UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

MATURAÇÃO *IN VITRO* DE OÓCITOS DE CANINOS DOMÉSTICOS (*CANIS FAMILIARIS*)

ARTUR EMILIO FREITAS E SILVA

Porto Alegre
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Tese apresentada ao curso de Doutorado do Programa de Pós-graduação em Ciências Veterinárias da Universidade Federal do Rio Grande do Sul, como requisito parcial para obtenção do grau de Doutor em Ciências Veterinárias na área de Reprodução Animal.

Orientador: Prof. Dr. José Luiz Rodrigues

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Aprovado em 29 de janeiro de 2010.
APROVADO POR:

_____

Prof. Dr. José Luiz Rodrigues
Orientador e Presidente da Comissão

_____

Profa. Dra. Maria Denise Lopes
Membro da Comissão

_____

Dra. Berenice de Ávila Rodrigues
Membro da Comissão

_____

Prof. Dr. Alexandre Tavares Duarte de Oliveira
Membro da Comissão

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SUMÁRIO

LISTA DE ABREVIATURAS

ºC	graus celsius
µg	micrograma(s)
µL	microlitro(s)
µM	micromolar
AI	anaphase I (anáfase I)
ACP®	água de coco em pó (ACP Biotecnologia ®, Fortaleza, Ceará, Brazil)
ACP-318®	água de coco em pó específica para uso em MIV de oócitos caninos
ATP	adenosine triphosphate (adenosina trifosfato)
BCB	brilhant cresil blue (azul cresil brilhante)
BME	β-mercaptoetanol ou beta-mercaptoetanol
BSA	bovine serum albumin (albumina sérica bovina)
CA	California, USA
CC(s)	cumulus cell (célula do cumulus)
CIV	cultivo *in vitro*
CO_2	dióxido de carbono
COBEA	Colégio Brasileiro de Experimentação Animal
COC(s)	*Cumulus*-oocyte complex (complexo cumulus oócito)
CYS	cysteine (cisteína)
DNA	deoxyribonucleic acid (ácido desoxiribonucléico)
EGF	epidermal growth factor (fator de crescimento epidérmico)
FBS	foetal bovine serum (soro fetal bovino)
FCS	foetal calf serum (soro fetal bovino)
FIV	fecundação *in vitro*
FSH	follicle stimulating hormone (hormônio folículo estimulante)
GSH	glutathione (glutationa)
GV	germinal vesicle (vesícula germinal)
GVBD	germinal vesicle breakdown (vesícula germinal descondensada)
hCG	human chorionic gonadotropin (gonadotrofina coriônica humana)
HCV	Hospita de Clínicas Veterinárias - UFRGS
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hST	human somatotropin (somatotrofina humana)

IAA	indole-3-acetic acid (ácido 3-indol-acético)
ICSI	intracytoplasmic sperm injection (injeção intracitoplasmática de espermatozóide)
IL	Illinois, USA
IU	international unit
IVC	*in vitro* culture
IVF	*in vitro* fertilization
IVM	*in vitro* maturation
IVP	*in vitro* production
LH	hormônio luteinizante(luteinizing hormone)
MI	metaphase I (metáfase I)
MII	metaphase II (metáfase II)
MAP	quinase mitogen activated protein kinase (Proteína-quinase ativada por mitógeno)
mg	miligrama(s)
MIV	maturação *in vitro*
mL	mililitro(s)
mM	milimolar
MO	Missouri, USA
MPF	maturation promoting factor (fator promotor da maturação)
MR	meiosis resumption OR meiotic resumption (retomada de meiose)
mRNA	messenger ribonucleic acid (ácido ribonucleico mensageiro)
mTCM-199	modified tissue culture medium 199 (meio de cultivo tecidual 199 modificado)
ng	nanograma(s)
O_2	superóxido
ON	Ontário, Canadá
OVH	ovariohysterectomy (ovário-histerectomia)
PBS	phosphate buffered saline (solução salina fosfatada)
PIV	produção *in vitro*
PVA	polyvinyl alcohol (álcool polivinilico)
RNA	ribonucleic acid (ácido ribonucléico)
ROS	reactive oxygen species (espécies reativas ao oxigênio)
SCNT	somatic cell nuclear transfer (transferência nuclear de célula somática)

SOF	sintetic oviduct fluid (fluido sintético de oviduto)
SPSS	Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA)
TCM 199	tissue culture medium 199 (meio de cultivo tecidual 199)
TYH	Krebs-Ringer Bicarbonato Modificado
UFRGS	Universidade Federal do Rio Grande do Sul
UK	United Kingdom (Reino Unido)
USA	United States of America (Estados Unidos da América)
VG	vesícula germinal
VGBD	vesícula germinal descondensada

LISTA DE TABELAS

RESUMO

Este estudo foi realizado com o objetivo de: 1) determinar a influência da tensão de oxigênio na viabilidade das células do cumulus oriundas de oócitos caninos maturados em meio com alta concentração de glicose (11,0 mM); 2) determinar a taxa de maturação *in vitro* de oócitos caninos mantidos em meio contendo altas concentrações de glicose (11,0 mM), bem como avaliar o efeito da tensão de oxigênio sobre a taxa de maturação *in vitro* de oócitos caninos; 3) determinar a influência da adição de água de coco em pó (ACP-318®) ao meio de maturação com alta concentração de glicose (11,0 mM) na taxa de maturação *in vitro* de oócitos caninos. O meio de maturação usado nos experimentos foi TCM 199 suplementado com 26,19 mM de bicarbonato de sódio, 50 µg/ml de gentamicina, 0,20 mM de ácido pirúvico, 20 µg/ml de estradiol, 0,5 µg/ml de FSH e 0,03 UI/ml hCG. O meio de maturação era modificado de acordo com a proposta experimental apresentada. Os resultados do primeiro experimento mostraram diferença estatística nas taxas de apoptose nas células do cumulus entre os três grupos avaliados. Foi concluído que células do cumulus oriundas de COCs caninos cultivados em meio contendo altas concentrações de glicose apresentaram significativamente menos apoptose do que as cultivadas em meio com soro fetal bovino, e que a baixa tensão de oxigênio foi eficiente em reduzir a ocorrência de apoptose nas células do cumulus. No segundo experimento deste estudo, os índices mais elevados ($p < 0,001$) de oócitos degenerados foram obtidos, quando TCM 199 era suplementado com 10 % de FCS. Uma influência positiva sobre a retomada de meiose e aquisição de metáfase I (MI) foi observada quando os oócitos caninos foram maturados em meio livre de soro e com altas concentrações de glicose. Foi concluído que a adição de FCS ao meio de maturação de oócitos caninos resulta em altas taxas de degeneração dos oócitos e que a redução da tensão de oxigênio não resultou em melhora da taxa de maturação nuclear dos oócitos caninos. Os resultados obtidos no terceiro experimento mostraram que nos grupos experimentais cujo meio foi suplementado com ACP-318®, houve melhora significativa ($p < 0,05$) na taxa de maturação nuclear dos oócitos caninos. Oócitos maturados em meio suplementado com 5% de ACP-318® mostraram-se menos susceptíveis a alterações na configuração típica da cromatina que os maturados com 10% de ACP-318® no meio de maturação. Os resultados sugerem que tanto a integridade da morfologia nuclear, como a progressão da meiose dos oócitos caninos são positivamente influenciadas quando estes são expostos ao TCM 199 com alta concentração de glicose e suplementado com 5% de ACP-318®.

Palavras chave: canino, oócito, *in vitro*, maturação, glicose, oxigênio, água de coco.

ABSTRACT

This study was designed: 1) determine the influence of oxygen tension on cumulus cell (CC) viability from canine oocytes in vitro matured in high glucose medium (11.0 mM); 2) determine the influence of two oxygen tensions on the nuclear maturation of canine oocytes in vitro matured in high glucose medium (11.0 mM); 3) determine the influence of powdered coconut water (ACP-318®) diluted in high glucose (11.0 mM) TCM199 in the achievement of nuclear in vitro maturation (IVM) of canine oocytes. Basic medium used in the experiments was TCM 199 suplemented with 26.19 mM sodium bicarbonate, 50 µg/ml gentamicin, 0.20 mM piruvic acid, 20 µg/ml oestradiol, 0.5 µg/ml FSH and 0.03 IU/ml hCG. Maturation medium was modified following the beyond described experimental proposals. The results of the first experiment showed that there was statistical difference in the rate of CCs apoptosis among the three groups. It was concluded that CCs of canine COCs cultured in high-glucose medium showed significantly less apoptosis than those cultured in medium with FCS. Low O_2 tension was efficient in reducing apoptosis in canine CCs. In the second experiment of this study, the highest rates ($p < 0.001$) of degenerated oocytes were achieved when TCM 199 was added with 10% FCS. A positive influence on the meiosis resumption and on the MI acquisition rate was observed when canine oocytes were matured in defined high-glucose medium. It was concluded that the addition of FCS in the maturation medium of canine oocytes result in a high level of degenerated oocytes, and that the low level of oxygen tension did not improve the nuclear maturation of canine oocytes. The results achieved on the third experiment showed that in the experimental groups with the medium supplemented with ACP-318® (groups 2 and 3) enhanced the rates of oocytes' nuclear maturation ($p < 0.05$). Ooocytes matured in medium added with 5% powdered coconut water (ACP-318®) were less prone to deviation of typical chromatin configuration than those matured in medium added with 10% ACP-318®. The results suggest that oocytes' nuclear morphology integrity and meiosis achievement were positively influenced when exposed to high glucose TCM199 supplemented with 5% powdered coconut water.
Key words: canine, oocyte, in vitro, maturation, glucose, oxygen, coconut water.

1. INTRODUÇÃO

Há mais de um século, foi observada pela primeira vez, a fecundação de um óvulo de estrela do mar com posterior formação da primeira célula do futuro embrião. A estrela do mar atraiu a atenção de cientistas por razões simples: ao contrário dos mamíferos, a fecundação ocorre externamente ao sistema reprodutor da fêmea, além do fato de que os óvulos da estrela do mar são fáceis de trabalhar, são quase transparentes e desenvolvem-se rapidamente apenas na presença de água do mar (Poster: The Sea Urchin, 2006). Os primeiros passos no sentido de se estabelecer metodologias que permitissem a manipulação de embriões foram dados por pesquisadores através do estudo dos aspectos relacionados à reprodução e ao desenvolvimento de organismos superiores. Neste sentido, os trabalhos experimentais pioneiros em embriologia de mamíferos foram realizados com coelhos, tendo em vista suas características biológicas favoráveis, como o tamanho relativamente grande do óvulo, o que facilita a manipulação, e a ovulação induzida pelo acasalamento, fato de elevada conveniência para determinação precisa da idade dos embriões (HOGAN *et al.*, 1986). No entanto, o primeiro oócito mamífero descrito em literatura foi o da espécie canina em 1827 por Karl Ernst von Baer (FARSTAD, 2000). Também, para esta mesma espécie foi notificada a primeira inseminação artificial em mamíferos, realizada pelo abade Lazzaro Spallanzani na Itália em 1780, da qual foram obtidos três filhotes vivos e sadios (HEAPE, 1897).

