h. Data were submitted to ANOVA and compared by Tukey or Qui-square test, according to the case. All tests were performed using

STATSOFT 7.0 software and the results were described as mean ± SEM. The mean for punctured follicles and COCs recovered was 15.1 ± 0.7 and 11.3 ± 0.8, respectively, resulting in a total recovery rate of 74.5%. There were no differences ($P>0.05$) among treatments for the number of visualized and punctured follicles. The average number of obtained COCs was similar ($P>0.05$) for FD (12.4 ± 1.0), TD (10.7 ± 1.0) or SD (10.8 ± 1.0). However, lower ($P<0.05$) recovery and maturation rates were obtained in TD (67.9%; 32.1%) when compared to FD (84.1%; 49.1%) or SD (72.4%; 46.2%). Therefore, TD treatment was less efficient on oocyte production and further IVM when compared to the other ones. Although the SD treatment produced a similar response to the FD, the former has the advantage of being more practical thus recommended for ovarian stimulation, and it may even be used in conservation programs for the Canindé breed. Financial support: CNPq and FUNCAP.

12.5 APÊNDICE E

RESUMO PUBLICADO: *Transgenic Research*, **21 (4)**: 21, 2012.

USE OF TWO METHODS FOR EMBRYO RECOVERY IN AN EMBRYO TRANSFER PROGRAM FROM A FOUNDER TRANSGENIC GOAT FOR HUMAN GRANULOCYTE COLONY-STIMULATING FACTOR (hG-CSF)

R.R. Moura[1], **J.M.G. Souza[1]**, C.H.S. Melo[1], E.S. Albuquerque[1], T.M. Almeida[1], M.P. Vieira[1], R.I.T.P. Batista[1], A.F. Pereira[1], D.I.A. Teixeira[1], L.M. Melo[1], J.F. Fonseca[2], V.J.F. Freitas[1]

[1]Faculty of Veterinary, State University of Ceará, Brazil; [2]Embrapa Goat and Sheep, Brazil

In a transgenesis program, after obtaining the founder, its breeding is fundamental to create a transgenic herd secreting the recombinant protein. This study aimed to compare the embryo recovery method (surgical vs. transcervical) to expedite the production of transgenic progeny from a founder transgenic doe for hG-CSF. It was used a transgenic Canindé doe, two non-transgenic Canindé bucks and nine undefined breed recipients. Four *in vivo* embryo production sessions were performed: two by surgical (laparotomy) and two by transcervical method. The embryo donor superovulated using ahormonal treatment consisting of vaginal sponges impregnated with progestagen coupled to pFSH and cloprostenol injections. From the second session, it was used flunixin-meglumine in the prevention of premature regression of corpora lutea (PRCL). The recipients received progestagen and cloprostenol associated with an eCG injection. Donor mating was performed at estrus onset and 24 h later. The embryo recovery was performed 7 days post-estrus and, just before, a laparoscopy was done to assess the number and quality of CL. Thetranscervical recovery was performed with a circuit and catheter for small ruminants and the donor received a cloprostenol injection at 12 h before collection. The transgenic kids were identified by PCR. During one surgical session and one transcervical, the collection was not performed due to the occurrence of PRCL and follicular cysts, respectively. In total, it was verified 24 CL and the recovery rate was 69.2% (9/13) and 72.7% (8/11), using surgical and transcervical method, respectively.

Sixteen embryos were transferred to the recipients and eight kids were born. Two males (surgical method) and 1 female (transcervical method) were identifying as transgenics. In summary, embryo recovery (surgical or transcervical), coupled with embryo transfer, expedited the production of progeny from a transgenic founder doe.

12.5 APÊNDICE F

RESUMO PUBLICADO: *Acta Scientiae Veterinariae*, **39 (Supl. 1):** 448, 2011.

USE OF EMBRYO TRANSCERVICAL COLLECTION IN A FOUNDER TRANSGENIC GOAT FOR HUMAN GRANULOCYTE COLONY-STIMULATING FACTOR (hG-CSF)

Moura, R.R.[1]; **Souza, J.M.G.[1];** Almeida, T.M.[1]; Vieira, M.P.[1]; Melo, C.H.S.[1]; Batista, R.I.T.P.[1]; Pereira, A.F.[1]; Teixeira, D.I.A.[1]; Melo, L.M.[1]; Fonseca, J.F.[2]; Freitas, V.J.F[1].