Durante o século XX, ocorreram avanços significativos no desenvolvimento e na utilização de biotécnicas ligadas à reprodução animal. Novas tecnologias foram desenvolvidas e aprimoradas no sentido de facilitar a manipulação, e aumentar a capacidade de utilização e preservação de gametas e embriões. A produção de embriões *in vivo* pode ser considerada como a primeira geração de tecnologias de embriões, como por exemplo, a superovulação das doadoras, coleta não-cirúrgica de embriões, criopreservação de embriões e transferência não-cirúrgica para receptoras sincronizadas (STRINGFELLOW *et al.*, 2004). A segunda geração de tecnologias de embriões inclui a produção *in vitro* (PIV). Os procedimentos fundamentais normalmente utilizados incluem: aspiração transvaginal de oócitos, maturação *in vitro* (MIV), capacitação dos espermatozóides, fecundação *in vitro* (FIV), e cultivo *in vitro* (CIV) dos embriões até o estádio de blastocisto. Por fim a terceira geração de tecnologias, desenvolvida mais

recentemente, inclui a injeção intra-citoplasmática de espermatozóide (ICSI), transferência nuclear e transgênese (STRINGFELLOW *et al.*, 2004).

Em mamíferos, o primeiro relato sobre a possibilidade de se cultivar embriões ocorreu ainda no final da década de 20 com coelhos (LEWIS e GREGORY, 1929). Entretanto, somente na década de 50 é que foi registrado o nascimento do primeiro animal gerado a partir da FIV (CHANG, 1959). O primeiro registro sobre MIV e FIV de oócitos foi no final da década de 70 (IRITANI e NIWA, 1977), em bovinos e somente no início dos anos 80 que se registrou nascimento do primeiro animal desta espécie (BRACKETT *et al.*, 1982).

Desde então, uma verdadeira revolução na aplicação destas novas biotecnologias foi observada e documentada, como o desenvolvimento da sexagem espermática (SEIDEL *et al.*, 1997; SEIDEL e GARNER, 2002), a criação de animais transgênicos, tais como murinos transgênicos (BRINSTER *et al.*, 1982), seguido da produção de bovinos, ovinos e suínos transgênicos (WALL *et al.*, 1992; EBERT e SCHINDLER, 1993), e finalmente a obtenção do primeiro mamífero oriundo da técnica de clonagem a partir de uma célula somática (transferência nuclear de célula somática – SCNT, do inglês somatic cell nuclear transfer), a ovelha Dolly (WILMUT *et al.*, 1997). Entretanto, o desenvolvimento e aplicação com sucesso da maioria destas tecnologias é inteiramente dependente de biotécnicas reprodutivas básicas tais como a maturação *in vitro* (MIV) de oócitos (KANE, 2003).

Anos após o nascimento de Dolly, várias outras espécies foram clonadas através da técnica de SCNT, dentre elas a canina (LEE *et al.*, 2005). Entretanto, a clonagem nesta espécie somente tornou-se realidade devido ao fato de que os oócitos utilizados foram obtidos pelo processo de maturação *in vivo*. A escassez de oócitos maduros é o principal fator limitante para a adoção desta biotecnologia em larga escala em caninos, visto que, somente um sistema eficiente de maturação *in vitro* poderia produzir o número suficiente de oócitos maduros para o uso na clonagem (FARSTAD, 2000). Os experimentos foram realizados com os seguintes objetivos: 1) determinar a influência da tensão de oxigênio na viabilidade das células do cumulus de oócitos caninos maturados em meio com alta concentração de glicose (11,0 mM); 2) determinar a taxa de maturação *in vitro* de oócitos mantidos em meio contendo altas concentrações de glicose (11,0 mM), bem como avaliar o efeito da tensão de oxigênio sobre a taxa de maturação *in vitro* de oócitos caninos; 3) determinar a influência da adição de água de

coco em pó (ACP-318®) ao meio de maturação com alta concentração de glicose (11,0 mM) na taxa de maturação *in vitro* de oócitos caninos.

2. REVISÃO BIBLIOGRÁFICA

2.1. Maturação Oocitária

Na maioria das espécies mamíferas, algumas horas antes da deiscência folicular, o oócito no folículo pré-ovulatório retoma a meiose, progredindo da prófase da primeira divisão meiótica para a metáfase da segunda divisão ou metáfase II (MII). Este processo, denominado maturação oocitária, envolve transformações tanto no núcleo quanto no citoplasma e está relacionado a uma série de alterações estruturais e bioquímicas. Estas alterações são progressivamente adquiridas pelo oócito durante o processo de maturação, tornando-o maduro, competente e apto a ser fecundado e suportar o desenvolvimento embrionário de forma adequada (LUVONI *et al.*, 2005; SIRARD *et al.*, 2006). Alguns estudos têm sugerido que a aquisição de competência para o completo desenvolvimento do oócito requer perfeita sincronia entre as maturações nuclear e citoplasmática (SUN e NAGAI, 2003; KRISHER, 2004). Insuficiente maturação citoplasmática do oócito pode resultar numa incorreta transição do controle de desenvolvimento materno para o embrionário e consequentemente em falha do desenvolvimento do embrião (VASSENA *et al.*, 2003). Entretanto, oócitos de camundongo podem adquirir competência para suportar a maturação citoplasmática independentemente da aquisição da competência de maturação nuclear (EPPIG, 1996). *In vivo*, este processo ocorre em resposta aos sinais associados com o pico pré-ovulatório de hormônio luteinizante (LH) (HYTTEL *et al.*, 1997; SIRARD, 2001). *In vitro*, entretanto, a maturação ovocitária pode ser iniciada quando os oócitos são removidos dos seus ambientes foliculares (EDWARDS, 1965; BEVERS *et al.*, 1997; SIRARD, 2001), processo denominado de maturação *in vitro* (MIV). Porém, nem todos os oócitos recuperados dos ovários têm habilidade de se desenvolver em um embrião viável, visto que a retomada da meiose pelo oócito somente é possível a partir de um determinado estado do seu crescimento (MERMILLOD, 2001). Este estado de competência meiótica corresponde igualmente a um determinado tamanho folicular, variável segundo a espécie. Segundo Fair *et al.* (1995) oócitos provenientes de folículos pré-antrais não podem completar a meiose devido ao fato de ainda estarem com os processos de maturação nuclear e citoplasmática incompletos.

A maturação meiótica ou nuclear envolve as alterações na cromatina ocorridas durante a passagem da fase de vesícula germinal (VG) para a metáfase II (MII) (BEVERS *et al.*, 1997; HYTTEL *et al.*, 1997; ABRIEU *et al.*, 2001; SIRARD *et al.*,

2006). Estes eventos estão programados para acontecer no oócito por ocasião da remoção de uma substância inibidora ainda indefinida (SIRARD *et al.*, 2006).

O primeiro sinal de reinício da meiose, observável sob microscópio, é a dissolução da membrana nuclear ou vesícula germinal descondensada (VGBD). A extrusão do primeiro corpúsculo polar (CP) e formação do segundo eixo meiótico ocorrem imediatamente após o oócito se apresentar maduro e as células do cúmulus que o circundam iniciarem a expansão (GORDON, 2003).

Os eventos envolvidos na maturação citoplasmática abrangem a transcrição de ácido ribonucléico mensageiro (mRNA) e a síntese protéica (VAN DEN HURK e ZHAO, 2005), além de todas as mudanças na distribuição e organização das organelas, tais como a migração das mitocôndrias para a região perinuclear e a migração dos grânulos corticais para a periferia próxima à membrana vitelínica, a fim de prevenir a polispermia (BEVERS *et al.*, 1997; HYTTEL *et al.*, 1997; SIRARD *et al.*, 2006). Ao contrário dos eventos que ocorrem na maturação nuclear que podem ser facilmente mensurados por meio do uso de técnicas de coloração, a avaliação da maturação citoplasmática exige técnicas mais apuradas como análise de ácidos ribonucléicos (RNAs) e microscopia eletrônica. Outra alternativa para se avaliar a maturação citoplasmática é por meio da obtenção de sucesso nas etapas de fecundação, desenvolvimento embrionário e fetal (KRISHER, 2004).

Ao contrário da maioria das espécies mamíferas, nas quais o oócito completa sua primeira divisão meiótica no folículo pré-ovulatório, a fêmea canina ovula oócitos imaturos em estádio de vesícula germinal (VG) (DOAK *et al.*, 1967; CONCANNON *et al.*, 1989) e a retomada de meiose ocorre em aproximadamente 48 horas no oviduto (TSUTSUI, 1989; REYNAUD *et al.*, 2005). Assim, enquanto que na maioria das espécies mamíferas o pico pré-ovulatório de LH representa o sinal estimulatório que induz, ainda antes da ovulação, tanto a retomada de meiose do oócito (da prófase I para a metafase II) quanto a mucificação das células do cúmulus devido ao acumulo de ácido hialurônico, na fêmea canina este processo é diferente. A ovulação ocorre após o pico de LH, porém os oócitos ainda se encontram em VG e somente algumas horas após o pico de LH que a mucificação se torna aparente nas células do cumulus (REYNAUD *et al.*, 2006). Portanto, a competência do oócito canino é adquirida nos ambientes intra e extra-folicular (HOLST e PHEMISTER, 1971). Todavia, os mecanismos que regulam a maturação citoplasmática e nuclear nesta espécie ainda não estão completamente esclarecidos. Muitas alterações ultra-estruturais do desenvolvimento oocitário canino

são comuns aos dos demais oócitos mamíferos (TESORIERO, 1982), porém com importantes diferenças. O oócito canino pode ser distinguido pela presença de grandes quantidades de gotas lipídicas (GURAYA, 1965), o que lhe confere a aparência escura e homogênea. A síntese destas gotas lipídicas ocorre no oócito canino durante o crescimento folicular e indica um dos primeiros sinais de maturação oocitária (TESORIERO, 1982).

Diversos fatores podem interferir no êxito da maturação oocitária *in vitro* nas diferentes espécies mamíferas, tais como o tempo gasto para transporte dos ovários até o laboratório (YANG *et al.*, 1990), fatores ambientais como temperatura (LENZ *et al.*, 1983) e tensão de oxigênio (O_2), fatores morfológicos como tamanho dos folículos (PAVLOK *et al.*, 1992; BLONDIN e SIRARD, 1995), estádios de desenvolvimento (HAGEMANN *et al.*, 1999) e diâmetro dos oócitos (HYTTEL *et al.*, 1997), composição do meio de cultura (ZUELKE e BRACKETT, 1990), utilização de soro como fonte de suplementação protéica (AVERY *et al.*, 1998; RIZOS *et al.*, 2003), concentração de substratos energéticos, como a glicose (SONGSASEN *et al.*, 2005), bem como o uso de antioxidantes no meio de maturação (CORDEIRO *et al.*, 2006). Dentre os fatores acima descritos, neste trabalho serão estudados diferentes composições do meio de cultura, assim como, a tensão de O_2 e a utilização de antioxidante no meio de maturação como forma de controlar o estresse oxidativo.

2.1.1. Composição do meio de cultura

Primeiramente serão discutidas as formulações básicas dos meios de cultura disponíveis, os quais podem ser classificados como simples e complexos (GORDON, 2003). Meios simples são usualmente sistemas tamponados com bicarbonato, contendo basicamente solução salina fisiológica acrescida de piruvato, lactato e glicose, e apresentam como principais diferenças variações na concentração iônica e energética. Os complexos têm como base os meios simples, porém suplementados com aminoácidos, vitaminas, purinas e outras substâncias, em concentrações similares às encontradas no soro sangüíneo.

Normalmente os meios utilizados na produção *in vitro* são suplementados com soro ou albumina, o que irá caracterizá-los como: definidos, semidefinidos ou indefinidos (HASLER, 1998). Os meios definidos são aqueles em que todos os constituintes têm sua composição química e concentração conhecidas, os semidefinidos

são aqueles suplementados com substâncias quimicamente definidas, porém que apresentam variabilidade na sua concentração entre lotes e os meios indefinidos são aqueles suplementados com substâncias quimicamente indefinidas e que variam em concentração (HASLER, 1998).

O uso da albumina sérica bovina (BSA) como suplementação protéica nos meios utilizados na produção *in vitro* permite caracterizar estes meios como semi-definidos ou definidos. Hasler (1998) propõe que os meios suplementados com BSA sejam classificados como semi-definidos, devido ao fato do processo de obtenção da albumina do soro gerar um produto final com contaminantes de natureza indefinida (THOMPSON, 1996), fato comprovado pela variabilidade encontrada entre diferentes lotes. Entretanto, ainda segundo Thompson (1996) o meio suplementado com BSA pode ser classificado como definido considerando-se que existem fontes de albumina relativamente puras. De acordo com Hasler (1998) o uso do soro como fonte de suplementação protéica faz com que este seja classificado como indefinido, pois o soro contém compostos variados como proteínas, hormônios, fatores de crescimento, vitaminas, quelantes de íons e várias moléculas definidas e indefinidas que variam em quantidade. O fato de sistemas indefinidos não poderem ser totalmente caracterizados faz com que também não possam ser efetivamente reproduzidos. Vanroose *et al.* (2001) consideram que o uso de meio definido apresenta vantagens como: a padronização dos sistemas de produção *in vitro*, a diminuição das variações entre os protocolos empregados, a prevenção da introdução de agentes patogênicos pela eliminação do uso de produtos de origem animal, e a determinação das necessidades exatas para o desenvolvimento oocitário.

Muito esforço tem sido feito no sentido de se desenvolver métodos eficientes de cultivo que suportem a maturação oocitária *in vitro* em caninos. Diferentes meios de cultura foram avaliados nesta espécie, tais como meio de cultivo tecidual 199 (TCM 199) (OTOI *et al.*, 2002; KIM *et al.*, 2005), Krebs-Ringer Bicarbonato Modificado (TYH) (YAMADA *et al.*, 1992), e Fluido Sintético de Oviduto (SOF) (HEWITT e ENGLAND, 1999; BOLAMBA *et al.*, 2002)**.** Vários protocolos de maturação utilizam o soro como fonte de proteína para o cultivo de oócitos caninos (BOLAMBA *et al.*, 1998; HEWITT *et al.*, 1998; SRSEN *et al.*, 1998; OTOI *et al.*, 2000; LUVONI *et al.*, 2003; RODRIGUES *et al.*, 2004; BOLAMBA *et al.*, 2006). Porém, a adição de soro no meio de maturação de oócitos caninos tem sido associada ao aumento da quantidade de degeneração dos oócitos (HEWITT *et al.*, 1998; SONGSASEN *et al.*, 2002) e alto

percentual de oócitos com material nuclear não identificável (HEWITT *et al.*, 1998; OTOI *et al.*, 1999).

2.1.2. Tensão de oxigênio

Outro importante fator envolvido no processo de maturação *in vitro* de oócitos é a tensão de oxigênio. A tensão de oxigênio influencia significativamente a maturação nuclear de oócitos murinos e bovinos, e alguns estudos sugerem que a tensão de 5% de oxigênio é preferível à tensão normal de 20% em ar (GORDON, 2003). O sistema de cultivo *in vitro* resulta em concentrações de oxigênio mais elevadas que sistemas *in vivo*, o que leva ao aumento dos níveis de espécies reativas ao oxigênio (ROS) (LUVONI *et al.*, 1996), ocorrência de estresse oxidativo, peroxidação lipídica das membranas celulares e apoptose (YUAN *et al.*, 2003; DALVIT *et al.*, 2005). O oócito também contribui com a produção de ROS através de reações enzimáticas e metabólicas (RILEY e BEHRMAN, 1991; HARVEY *et al.*, 2002), as quais têm efeitos deletérios para a sua qualidade (AGARWAL *et al.*, 2005).

Vários pesquisadores têm avaliado o efeito da concentração de oxigênio, comparando resultados obtidos em baixas tensões de oxigênio (5%), que mimetizam as concentrações (2-6% de oxigênio) encontradas no aparelho reprodutivo de algumas espécies mamíferas (FISCHER e BAVISTER, 1993), com os obtidos sob a tensão encontrada na atmosfera (20%) (NASR-ESFAHANI *et al.*, 1992; SONGSASEN *et al.*, 2001; VAN SOOM *et al.*, 2002; YUAN *et al.*, 2003; BOOTH *et al.*, 2005; LEIVAS *et al.*, 2006). O uso de baixas tensões de oxigênio em sistemas de cultivo *in vitro* pode prevenir a excessiva formação de ROS, a qual é diretamente dependente da concentração de oxigênio no ambiente em que se encontram (FREEMAN e CRAPO, 1981; GOTO *et al.*, 1993).

Apesar das vantagens implicadas com a redução da tensão de oxigênio durante o período de maturação *in vitro*, não existe evidência de que esta medida exerça algum efeito significativo na maturação de oócitos caninos (SONGSASEN *et al.*, 2001; 2002).

2.1.3. Uso de antioxidantes

Por fim, outra ferramenta comumente utilizada para minimizar os efeitos do estresse oxidativo durante a maturação *in vitro* é a utilização de antioxidantes, tais como beta-mercaptoetanol (BME), cisteína (CYS), cistina, cisteamina, ácido ascórbico, alfa-

tocoferol, e até mesmo água de coco, sendo que o uso destes têm apresentado algum resultado significativo (ALI *et al.*, 2003; KITAGAWA *et al.*, 2004; TAO *et al.*, 2004; DALVIT *et al.*, 2005; MARTINS *et al.*, 2005; CORDEIRO *et al.*, 2006; COSTA *et al.*, 2006; LEE *et al.*, 2007).

A cisteína, cistina, cisteamina e o beta-mercaptoetanol são elementos precursores para a síntese do principal composto sulfidril não-protéico existente nas células mamíferas, a glutationa (GSH), a qual tem papel importante em várias funções celulares, tais como síntese de ácido desoxiribonucléico (DNA), transporte, atividades enzimáticas, além da proteção contra o estresse oxidativo (MEISTER e ANDERSON, 1983; LUBERDA, 2005; RAUSSEL e TARIN, 2005).

Compostos Thiol de baixo peso molecular, tais como beta-mercaptoetanol, cisteína, cistina, e cisteamina têm sido utilizados como antioxidantes na suplementação de meios de maturação oocitária, em algumas espécies, como em bovinos (DE MATOS *et al.*, 1995; DE MATOS *et al.*, 1997), suínos (ABEYDEERA *et al.*, 1998; 1999; KITAGAWA *et al.*, 2004; FUNAHASHI, 2005; SONG e LEE, 2007) e também em caninos (SONGSASEN *et al.*, 2002; SONGSASEN *et al.*, 2003; KIM *et al.*, 2004; HOSSEIN *et al.*, 2007; KIM *et al.*, 2007). Todavia, o emprego de antioxidantes no meio de maturação de oócitos caninos ainda tem sido pouco estudado e os resultados obtidos são controversos.

Kim *et al.* (2004) testaram 3 concentrações de beta-mercaptoetanol na suplementação do meio de maturação de oócitos caninos (25, 50 e 100 μM) e concluíram que as concentrações de 50 e 100 μM resultaram em aumento significativo no percentual de oócitos que alcançaram o estádio de MII, sendo que a concentração de 25 μM somente exerceu efeito no aumento do percentual de oócitos que alcançaram metáfase I (MI). Por outro lado, (SONGSASEN *et al.*, 2002) em experimento realizado testando também o uso deste mesmo antioxidante, concluíram que o beta-mercaptoetanol na concentração de 25 μM não exerceu nenhum efeito sobre a maturação nuclear de oócitos caninos.

Em relação ao uso de cisteína e cisteamina na maturação *in vitro* de oócitos caninos, Hossein *et al.* (2007) avaliaram três concentrações de cada uma destas substâncias (0,1, 0,5 e 1,0 mM cisteína e 50, 100 e 200 μM cisteamina), e concluíram que tanto a cisteína quanto a cisteamina em qualquer uma das concentrações testadas foram eficientes em promover maturação nuclear de oócitos caninos. Entretanto, os autores afirmam que os efeitos do uso da cisteína e cisteamina na maturação *in vitro*

nesta espécie foram dependentes do estágio reprodutivo das fêmeas, visto que melhores resultados foram obtidos com oócitos oriundos de cadelas em fase folicular.

2.1.3.1. Água de coco

A água de coco tem sido utilizada em várias biotecnologias relacionadas com a reprodução animal, tais como preservação de sêmen, cultura de folículos primordiais, pré-antrais e também na manutenção de oócitos. Com relação à preservação de sêmen, resultados satisfatórios têm sido obtidos com a utilização da água de coco em estudos com animais domésticos como caprinos (SALLES, 1989; TONIOLLI, 1989b; ARAÚJO e NUNES, 1991; RODRIGUES *et al.*, 1994), ovinos (FREITAS, 1992; CRUZ, 1994; SOUSA *et al.*, 1994), suínos (TONIOLLI, 1989a; TONIOLLI e MESQUITA, 1990) e caninos (UCHOA *et al.*, 2002; CARDOSO *et al.*, 2003; CARDOSO *et al.*, 2006; SILVA *et al.*, 2006; CARDOSO *et al.*, 2007).

Na preservação de sêmen, inicialmente a água de coco foi empregada como diluidor na espécie caprina (SALLES e NUNES, 1992), sendo posteriormente adaptada para seu uso na espécie canina (CARDOSO *et al.*, 2002), mostrando-se eficiente para conservação de sêmen nesta espécie (CARDOSO *et al.*, 2003; CARDOSO *et al.*, 2006).

A água de coco (*Cocos nucifera* L.) é uma solução estéril, ligeiramente ácida, contendo proteínas, sais, açúcares, vitaminas, fatores de crescimento (fitormônios) e muito pouco fosfolipídeo (SANTOSO *et al.*, 1996; LAGUNA e NUNES, 1997). Ela contém ainda outros compostos que mostram atividades semelhantes as citocininas e que são derivados das purinas (difeniluréia), possuindo atividades biológicas consideradas importantes para as células (NUNES e SALGUEIRO, 1999), além de conter eletrólitos que contribuem na manutenção e sobrevivência de gametas femininos (MARTINS *et al.*, 2005).

Em 1974, foi realizado o isolamento da citocinina na água de coco (LETHAM, 1974). Posteriormente, o fracionamento da água de coco permitiu o isolamento de um componente, nomeado de JYP, o qual, segundo os autores, aparenta ser responsável pelo efeito favorável da água de coco para os gametas (SALLES e NUNES, 1992). Mais tarde, o ácido 3-indol-acético (IAA), uma molécula pertencente ao grupo das auxinas, foi identificado como sendo a principal substância ativa da fração JYP (COMBARNOUS e NUNES, 1995).