[1]UECE, Fortaleza/CE, BRASIL; [2]EMBRAPA Caprinos e Ovinos, Sobral/CE, BRASIL

Goats are used as model of transgenic animals secreting recombinant proteins in their milk. After the founder production, its multiplication is indispensable in order to obtain a transgenic herd, which can be achieved by the transfer of *in vivo* produced embryos. However, embryo collection in goats is usually done by surgery, which can promote adhesions, limiting the number of times that it can be applied in the same animal. Thus, the study aimed to use the embryo transcervical collection in a founder goat obtained by our group (Freitas et al., 2010, Transgenic Res, 19, 146) for embryo transfer into synchronized recipients. This study was approved by UECE biosafety and ethic committee. One transgenic founder Canindé doe as a donor, two non-transgenic bucks of the same breed and four u breed recipients were used. The embryo donor goat was submitted to estrus synchronization and superovulation using progestagen (Progespon®, Buenos Aires, Argentina), pFSH injections (Folltropin®, Ontario, Canada) and cloprostenol (Prolise®, Buenos Aires, Argentina). In the prevention of premature regression of corpora lutea (CL), flunixin-meglumine (Flumedin®, Varginha, Brazil) was used. Donor fertilization was performed at estrus onset and 24 h later. The number of CL was assessed by laparoscopy before embryo collection. Embryo transcervical collection was performed seven days post-estrus. Twelve hours before collection, the donor received 37.5 μg cloprostenol for cervix dilatation. The embryo collection was performed with a circuit and catheter for small ruminants (Circuit/catheter to collect embryos for sheep and goats®, Embrapa, Brasília, Brazil). It was possible to recover almost all the collection medium at the end of process. The transgenic donor goat had

11 CLs and eight structures were recovered, with a collection rate of 72.7%. Five blastocysts grade I, one grade II and one compacted morula were transferred. The recovered embryos were transferred by semi-laparoscopy into recipients that received progestagen and cloprostenol associated with an eCG injection (Novormon®, Buenos Aires, Argentina). Pregnancy diagnosis was assessed by ultrasonography 30 and 45 days post-estrus. Identification of transgenic offspring was performed by PCR. On day 30 post-estrus, pregnancy rate was 75.0% (3/4) and dropped to 50.0% (2/4) at 45 days. From the two pregnant recipients, one showed twin pregnancy, achieving a total of three kids. One transgenic female was identified by PCR. In this study, 33% of kids born were transgenic. In conclusion, embryo transcervical collection showed to be an efficient method for propagation of founder transgenic goat for hG-CSF.

Keywords: transgenesis, goat, hg-csf.

12.7 APÊNDICE G

RESUMO PUBLICADO: *Acta Scientiae Veterinariae*, **39 (Supl. 1):** 440, 2011.

ESTABLISHMENT OF AN hG-CSF TRANSGENIC GOAT LINE ORIGINATED FROM A MALE FOUNDER AND DETECTION OF AGE AT PUBERTY IN F1

Batista, R.I.T.P*; **Souza, J.M.G**.; Oliveira, C.R.G.; Almeida, T.M.; Vieira, M.P.; Melo, C.H.S.; Albuquerque, E.S.; Luciano, M.C.S.; Felix, J.B.; Melo, L.M.; Pereira, A.F.; Teixeira, D.I.A.; Freitas, V.J.F.

Laboratório de Fisiologia e Controle da Reprodução, Faculdade de Veterinária, Universidade Estadual do Ceará – Fortaleza-CE, Brasil. E-mail: vjff@pq.cnpq.br

The growing worldwide demand for human Granulocyte Colony Stimulating Factor (hG-CSF) stimulated our laboratory to produce transgenic goats harboring this gene (Freitas *et al*., 2010; Transgenic Res., 19, 146). From the birth of founders, obtained by pronuclear microinjection, the imminent aim was the establishment of a transgenic herd for a commercial scale production of the protein. The objectives of this study were: a) to obtain transgenic goats from a male founder and b) to determine the age at puberty of the progeny (F1). For this, seven non-transgenic Canindé females were fertilized by the transgenic founder male. The characterization of F1, for the presence of foreign gene was performed by conventional PCR. All animals received breastfeeding and supplementation with commercial concentrate and Tifton hay until three months of age (weaning). Thereafter, the detection of puberty was performed on both males and females. In males, sexual behavior was evaluated weekly, using a female in estrus until the onset of the first ejaculate containing spermatozoa. In females, blood samples were taken weekly to determine serum progesterone (P4). A total of 12 kids were born, with a foreign gene transmission rate of 50.0% (6/12) and the same ratio sex (three females and three males). Concerning the non-transgenic animals born, 66.7% (4/6) were males. In total, five kids from two pregnancies (one triple and one double) were stillborn, one of which was transgenic. The offspring born live remain healthy until now. Both transgenic males had motile spermatozoa in the ejaculate for the first time at 144 days of age, with 17.2 and 15.8 kg, corresponding to 42.2 and 38.7% of the adult weight of an adult Canindé male, respectively. Concerning the three non-transgenic males, the same found occurred at 119, 119 and 165 days, with 15.8, 12.6 and 13.6 kg,