O IAA é um hormônio que atua no crescimento de vegetais (BARBIER-BRYGOO, 1995), ligando-se às proteínas solúveis da seiva, sendo transportado a receptores transmembranários, provocando, direta ou indiretamente, respostas celulares variadas (TONIOLLI *et al.*, 1996), tais como o aumento da plasticidade da parede celular, aumento da entrada de água na célula e alteração do metabolismo dos ácidos nucléicos e da respiração celular (GALSTON e PURVES, 1960), o que promove o crescimento da célula (BARBIER-BRYGOO, 1995).

O ácido 3-indol-acético oriundo da água de coco tem se mostrado eficiente em promover a sobrevivência espermática após preservação *in vitro* em suínos (TONIOLLI *et al.*, 1996). Segundo os autores, uma das principais características observadas no IAA é o seu efeito na manutenção da integridade do acrossoma do espermatozóide, bem como também seu efeito benéfico no metabolismo espermático, através do aumento da motilidade e das taxas de fertilidade após preservação do sêmen (NUNES e CAMBARNOUS, 1995). Além disso, no que diz respeito ao seu uso para preservação de gametas femininos, êxito tem sido obtido no cultivo e preservação de folículos pré-antrais caprinos a 4 ºC durante o período de transporte dos mesmos até o laboratório por até 12 h (FERREIRA *et al.*, 2001), além de prevenir a degeneração de folículos pré-antrais em ovinos quando adicionado ao meios de cultura em presença de hormônio folículo estimulante (FSH) ou fator de crescimento epidérmico (EGF) (ANDRADE *et al.*, 2005).

Com relação ao uso da água de coco, esta também tem sido utilizada em vários trabalhos e se mostrado eficiente no cultivo e preservação de folículos pré-antrais em caprinos (SILVA *et al.*, 2000; SILVA *et al.*, 2004), ovinos (ANDRADE *et al.*, 2002; COSTA *et al.*, 2006), na manutenção e maturação de oócitos bovinos (BLUME *et al.*, 1997a; CORDEIRO *et al.*, 2006), assim como também no cultivo de embriões bovinos (BLUME *et al.*, 1997b) e murinos (BLUME e MARQUES JR, 1994).

No entanto, a utilização da água de coco como diluidor de sêmen ou como componente de meio de cultura enfrenta algumas dificuldades de ordem prática, tais como a disponibilidade de frutos com características ideais (fruto com 6 meses de maturação), a impossibilidade de armazenamento do produto, bem como a padronização da mesma, o que seria fundamental para sua utilização em processos biotecnológicos. Desse modo, Salgueiro et al. (2002) desenvolveram, através de um processo de pulverização a seco (Spray Dryer), a água de coco padronizada na forma de pó (ACP®),

permitindo a conservação das suas características físico-químicas e facilitando o seu uso em regiões onde não se dispõe do fruto de forma padronizada e estabilizada.

Desta forma, a água de coco em pó (ACP®) foi avaliada com resultados satisfatórios em diversos experimentos com sêmen em caprinos (SALGUEIRO *et al.*, 2002) e em caninos (UCHOA, 2004; CARDOSO *et al.*, 2005; 2006; CARDOSO *et al.*, 2007).

No entanto, relatos na literatura sobre o uso de água de coco em pó (ACP®) na maturação *in vitro* de oócitos na espécie canina ainda são desconhecidos.

Em suma, a compreensão do processo de maturação *in vitro* dos oócitos caninos é importante no sentido de que possibilita criar um ambiente artificial para a maturação dos mesmos, da forma mais semelhante possível ao fisiológico. Apesar de vários trabalhos realizados no sentido de aprimorar o processo de maturação *in vitro* de oócitos caninos, a baixa taxa de maturação *in vitro* dos oócitos nesta espécie ainda é um dos principais obstáculos para a produção *in vitro* de embriões.

3. ARTIGO 1

The influence of oxygen tension on cumulus cell viability of canine COCs matured in high-glucose medium

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The Influence of Oxygen Tension on Cumulus Cell Viability of Canine COCs Matured in High-Glucose Medium

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Abridged title: **OxygenTtension on Cumulus Cells of Canine COCs**

Contents

High *in vitro* oxygen (O_2) tensions are associated with enhanced levels of reactive oxygen species (ROS), and cumulus oocyte complex (COC) apoptosis. The objective of this study was to determine the influence of O_2 tension on cumulus cell (CC) viability from canine oocytes. Cumulus oocyte complexes were distributed into three groups (CG, T20, T5) and two O_2 tension levels (20% and 5%). The control group (CG) was matured *in vitro* in a humidified atmosphere with 5% CO_2 in air in TCM199 with 26.19 mM sodium bicarbonate, 10% (v/v) foetal calf serum (FCS), 0.10 mM gentamicin, 0.20 mM pyruvic acid, 20 μg/ml oestradiol, 0.5 μg/ml follicle-stimulating hormone (FSH), 0.03 IU/ml human chorionic gonadotropin, and 1.0 μg/ml human somatotropin. Groups T20 and T5 were matured under 20% or 5% O_2 tensions respectively in a high-glucose medium, without FCS. T20 and T5 were as CG, and supplemented with 0.1% Polyvinyl Alcohol (PVA), and 5.5 mM glucose. After 48h of IVM, CCs from COCs were stained with propidium iodide (1.50 mM). The results showed that viability in CCs (cytoplasmic features and nuclear morphological integrity) was different for the three groups. Rates of apoptosis were at 57.9% (521/900) for CG, 54.4% (490/900) for T20 and 38.9% (350/900) for T5 ($p<0.001$). Predominant features in apoptotic cells (n = 1361) were DNA nuclear fragments (94.0%). It was concluded that CCs of canine COCs cultured in high-glucose medium showed significantly less apoptosis than those cultured in medium with FCS. Low O_2 tension was efficient in reducing apoptosis in canine CCs.

Introduction

In canids, *in vitro* maturation has shown limited success with rates varying from 0% to 58% for oocytes matured to Metaphase I, Anaphase I and Metaphase II (MI / AI / MII) (Farstad 2000). Oocyte nuclear maturation to the MII stage has been achieved in different culture systems and media (Songsasen et al. 2005). Yet, oocyte meiosis *in vitro* is not efficient enough and due to the low rates of meiotic maturation of canine oocytes, much effort has been made with the aim of improving efficacy of culture sytems in supporting the developmental capacity of canine COCs. Researchers have tested different components in media and various protocols use serum as a source of protein to achieve maturation of canid oocytes (Otoi et al. 2000; Rodrigues et al. 2004). Yet, it has been suggested that the addition of serum to culture medium can increase the rate of oocyte degeneration in dogs (Songsasen et al. 2002), as well as increase the incidence of cell death (apoptosis) in cultured embryos of other species, as for example the bovine (Byrne et al. 1999) and the swine (Cui et al. 2004).

Cumulus cells surround and intercommunicate with oocytes during follicular development and after ovulation, suggesting that the incidence of apoptosis in CCs can influence the developmental capacity of the oocyte. As previously reported by Ruvolo et al. (2007), apoptosis in cumulus cells (CCs) can be used as an indicator of oocyte quality.

Reactive oxygen species (ROS) production is a normal process of cell metabolism (Cetica et al. 2001). Nevertheless, high levels of ROS, due to enhanced oxygen tensions, have been implicated in the occurrence of apoptosis (Yuan et al. 2003). *In vitro* culture systems produce higher oxygen concentrations than *in vivo* ones. Intracellular increases in ROS levels cause lipid peroxidation of cellular membranes, thereby inflicting deleterious effects on oocyte quality (Luvoni et al. 1996; Agarwal et al. 2005; Dalvit et al. 2005).

The oocyte response to reduced oxygen tension, as compared to atmospheric tension (20%), has been examined in studies with oocytes from different species (Van Soom et al. 2002; Booth et al. 2005; Leivas et al. 2006), to prevent excessive formation of ROS, by mimicking atmospheric concentrations recorded in the reproductive tract of mammals (2–6% O_2) (Fischer and Bavister 1993). The only study conducted so far, evaluating the effect of the O_2 concentrations on nuclear maturation of dog COCs, indicated that oxygen tension was not essential for *in vitro* meiosis of canine oocytes (Songsasen et al. 2001).

The objective of this study was to determine the influence of two O_2 tension levels on CC viability of canine oocytes matured in TCM 199 supplemented with foetal calf serum (FCS) or glucose at high concentrations.

Materials and Methods

Ovary and oocyte retrieval

Canine ovaries were obtained after elective ovariohysterectomy surgery from nine bitches at unknown reproductive stages, aged between 1 and 12 years (mean age was 4.6 years). Ovaries were obtained at the Veterinary Hospital of Federal University of Rio Grande do Sul (UFRGS) (Av. Bento Gonçalves, 9090/ Porto Alegre/ RS; Brazil), and Zoonoses Control Center of Porto Alegre (Estrada Bérico José Bernardes, 3489/ Porto Alegre/ RS; Brazil). The ovaries were transported to the laboratory at room temperature in phosphate buffered saline (PBS) solution and were processed within 2h of collection. The ovarian cortex was sliced and washed in PBS supplemented with 1% FCS (Nutricell, São Paulo, Brazil) to release COCs. COCs were placed in TCM199 buffered with 25 mM HEPES (M-2520; Sigma, St Louis, MO, USA), supplemented with 2.38 mM sodium bicarbonate (S-5761; Sigma), 0.10 mM gentamicin (G-1264; Sigma), 0.20 mM pyruvic acid (P-4562; Sigma), and 15.05 mM of bovine serum albumin (BSA) (A-3311; Sigma). Only COCs with a multilayered compact cumulus-oophorus and homogeneous dark cytoplasm (Grade 1) (Otoi et al. 2000) were matured *in vitro*. This study followed the guidelines for ethical conduct in the care and use of animals instituted by COBEA (Colégio Brasileiro de Experimentação Animal).

In vitro maturation

The COCs (n = 405) were randomly distributed in groups of 6-10 in 100 µL drops (four drops per dish) under mineral oil at 37ºC in a humidified atmosphere, and then allocated into three maturation media under two different oxygen tension levels (20% or 5% O_2). Cumulus oocyte complexes in the control group (CG) were matured in TCM199 with 26.19 mM sodium bicarbonate (1115059; Gibco, Long Island, New York, NY, USA) and supplemented with 10% (v/v) FCS, 50 µg/ml gentamicin (G-1264; Sigma), 0.20 mM pyruvic acid (P-4562; Sigma), 20 µg/ml oestradiol (E-8875; Sigma), 0.5 µg/ml follicle-stimulating hormone (FSH) (Folltropin-V; Vetepharm Inc., ON, Canada), 0.03 IU/ml human chorionic gonadotropin (hCG) (Chorulon®; Intervet, Milton Keynes, UK), and 1.0 µg/ml human somatotropin (hST) (Humatrope; Lilly, France) in high

oxygen tension (20% O_2) with 5% CO_2 in air for 48h. The COCs in the two other groups, which were designated as T20 and T5, were matured in a high-glucose medium (11.0 mM glucose), which was composed of TCM199 buffered with 26.19 mM sodium bicarbonate, and supplemented with 0.1% Polyvinyl Alcohol (PVA) (P-8136; Sigma), 5.5 mM glucose (108337; Merck, Darmstadt, Germany), 50 μg/ml gentamicin, 0.20 mM pyruvic acid, 20 μg/ml oestradiol, 0.5 μg/ml follicle-stimulating hormone (FSH), 0.03 IU/ml human chorionic gonadotropin (hCG), under 20% or 5% oxygen tensions, respectively. As TCM199 medium contains 5.5 mM glucose, the final concentration of energy substrate in these groups was 11.0 mM glucose.

Evaluation of viability in cumulus cells

After 48h of IVM, CCs were removed from COCs by mechanical displacement under repeated passage of the oocytes through a small-diameter glass micropipette in modified PBS. A pooled sample of CCs from COCs at each experimental group was then pipetted onto a microscope slide, mounted with coverslip, stained with 1.50 mM propidium iodide (P-4170; Sigma) in PBS, and thereafter sealed with incolor nail polish. Counts in CCs (n = 100) were performed immediately after removal, at various randomly selected areas of the cumulus mass. Apoptosis in cumulus cells was evaluated by assessing cytoplasmic features and integrity in nuclear morphology, as previously reported (Yang and Rajamahendran 2000). Incidences of apoptosis (marginated chromatin, pyknotic appearance, multiple nuclear fragments, and apoptotic bodies) (Yang and Rajamahendran 2000) in cumulus cells were expressed as percentages. Cumulus mass areas were observed by using a contrast microscope (Olympus BX41) at magnification of 1000X. Statistical analysis was performed using the data analysis software SPSS, version 13.0. The Chi-square test, with adjusted residual, was used to assess differences in cumulus cell viability. Differences at p-value < 0.05 were considered significant. The experiment was done in nine replicates.

Results

The results showing the viability of CCs from oocytes matured in three media and two oxygen tension levels are summarized in Table 1. Cumulus cells were removed from a total of 405 COCs: (i) CG (n = 136); (ii) T20 (n = 134); (iii) T5 (n = 135). A total of 2700 CCs were counted, i.e. 900 for each group. Rates of CC apoptosis were statistically different among the three groups, at 57.9% (521/900) for CG, 54.4%

(490/900) for T20, and 38.9% (350/900) for T5 ($p < 0.001$). The predominant feature of apoptosis (Table 2) was the presence of multiple nuclear fragments (94.0%; 1280/1361) ($p < 0.001$).

Table 1: Viability of cumulus cells in canine COCs in three different maturation systems

Treatment	CCs	Normal CCs	Apoptotic CCs
	n	n (%)	n (%)
CG	900	379 (42.1) [a]	521 (57.9) [d]
T20	900	410 (45.6) [b]	490 (54.4) [e]
T5	900	550 (61.1) [c]	350 (38.9) [f]

[a,b,c,d,e,f] Values with different superscripts in the same column differ significantly (Chi-square analysis with adjusted residual) ($p < 0.001$).
CG : control group TCM 199 wit serum; T20 : TCM 199 with 11.0 mM glucose under 20% oxygen tension; T5 : TCM 199 with 11.0 mM glucose under 5% oxygen tension; CCs: cumulus cells; COCs: cumulus oocyte complexes.

Table 2: Features of cumulus cells apoptosis in canine oocytes in three different maturation systems

Treatment	n	Fragmentation	Apoptotic body	Pyknosis	Marginated chromatin
		n (%)	n (%)	n (%)	n (%)
CG	521	486 (93.3) [a]	17 (3.2) [d]	16 (3.1)	2 (0.4) [f]
T20	490	455 (92.8) [b]	17 (3.5) [d]	18 (3.7)	0 (0.0) [g]
T5	350	339 (96.8) [c]	1 (0.3) [e]	10 (2.9)	0 (0.0) [g]
Total	1361	1280 (94.0)	35 (2.6)	44 (3.2)	2 (0.2)

[a,b,c,d,e,f,g] Values with different superscripts in the same column differ significantly (Chi-square analysis with adjusted residual) ($p < 0.001$).
CG : control group TCM 199 with serum; T20 : TCM 199 with 11.0 mM glucose under 20% oxygen tension; T5 : TCM 199 with 11.0 mM glucose under 5% oxygen tension.

Discussion

Degeneration of canine COCs is high in *in vitro* studies. Data from previous (Songsasen et al. 2002) and recent experiments (Rodrigues et al. 2009) showed that degeneration of COCs are thought to be related to detrimental factors inflicted by the supplementation of maturation medium with serum, e.g, foetal bovine serum (FBS).Yet, the mechanism

by which the presence of serum in medium raises the percentage of oocyte *in vitro* degeneration is not clear. Nevertheless, serum has adverse effects on the structure of mitochondria, by causing the accumulation of cytoplasmic lipids (Abe and Hoshi 2003). While information on oocyte degeneration is regularly found in the literature, that related to apoptosis in CCs of dog oocytes is rare, except for a few recent reports (Lopes et al. 2007 ; Rodriguez et al. 2008, Rodrigues et al. 2009).

Different molecules and nutrients in maturation media can greatly influence oocyte nuclear and cytoplasmic maturation (Chung et al. 2002). Metabolism of glucose is important during canine oocyte *in vitro* maturation, because glucose is the predominant energy substrate used by dog oocytes (Songsasen et al. 2005). Glucose concentration in medium operates at an optimal level, whereby too much or too litte produces negative effects during oocyte maturation (Thompson 2006). The concentration of extracellular glucose in conjunction with a low O_2 tension level was associated, in a study (Hashimoto et al. 2000), with an improvement in the developmental potential of bovine COCs. Paradoxically, Krisher (2004) reported about the inhibition of adenosine triphosphate (ATP) production and progression to MII in a low oxygen environment. Yet, the results of this study showed that oxygen tension level has a significant influence on CC viability of dog COCs, with higher levels of cell death observed in oocytes matured under high oxygen atmosphere as opposed to low oxygen atmosphere. These findings are in agreement with previous reported results that high oxygen tensions are associated with the establishment of expressive levels of ROS, which in turn lead to cell apoptosis (Yuan et al. 2003). The oocytes themselves also produce endogenous ROS by various enzymatic actions and metabolic pathways (Riley and Behrman 1991; Harvey et al. 2002). Impairement of glutathione (GSH) synthesis as a result of uncoupling between the oocyte an its CCs, is one of the pathways leading to oocyte degeneration *in vitro (*de Matos et al. 1997). Appropriate GSH status is usually achieved by the inclusion of its constituent aminoacids, cysteine, glycine and glutamine, within the incubation medium (Harvey et al. 2002). Therefore, metabolic variables might be implicated in the results of CC integrity observed in the present experiment. As showed by the results in this study, a deleterious influence of FBS might have been responsible for the increase in the incidence of apoptosis in CCs of COCs matured in the control group. Overall, the maintenance of higher rates of CC viability in medium with low oxygen tension level (T5), suggests that reduction-oxidation status in oocytes matured *in vitro* in T5 medium, was preserved in a better way than that verified in

COCs matured in CG and T20 media. As GSH plays a major role in regulating ROS concentrations (Harvey et al. 2002), and since apoptosis in CCs during the IVM period was by far more expressive in medium added with serum (CG), a negative effect of medium on COC quality reinforces similar assertions from other authors (Songsasen et al. 2002; Hewitt et al. 1998).

Apoptotic cell death is characterized by cell shrinkage, cytosolic and nuclear condensation, plasma membrane blebbing, breakdown of nuclear DNA and, finally, separation of apoptotic bodies (Stastna and Sedlackova 2001). In the present study, presence of high levels of glucose and piruvate in medium seemed to have positively influenced the CC integrity of dog COCs. Nevertheless, the preservative effect of glucose in medium on CCs was clearly oxygen-tension dependent, with significantly lower rates of apoptotic bodies observed in T5 medium.

In conclusion, CCs of canine COCs cultured in high-glucose medium had significantly lower rates of apoptosis than those cultured in medium with FCS. The FCS might have been responsible for the high level of apoptosis observed in CG. Additionaly, low levels of oxygen tensions were efficient in reducing the occurrence of apoptosis in canine CCs. As cumulus quality is thougth to participate in the establishment of developmental competence in the oocyte, apoptosis in CCs may be a relevant biomarker of COC fitness.

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4. ARTIGO 2

High-glucose but not low oxygen tension enhances *in vitro* maturation of canine oocytes

Artigo a ser submetido para publicação.

High-glucose but not low oxygen tension enhances *in vitro* maturation of canine oocytes

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Abridged title**: *In vitro* maturation at defined high-glucose medium**

Contents

The objective of this study was to determine the influence of two oxygen tensions on the nuclear maturation of canine oocytes. Cumulus oocyte complexes (COCs) (n = 843) were distributed into three groups (CG, T20, T5) and two O_2 tension levels (20% and 5%). The control group (CG) was matured *in vitro* in a humidified atmosphere with 5% CO_2 in air in TCM199 with 26.19 mM sodium bicarbonate, 10% (v/v) foetal calf serum (FCS), 0.10 mM gentamicin, 0.20 mM pyruvic acid, 20 µg/ml oestradiol, 0.5 µg/ml follicle-stimulating hormone (FSH), and 0.03 IU/ml human chorionic gonadotropin (hCG). T20 and T5 were as CG, but without FCS, and supplemented with 0.1% Polyvinyl Alcohol (PVA) and 5.5 mM glucose. Groups T20 and T5 were matured under 20% or 5% O_2 tensions respectively in a high-glucose medium. After 48h of IVM, the chromatin configuration in oocytes were evaluated by Hoechst 33342 staining. The results showed that the medium used for IVM significantly influenced nuclear maturation of canine oocytes. High percentages of degenerated oocytes were observed in the serum-supplemented medium (CG) when compared to those cultured in defined high-glucose medium (T20) (154/211, 73.0% vs 134/239, 56.1%). Meiotic resumption percentage for each experimental treatment matured in high oxygen tension (20%) was measured to be 14.7% for CG and 31.4% for T20. The oxygen tension did not influenced nuclear maturation of canine oocytes (P = 0.809). It can be concluded that the oocyte nuclear maturation in canids is influenced by the medium used for IVM. The addition of FCS in the maturation medium of canine oocytes result in a high level of degenerated oocytes, and that the low level of oxygen tension did not improve the nuclear maturation of canine oocytes.

Introduction

Otherwise to majority of mammalian species in which the oocyte completes its first meiotic division in the preovulatory follicle, the domestic bitch ovulates immature oocytes in the germinal vesicle (GV) stage (Doak et al. 1967; Concannon et al. 1989) and meiotic resumption occurs after about 48 h spent in the oviduct (Tsutsui 1989; Reynaud et al. 2005). Most studies have tracked the *in vitro* maturation of oocytes by evaluating their meiosis resumption (Bolamba et al. 1998; Hewitt et al. 1998; Bolamba et al. 2006; Santos et al. 2006; Lopes et al. 2007), but the low rate of meiotic maturation of canine oocytes cultured *in vitro* still is the main obstacle to the *in vitro* production of canine embryos. Much effort has been made to develop effective culture methods that support oocyte maturation in vitro, but the rate of canine oocytes that achieve the metaphase II (MII) stage rarely exceed 20% (Otoi et al. 1999; Luvoni et al. 2001; Bogliolo et al. 2002; Rodrigues and Rodrigues 2003; Kim et al. 2004; De los Reyes et al. 2005; Luvoni et al. 2005; Santos et al. 2006; Vannucchi et al. 2006). Many protocols use serum as a source of protein to culture oocytes in canids (Bolamba et al. 1998; Hewitt et al. 1998; Srsen et al. 1998; Otoi et al. 2000; Luvoni et al. 2003; Rodrigues et al. 2004a; Bolamba et al. 2006). It has been suggested that the addition of serum to maturation media in canids can increase the rate of degenerated oocytes (Hewitt et al. 1998; Songsasen et al. 2002), resulted in high percentages of canine oocytes with unidentified nuclear material (Hewitt et al. 1998; Otoi et al. 1999), as well as increase the incidence of cell death (apoptosis) in cultures embryos of others species, as for example the bovine (Byrne et al. 1999) and the swine (Cui et al. 2004). Furthermore, contrary to defined culture systems, the addition of serum in media may make difficult to study cooperative interactions among nutrients during culture, as that supplement contain unknown factors.