respectively. The transgenic females showed serum levels of P4 > 1 ng/mL (suggestive of ovulation) at 119 and 150 days of age, being 48% (14.9 kg) and 58% (17.9 kg) of the adult weight of an adult Canindé female, respectively. These results are consistent with the average age at puberty of naturalized breeds in Northeast Brazil. Moreover, indicate that the presence of foreign gene in F1, transmitted from the transgenic male thorough Mendelian inheritance, does not compromise the age at puberty in both males and females. Additional studies with a greater number of animals born in this line, as well as verification of fertility, are still needed.

12.8 APÊNDICE H

RESUMO PUBLICADO: *Animal Reproduction*, **9(3)**: 587, 2012.

EFFECT OF THE NUMBER OF LAPAROSCOPIC OOCYTE RECOVERY SESSIONS IN CANINDÉ GOATS ON THE EFFICIENCY OF OOCYTE PRODUCTION IN AN IVEP SYSTEM

Freitas, V.J.F.*; **Souza, J.M.G.;** Batista, R.I.T.P.; Melo, C.H.S.; Vieira, M.P.; Alcântara Neto, A.S.; Campelo, I.S.; Pereira, A.F.; Melo, L.M.; Teixeira, D.I.A.

Laboratório de Fisiologia e Controle da Reprodução, Faculdade de Veterinária, Universidade Estadual do Ceará – Fortaleza-CE, Brasil. E-mail: vjff@pq.cnpq.br

In vitro embryo production (IVEP) can become an excellent tool for genetic improvement and preservation of goat breeds. However, for this, it is necessary the use of gametes (spermatozoa and oocytes) derived from genetically superior and pure specimen. Concerning the female, laparoscopic oocyte recovery (LOR) could be the appropriate method. However, in Canindé goats there is no study on the effect of repeated LOR in the same oocyte donor. The objective of this study was to observe the response of donors after successive sessions for the oocyte production in an IVEP system. For this purpose, it was used 16 adult and cyclic Canindé goats, which were submitted to two or three treatments for hormonal ovarian stimulation followed by LOR. All oocyte donors received intravaginal sponges containing 60 mg MAP (Progespon®, Syntex, Buenos Aires, Argentina) for 11 days, combined with an intramuscular (im) injection of 50 μg d-cloprostenol (Ciosin®, Coopers, São Paulo, Brazil) on day 8 of progestagen treatment. For ovarian stimulation, goats received a single im injection of 70 mg NIH-FSH-P1 (Folltropin-V®, Vetrepharm, Belleville, Canada) plus 200 IU eCG (Novormon®, Syntex, Buenos Aires, Argentina) 36 h before sponge removal. The interval between each hormonal treatment/LOR was 14 days. LOR was performed under volatile anesthesia and according the procedure cited by Avelar *et al* (2012 Anim Reprod, *in press*). The vacuum pressure was set at 30 mmHg and all follicles larger than 2 mm were aspirated. The collection medium used was TCM199 supplemented with HEPES, heparin and gentamicin. Once the LOR was completed,

each ovary was gently flushed with a heparinized saline. The effect of repeated LOR was analyzed using repeated-measures ANOVA and Tukey's test. There were no statistical differences among the three LOR sessions in the number of visualized/punctured follicles (15.3 ± 5.1/12.7 ± 4.5, 15.5 ± 4.2/12.8 ± 3.9 and 14.7 ± 6.4/11.9 ± 4.9, $P > 0.05$). Concerning the recovery rate, there was also no statistical difference between the different sessions, with an average of 71.2, 74.8 and 74.4% ($P > 0.05$) for the first, second and third session, respectively. In conclusion, three LOR sessions were not enough to decrease the oocyte production in Canindé goats submitted to hormonal ovarian stimulation aiming subsequent IVEP.