Chemically defined media facilitate the study of beneficial or suppressive factors in oocyte maturation and have been used for the maturation of canine oocytes (Songsasen et al. 2002; Kim et al. 2005). The use of defined culture conditions allows the identification of essential factors required for the nuclear maturation of canine oocytes, leading to the development of reliable culture conditions.

Energy substrates like glucose, pyruvate and glutamine are known to play a fundamental role in oocyte maturation in some mammals. Among them, glucose is the predominant energy substrate used for dog oocytes (Songsasen et al. 2005b). However, culturing canine oocytes in a medium containing a high glucose concentration may be

detrimental to nuclear maturation in high oxygen tension (Songsasen et al. 2002). On the other hand, it has been reported that bovine oocytes cultured in high glucose medium develop to the MII stage when submitted to low oxygen tension (5%) (Hashimoto et al. 2000a).

Oxygen tension significantly influences nuclear maturation of mouse and bovine oocytes, and some evidence has suggested that a 5% oxygen level may be preferable to the normal 20% level in air (Gordon 2003). High levels of reactive oxygen species (ROS) production, which is a normal process of cell metabolism (Cetica et al. 2001), have been implicated in the occurrence of cell apoptosis *in vitro* (Yuan et al. 2003). *In vitro* culture systems produce higher oxygen concentrations than *in vivo* ones. Intracellular increases in ROS levels cause lipid peroxidation of cellular membranes, thereby inflicting deleterious effects on oocyte quality (Luvoni et al. 1996; Agarwal et al. 2005; Dalvit et al. 2005). In order to mimic the concentrations (2–6% oxygen) recorded in the reproductive tract in several mammalian species (Fischer and Bavister 1993), and prevent excessive formation of ROS, the oocyte response to reduced oxygen tension compared to that existing atmospherically (20%) has been examined (Nasr-Esfahani et al. 1992; Songsasen et al. 2001; Van Soom et al. 2002; Yuan et al. 2003; Booth et al. 2005; Leivas et al. 2006). Nevertheles, the only study conducted so far, evaluating the effect of the O_2 concentrations on nuclear maturation of dog cumulus-oocyte complexes (COCs), indicated that oxygen tension was not essential for *in vitro* meiosis of canine oocytes (Songsasen et al. 2001).

The objectives of the present study were to observe whether a defined high-glucose medium supports the meiotic maturation of canine oocytes, and additionally to determine the influence of oxygen tension on the nuclear maturation of canine oocytes.

Materials and Methods

Ovaries and oocyte retrieval

Canine ovaries were obtained after elective ovariohysterectomy (OVH) surgery from 16 bitches at unknown reproductive stages, aged between 1 and 12 years (mean age was 4.6 years). Ovaries were obtained at the Veterinary Hospital of Federal University of Rio Grande do Sul (UFRGS) (Av. Bento Gonçalves, 9090/ Porto Alegre/ RS; Brazil), and Zoonoses Control Center of Porto Alegre (Estrada Bérico José Bernardes, 3489/ Porto Alegre/ RS; Brazil). The ovaries were transported to the laboratory at room temperature in phosphate buffered saline (PBS) solution and were processed within 2h

of collection. The ovarian cortex was sliced and washed in PBS supplemented with 1% FCS[1] to release COCs. Only COCs with a multilayered compact cumulus-oophorus and homogeneous dark cytoplasm (Grade 1) (Otoi et al. 2000; Luvoni et al. 2001; Otoi et al. 2007) were selected for maturation. A total of 843 COCs were selected and matured *in vitro*. COCs were placed in TCM199 buffered with 25 mM HEPES[2], supplemented with 2.38 mM sodium bicarbonate[3], 0.10 mM gentamicin[4], 0.20 mM pyruvic acid[5], and 15.05 mM BSA[6]. This study followed the guidelines for ethical conduct in the care and use of animals instituted by COBEA (Colégio Brasileiro de Experimentação Animal).

In vitro maturation

The COCs (n = 843) were randomly distributed in groups of 6-10 in 100 µL drops (four drops per dish) under mineral oil at 37 ºC in a humidified atmosphere, and then allocated into two maturation groups under two different oxygen tension level (20% or 5% O_2). COCs in the control group (CG) were matured in TCM199 with 26.19 mM sodium bicarbonate[7] and supplemented with 10% (v/v) FCS, 0.10 mM gentamicin, 0.20 mM pyruvic acid, 20 µg/ml oestradiol[8], 0.5 µg/ml follicle-stimulating hormone (FSH)[9], and 0.03 IU/ml human chorionic gonadotropin (hCG)[10] in high oxygen tension (20% O_2) with 5% CO_2 for 48 hours. The COCs in the two others groups, which were designed as T20 and T5, were matured in a high-glucose medium (11.0 mM glucose), which was composed by TCM199 buffered with 26.19 mM sodium bicarbonate, and supplemented with 0.1% Polyvinyl Alcohol (PVA)[11], 5.5 mM glucose[12], 0.10 mM gentamicin, 0.20 mM pyruvic acid, 20 µg/ml oestradiol, 0.5 µg/ml follicle-stimulating hormone (FSH), 0.03 IU/ml human gonadotropin (hCG), under 20% or 5% oxygen tensions, respectively for 48 hours. Since TCM199 medium contains 5.5 mM glucose, the final concentration of energy substrates in these groups were 11.0 mM glucose.

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[1] Nutricell, São Paulo, Brazil
[2] Sigma, M-2520, St. Louis, MO, USA
[3] Sigma, S-5761, St. Louis, MO, USA
[4] Sigma, G-1264, St. Louis, MO, USA
[5] Sigma, P-4562, St. Louis, MO, USA
[6] Sigma, A-3311, St. Louis, MO, USA
[7] Gibco, 1115059, Long Island, New York, USA
[8] Sigma, E-8875, St. Louis, MO, USA
[9] Folltropin-V, Vetepharm Inc., Ontário, Canada
[10] Chorulon®, Intervet, Milton Keynes, UK
[11] Sigma, P-8136, St. Louis, MO, USA
[12] Merck, 108337, Darmstadt, Germany

Chromatin evaluation

The chromatin configuration was characterized according to Bruck et al. (2000) by one of the following nuclear maturation stages: a) germinal vesicle (GV): finely granulated nucleoplasm with filamentous chromatin configurations; b) germinal vesicle breakdown (GVBD): condensed filamentous chromatin within finely granulated nucleoplasm; c) metaphase I (MI): condensed chromosomes oriented in a metaphase plate; d) metaphase II (MII): two sets of condensed chromosomes of which one set with orientation in a metaphase plate, while the second (polar body) more or less randomly oriented in a cluster; e) others: no chromatin (degenerated) or small spots of dense chromatin. Chromatin configuration in oocytes after IVM was evaluated by Hoechst 33342 (0.02 mM) staining as previously described (Rodrigues et al. 2004b).

Statistical Analysis

Statistical analyses were performed using the data analysis software SPSS, version 13.0 (SPSS Inc., Chicago, IL, USA). The Chi-square test, or Fisher exact test, when appropriate, with adjusted residual were used to assess differences in oocyte nuclear maturation. Differences at p value < 0.05 were considered significant. The experiment was done in 16 replicates.

Results

Effects of maturation media and oxygen tension

The results showing the meiotic progression after the oocytes were exposed to the different medium during the IVM period are summarized in Table 1. The medium used for IVM significantly influenced nuclear maturation of canine oocytes ($P < 0.001$).

Significantly high percentages ($P < 0.001$) of degenerated oocytes were observed in the serum-supplemented medium (CG) when compared to those cultured in defined high-glucose medium (T20) (154/211, 73.0% vs 134/239, 56.1%).

Meiotic resumption percentage for each experimental treatment matured in high oxygen tension (20%) was measured to be 14.7% for CG and 31.4% for T20. The highest rates of meiotic resumption (75/239, 31.4%) (Table 2), as well as the rates of oocytes undergoing nuclear maturation to MI stage (58/239, 24.3%) were achieved in defined high-glucose medium (T20) when comparison was established with the CG. The rate of oocytes undergoing MI maturation with high-glucose in medium was statistically different from those observed in the serum-supplemented group ($P<0.001$).

The results showing the meiotic progression after the oocytes were exposed to the different oxygen tension levels during the IVM period are summarized in Table 3. The oxygen tension did not influenced nuclear maturation of canine oocytes (P = 0.809).

Discussion

The results obtained in this study showed that the defined high-glucose medium supports resumption of meiosis of canine oocytes from GVBD to MII stage (75/239, 31.4%). These findings are similar to those previously reported in the literature, where rates of meiosis resumption (MR) (GVBD to MII) in dogs oocytes achieved percentages of 20.1% to 34.7% (Bolamba et al. 1998), 27.6% (Rodrigues and Rodrigues 2003) and 0 to 58% (Farstad 2000).

The meiosis resumption was different between the two culture media used for *in vitro* maturation in this study. In the serum-supplemented medium (CG), meiosis resumption was lower than the defined high-glucose medium supplemented with PVA (T20) (14.7% vs 31.4%) (Table 2). These results appear to be in contradiction with other study showing that FBS supplementation improved meiotic resumption in canine oocytes when compared to PVA supplementation (Cui et al. 2006). Further, the number of degenerated oocytes after IVM was higher in serum-supplemented medium than those of oocytes cultured in defined high-glucose medium. In accordance to other studies (Hewitt et al. 1998), the present data confirm that the addition of serum in the maturation media of canine oocytes increases the rate of degenerated oocytes. The reason for this high level of degeneration is unclear. Nevertheless, serum has adverse effects on the structure of mitochondria, by causing the accumulation of cytoplasmic lipids in bovine (Abe and Hoshi 2003) and ovine (Gardner 1994; Thompson et al. 1995) oocytes. Since the canine oocyte is characterized by the presence of large amounts of lipid yolk material, that gives a dark and homogeneous appearance to the oocyte in this specie (Guraya 1965), they might be more susceptible to lipid peroxidation. The lipid accumulation induced by the presence of serum in the maturation media may become the canine oocytes still more susceptible to the occurrence of lipid peroxidation. In addition, the presence of serum in maturation media is also responsible for the occurrence of high level of apoptosis in cumulus cells of canine oocytes (Silva et al. 2008). Degeneration of canine cumulus oocyte complexes (COCs) is high in *in vitro* studies. Data from previous (Hewitt et al. 1998; Songsasen et al. 2002), and recent experiments (Rodrigues et al. 2009) showed that degeneration of COCs are thought to

be related to detrimental factors inflicted by the supplementation of maturation medium with serum, e.g, foetal bovine serum (FBS). As previously reported by Ruvolo et al. (2007), apoptosis in cumulus cells is used as an indicator of oocyte quality, since they surround and intercommunicate with oocytes during follicular development and after ovulation. In this way, apoptosis in cumulus cells can influence the developmental capacity of oocyte to resume meiosis and leads the oocyte to a degeneration process. Thereby, the cytoplasmic lipid accumulation, which leads to lipid peroxidation, associated with the occurrence of apoptosis in cumulus cells, both induced by the presence of serum in the maturation media, might explain the high level of degenerated oocytes found in the serum-supplemented medium.

The metabolism of glucose is important during oocyte *in vitro* maturation in canines, because glucose is the predominant energy substrate used by dog oocytes (Songsasen et al. 2005a). Since glucose is the main substrate energetic used by dog oocytes and as previous reported by (Sutton et al. 2003), the glucose requirements of the COC increase through *in vitro* maturation period, a high concentration of this substrate may be desirable in maturation media. Glucose concentration in medium operates at an optimal level, whereby too much or too litte produces negative effects during oocyte maturation (Thompson 2006). However, the presence of a high concentration of glucose was found to be harmful for the embryo development in some species, like in bovines, especially in the early stages (Pinyopummintr and Bavister 1991; Hashimoto et al. 2000b), and in hamster (Schini and Bavister 1988). According to Hashimoto el al. (2000b) this impaired effect is probably associated to the increase of ROS and the decrease in the intracellular glutathione content of oocytes triggered by the excessive concentration of glucose (28 mM) during *in vitro* maturation period. It has been show that exposure of mouse endothelial cells to high glucose concentration (28mM) impairs the expression of γ-glutamylcysteine synthetase, which catalyzes the intracellular synthesis of glutathione (Meister 1983), resulting in a decrease of intracellular glutathione (Urata et al. 1996). On the other hand, it has been reported that bovine oocytes cultured in high glucose medium can develop to the MII stage if they are cultured under low oxygen tension (5%) (Hashimoto et al. 2000a). In the present study, we found that the high-glucose medium could afford the maturation of canine oocytes until MI stage in the two tested oxygen tension levels (20% and 5%). However, despite the superiority of high-glucose at that development point, in this study, the glucose at a concentration of 11 mM was unable to support further maturation to the MII stage in the same proportion. Oocytes

that are capable of resuming meiosis do not necessarily develop to the MII stage *in vitro* (Songsasen et al. 2003). In bitches, various oocytes selected for in vitro culture are collected from atretic follicles during the slicing procedure. It is noteworthy that oocytes from atretic follicles undergo meiosis-like changes (germinal vesicle breakdown), which are followed by oocyte fragmentation and disruption of the oocyte-cumulus connection (Tsafriri and Braw 1984). Canine oocytes, like those of other species, are able to resume meiosis *in vitro*, but they are not fully competent to complete nuclear maturation (Nickson et al. 1993; Hewitt and England 1998b; Hewitt and England 1998a; Otoi et al. 2000; Songsasen et al. 2002).

In this study, no differences between the two experimental groups (T20 and T5) with two different oxygen levels (20% and 5%, respectively) were observed. These findings are in agreement with those previously reported in literature (Songsasen et al. 2001), confirming that the oxygen tension is not essential for *in vitro* meiosis of canine oocytes. Yet, in a recent study about interactions between oxygen tension and glucose concentration, de Castro and Hansen (2007) reported that the development of bovine oocytes was not affected by oxygen tension when they were matured in high glucose concentration. However, the influence of oxygen tension on nuclear maturation seems to be influenced by others details of culture. Fukui et al. (1991) showed that the different types of culture media affected responses of bovine oocytes to oxygen tension. In that way, de Castro and Hansen (2007) reported for bovine oocytes matured in mTCM-199, an oocyte development greater in high oxygen than in low oxygen atmosphere, when 5.6 mM glucose was used. Nevertheles the development was not affected by oxygen when 20 mM glucose was used. Eppig and Wigglesworth (1995) reported that high oxygen tension is detrimental to mouse oocytes obtained from pre-antral follicles. However, Hu et al. (2001) reported that the percentage of pre-antral mouse oocytes cultured in presence of 5% FBS which had undergone nuclear maturation was significantly reduced when the oxygen tension in medium was decreased. Under the low oxygen atmosphere the oocyte itself experiences a deficit in oxygen availability (de Castro and Hansen 2007). Exposing the cultured cell to a low oxygen tension causes a decrease in ATP via mitochondrial oxidative metabolism (Czyzyk-Krzeska 1997; Wenger and Gassmann 1997). Such decrease is compensated by an increase in glucose metabolism, as shown by increases in the expressions of glucose transporter 1 (Bashan et al. 1992; Ebert et al. 1995) and glycolytic enzymes (Firth et al. 1994; Semenza et al. 1994). Certain amount of ATP is required for the

meiotic maturation of oocytes irrespective of the oxygen tension, and ATPs are mainly supplied by the mitochondrial respiratory chain under aerobic conditions or by glycolysis under anaerobic conditions (Hashimoto et al. 2000a). The same authors reported that the mechanisms controlling the achievement of meiotic maturation and the production of ATP are different between COCs cultured under low oxygen tension ands those cultured under high oxygen tension. Because glycolysis is less efficient than oxidative phosphorylation for ATP production, it is likely that ATP production by the oocyte becomes insufficient in oxygen-restricted environments (de Castro and Hansen 2007). Indeed, oocyte ATP content was reduced in oocytes matured in 5% oxygen as compared to those matured in atmospheric oxygen (Hashimoto et al. 2000a). In this study, the high glucose in the maturation media appears to provide the sufficient glucose needed by the increase of glucose metabolism induced by the low oxygen tension.

Conclusion

This study has yield the following findings: (a) oocyte nuclear maturation in canids is influenced by the medium used for IVM; (b) the addition of FCS in the maturation medium might be responsible for the high level of degenerated oocytes observed in the control group; (c) low level of oxygen tension did not improve the nuclear maturation of canine oocytes.

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Table 1. The *in vitro* maturation rates of canine oocytes exposed to different maturation medium.

Treatment	n	GV	GVBD	MI	MII	Others
		n (%)	n (%)	n (%)	n (%)	n (%)
CG	211	26 (12.3)	12 (5.7)	16 (7.6)[b]	3 (1.4)	154 (73.0)[c]
T20	239	30 (12.6)	14 (5.9)	58 (24.3)[a]	3 (1.3)	134 (56.1)[d]

Values with different superscripts in the same column [(ab, cd)] differ significantly (Chi-square analysis with adjusted residual) ($p < 0.001$).
CG: control group TCM 199 with serum; T20: TCM 199 with 11.0 mM glucose and 0.1% PVA; GV: germinal vesicle, GVBD: germinal vesicle breakdown, MI: metaphase I, MII: metaphase II, Others: unidentified or degenerated.

Table 2. Meiosis resumption of canine oocytes exposed to different maturation medium.

Treatment	n	GV n (%)	MR n (%)	Others n (%)
CG	211	26 (12.3)	31 (14.7)[b]	154 (73.0)[c]
T20	239	30 (12.6)	75 (31.4)[a]	134 (56.1)[d]

Values with different superscripts in the same column [(ab, cd)] differ significantly (Chi-square analysis with adjusted residual) ($p < 0.001$).
CG: control group TCM 199 with serum; T20: TCM 199 with 11.0 mM glucose and 0.1% PVA; GV: germinal vesicle, MR: meiosis resumption, Others: unidentified or degenerated.

Table 3. The *in vitro* maturation rates of canine oocytes exposed to different oxygen tension.

Treatment	n	GV n (%)	GVBD n (%)	MI n (%)	MII n (%)	Others n (%)
T20	239	30 (12.6)	14 (5.9)	58 (24.3)	3 (1.3)	134 (56.1)
T5	233	25 (10.7)	19 (8.2)	52 (22.3)	2 (0.9)	135 (57.9)

T20: TCM 199 with 11.0 mM glucose and 0.1% PVA under 20% oxygen tension; T5: TCM 199 with 11.0 mM glucose and 0.1% PVA under 5% oxygen tension; GV: germinal vesicle, GVBD: germinal vesicle breakdown, MI: metaphase I, MII: metaphase II, Others: unidentified or degenerated.

5. ARTIGO 3

The influence of powdered coconut water (ACP-318®) in *in vitro* maturation of canine oocytes

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The Influence of Powdered Coconut Water (ACP-318®) in *In Vitro* Maturation of Canine Oocytes

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Abridged title: **Powdered Coconut Water on Canine Oocytes**

Contents

The objective of this study was to determine the influence of powdered coconut water (ACP-318®) diluted in high glucose (11.0 mM) TCM199 in the achievement of nuclear *in vitro* maturation (IVM) of canine oocytes. Cumulus oocyte complexes (COCs) (n=632) were randomly allocated into three experimental groups named as group 1 (control group), group 2 (5% powdered coconut water) and group 3 (10% powdered coconut water). The percentage of meiotic resumption (MR) (GVBD to MII) was 39.1% (81/207), 50.2% (108/215) and 46.6% (98/210) for groups 1, 2 and 3 respectively ($p < 0.05$). There were no differences in MR rates among groups 2 and 3. The medium with ACP-318® slightly enhanced the nuclear maturation of canine oocytes when a comparison was established with rates of maturation exhibited by oocytes in the experimental group 1 without ACP-318® ($p < 0.05$). The results suggest that oocytes' nuclear morphology integrity and meiosis achievement were positively influenced when exposed to high glucose TCM199 supplemented with 5% powdered coconut water. Further investigation must be performed for a better understanding of powdered coconut water influence in cellular events during *in vitro* maturation of dog oocytes.

Introduction

Coconut water (endosperm from *Cocos nicifera*) is a sterile and weak acid solution. It contains proteins, salts, carbohydrates, vitamins, growth factors (phytohormones) and traces of phospholipids (Laguna 1996). Studies performed with gametes of various species have shown that coconut water can be successfully used for the preservation of preantral follicles in caprine (Silva et al. 2000, 2004; Martins et al. 2005), and in ovine

species (Andrade et al. 2002; Costa et al. 2006). Coconut water has also been tested as a holding medium for immature oocytes from bovine ovaries (Cordeiro et al. 2006), and as *in vitro* maturation medium for bovine oocytes (Blume et al. 1997a). Furthermore, coconut water has been used for culturing mouse (Blume and Marques 1994) and bovine embryos (Blume et al. 1997b).

Besides its nutrients (Santoso et al. 1996; Laguna and Nunes 1997) and saccharose concentrations, coconut water is endowed with antioxidants (Leong and Shui 2002), such as ascorbic acid in concentrations that vary between 1.91 to 2.03 mg/ml depending on stage of fruit maturation (Aroucha et al. 2005). Ascorbic acid is thought to contribute largely to coconut water's antioxidant activity (Leong and Shui 2002). Two important ingredients of coconut water are the indole-3-acetic acid (IAA) and the zeatin, one of the most frequent cytokinin in plants (Toniolli et al. 1996; Taiz and Zeiger 2002). According to Taiz and Zeiger (2002), a balance between these two components is necessary to induce cellular division in vegetal cells, as the IAA induces cellular growth and differentiation, while the cytokinin induces cellular division. It is known that IAA has the power to preserve caprine preantral follicles *in vitro* (Ferreira et al. 2001). However, the way these substances act on animal cells is not well understood and information related of their effects in IVM of canine oocytes is lacking.

The biochemical properties of coconuts can vary among fruits, directly affecting its ability to support cells in culture, and additionally the maintenance of the biological characteristics of coconut water during storage is restricted to a defined interval of time (Cardoso et al. 2005). To reduce this variability, studies were conducted to evaluate the effects of dehydration on coconut water stability (Salgueiro et al. 2002). ACP® consists of a thin and uniform dehydrated powdered formulation of coconut water obtained after selection of the fruits, collection of the endospermic liquid and submission to heat dehydration. Different forms of ACP® exist. Each has been adjusted according to the purpose of use for pH and osmolarity resulting i.e. in a medium like ACP-318®, developed specifically for *in vitro* maturation (IVM) of canine oocytes. ACP® has already been tested for the preservation of goat (Salgueiro et al. 2002), stallion (ACP-105®) (Sampaio Neto et al. 2002), and dog semen (ACP-106®) (Cardoso et al. 2005).

As opposed to the watery form, the powdered coconut water can be easily stored and readily sent to regions where fresh coconuts are not available. In addition, the composition is standardized, since it is obtained from fruits of the same plantation

(Cardoso et al. 2005). After solubilization, the biochemical characteristics of ACP® are very similar to those found in fresh coconut water (Cardoso et al. 2006).

To the best of our knowledge, there was no report on the efficiency of the use of powdered coconut water in the IVM of canine oocytes. For this reason, the objective of this study was to determine the influence of a high glucose TCM199 with diluted powdered coconut water (ACP-318®) in the achievement of nuclear IVM of canine oocytes.

Materials and Methods

Ovary and oocyte retrieval

Canine ovaries were obtained after elective ovariohysterectomy (OVH) surgery from 16 sexually mature females at unknown reproductive stages, aged between 1 and 8 years (mean age was 3.3 years). Ovaries were obtained at the Veterinary Hospital of Federal University of Rio Grande do Sul (UFRGS) (Av. Bento Gonçalves, 9090/ Porto Alegre/ RS; Brazil), and Zoonoses Control Center of Porto Alegre (Estrada Bérico José Bernardes, 3489/ Porto Alegre/ RS; Brazil). The ovaries were transported to the laboratory at room temperature in modified phosphate buffered saline (PBS) (Whittingham 1971) and were processed within 2 hours of collection. The ovarian cortex was sliced and washed in PBS supplemented with 1% foetal calf serum (FCS) (Nutricell, São Paulo, Brazil) to release cumulus oocyte complexes (COCs). COCs were placed in TCM199 buffered with 25 mM HEPES (Sigma, M-2520, St Louis, MO, USA), supplemented with 2.38 mM sodium bicarbonate (Sigma, S-5761), 0.10 mM gentamicin (Sigma, G-1264), 0.20 mM pyruvic acid (Sigma, P-4562), and 15.05 mM bovine serum albumin (BSA) (Sigma, A-3311). Only COCs with a compact multilayered cumulus-oophorus and homogeneous dark cytoplasm (Grade 1) (Otoi et al. 2000) were matured *in vitro*. This study followed the guidelines for ethical conduct in the care and use of animals instituted by COBEA (Colégio Brasileiro de Experimentação Animal).

In vitro maturation

The COCs (n = 632) were randomly distributed into experimental groups in Petri dishes, where 6-10 oocytes were placed into 100 µL drops (four drops per dish) of maturation medium under mineral oil at 37ºC in a humidified atmosphere containing 5% CO_2 in air for 48 hours. COCs in the group 1 (control group) were matured in

TCM199 buffered with 26.19 mM sodium bicarbonate (Gibco Invitrogen Corporation, 11150059, Carlsbad, CA, USA) (pH 7.36), and supplemented with 0.1% Polyvinyl Alcohol (Sigma, P-8136), 5.5 mM glucose (Merck, 108337, Darmstadt, Germany), 0.10 mM gentamicin, 0.20 mM pyruvic acid, 20 µg/ml oestradiol (Sigma, E-8875), 0.5 µg/ml follicle-stimulating hormone (FSH) (Folltropin-V, Vetepharm Inc., ON, Canada), 0.03 IU/ml human chorionic gonadotropin (hCG) (Chorulon®, Intervet, Milton Keynes, UK). The two others groups were matured in the same medium as in the control but added of 5% (group 2) and 10% (group 3) of powdered coconut water (ACP-318®) (ACP Biotecnologia ®, Fortaleza, Ceará, Brazil). The pH and osmolarity of ACP-318® were 7.35 and 300 mOsm/Kg respectively. The experiment was performed in 10 replicates.

Chromatin evaluation

Chromatin configuration in oocytes after IVM was evaluated by Hoechst 33342 (Sigma, B-2261) (10µg/ml) staining as previously described (Rodrigues et al. 2004). The chromatin configuration was characterized according to Bruck et al. (2000) by one of the following nuclear maturation stages: (i) germinal vesicle (GV): finely granulated nucleoplasm with filamentous chromatin configurations; (ii) germinal vesicle breakdown (GVBD): condensed filamentous chromatin within finely granulated nucleoplasm; (iii) metaphase I (MI): condensed chromosomes oriented in a metaphase plate; (iv) metaphase II (MII): two sets of condensed chromosomes of which one set with orientation in a metaphase plate, while the second set (polar body) more or less randomly oriented in a cluster; (v) others: no chromatin or small spots of dense chromatin.

Statistical Analysis

Statistical analysis was performed using the data analysis software SPSS, version 13.0 (SPSS Inc., Chicago, IL, USA). For comparison purpose of nuclear maturation in oocytes, data were analysed by Fisher's Exact Test. Differences at p value < 0.05 were considered of statistical significance.

Results

The meiosis resumption and progression of maturation results after exposition of canine oocytes to different medium are summarized in Table 1. The medium supplemented

with ACP-318® (groups 2 and 3) enhanced the rates of oocytes' nuclear maturation in comparison with the rates observed in group 1 without ACP-318® ($p < 0.05$). Additionally, oocytes matured in group 2 (with 5% powdered coconut water) were less prone to deviation of typical chromatin configuration (73/215, 34.0% versus 85/207, 41.1% and 90/210, 42.9% for group 1 and 3 respectively). The percentage of meiosis resumption (GVBD to MII) was similar between oocytes in experimental groups 2 (50.2%; 108/215) and 3 (46.6%; 98/210), while a different and lower response was observed for group 1 oocytes (39.1%; 81/207) ($p < 0.05$).

Discussion

The present results concerning *in vitro* meiosis resumption are similar to those previously reported in the literature for dog oocytes (Bolamba et al. 1998; Farstad 2000; Rodrigues and Rodrigues 2003). Even when at low rates, high glucose TCM199 supplemented with powdered coconut water (ACP-318®) supports the nuclear maturation of canine oocytes *in vitro*. In IVM of dog oocytes, the advancement of meiosis has been reported as a regulated time dependent event (De los Reyes et al. 2005). Despite the fact that an interval time of 48 hours seems to be sufficient for IVM of dog oocytes (Otoi et al. 2004; Rodrigues et al. 2004), under the culture conditions used herein, and as previously reported by others (De los Reyes et al. 2005), an interval beyond 48 hours of culture might have been necessary for the increase in the number of oocytes to the MII stage. Yet, a prolonged incubation time determines high rates of degeneration of canine oocytes which are particularly sensitive to this phenomenon (Luvoni et al. 2003).

The positive effects of powdered coconut water observed in the present study can be related to the high concentration in ascorbic acid as described by Leong and Shui (2002). Ascorbic acid has indeed been demonstrated to be one the most important oxygen scavenger in extracellular fluids (Levine 1986; Buettner 1993; Rose and Bode 1993) protecting the oocytes against oxidative stress (Wang et al. 2002) characterized by a significantly increased production of reactive oxygen species (ROS) *in vitro* (Luvoni et al. 1996).

Ascorbic acid also contributes to follicle integrity and survival in intact mouse ovarian follicles *in vitro* (Murray et al. 2001), facilitates meiotic maturation of porcine oocytes, and protects the cumulus cells (CCs) from DNA fragmentation (Tao et al. 2004).One of the main properties of coconut water has been attributed to the antioxidant activity due

to its content on ascorbic acid (Leong and Shui 2002). Yet, there is no information on the specific concentration of ascorbic acid in the powdered coconut water (CCM Salgueiro, 2008 personal communication). However as the biochemical characteristics of ACP® are very similar to those found in fresh coconut water (Cardoso et al. 2006), ascorbic acid values in the vicinity of 1.91 to 2.03 mg/ml, which are the usual concentrations on coconut fruit (Aroucha et al. 2005), are expected to be found in the powder as well. However, ascorbic acid being highly sensitive to denaturation, this assumption needs to be validated in future studies.

Despite the fact that our study found no differences in meiosis progression till the MII stage among oocytes in ACP medium, surprisingly ACP medium with 5% powdered coconut water contributed to a discreetly higher percentage of MII oocytes than ACP medium with 10% powdered coconut water. If caution must be used when identifying chromatin separation and condensation of oocytes, unclassifiable chromatin morphology may be viewed as a step toward degeneration. Therefore, and warranting confirmation, ACP medium with 5% powdered coconut water may exert a beneficial effect both on the maintenance of a typical chromatin configuration and on MII stage achievement of canine oocytes. The authors hypothesize on glutathione (GSH) and ascorbic acid as potential contributors to these findings, due to their well established cellular antioxidative actions (Meister 1994; Leong and Shui 2002).

Although there were minor differences in meiosis among groups, it seems pertinent that inferior results of MII achievement and oocyte´s usual chromatin configuration in group 3 might have been generated by the higher concentration of powdered coconut water in this group (bell-shape effect). Indeed, the addition of excessive amounts of ascorbic acid in medium was previously viewed as inductor of degeneration both in *in vitro* matured Nili ravi buffalo oocytes (Ullah et al. 2006), and in cryopreserved mouse embryos (Lane et al. 2002).

The apparently reduced meiosis resumption in group 3 may also have resulted from a negative interaction effects between IAA (auxins), nutritional factors and high glucose concentration. Indeed, Nanda and Jain (1971) reported that, in a high glucose environment, the cell development effects of auxins are reduced in an inversely proportional manner to its concentration. This is also consistent with Raven et al. (2003) observation that high concentration of auxin may have a deleterious role on meiosis resumption. These authors indeed postulated that increased IAA concentration in the

culture medium inhibits the process of cellular division. Our results may confirm these results, but require further evaluation.

Conclusions

This study showed that canine oocytes can resume meiosis and, achieve nuclear maturation *in vitro* in high glucose TCM199 supplemented with ACP-318®. Although there were differences of IVM among the experimental groups, numerical improvement in MII achievement and maintenance of usual chromatin morphology in oocytes were observed in medium supplemented with 5% powdered coconut water. Further investigation are necessary to confirm our observations, to identify the unknown variables affecting the data herein, and to improve the understanding of the function of powdered coconut water in *in vitro* maturation of canine oocytes.

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Table 1. *In vitro* maturation of canine oocytes exposed to different maturation media.

Treatment	Oocytes n.	GV n (%)	GVBD n (%)	MI n (%)	MII n (%)	Others n (%)
Group 1	207	41 (19.8) [a]	11 (5.3) [d]	68 (32.9)	2 (1.0)	85 (41.1) [f]
Group 2	215	34 (15.8) [b]	25 (11.6) [e]	78 (36.3)	5 (2.3)	73 (34.0) [g]
Group 3	210	22 (10.5) [c]	20 (9.5) [e]	76 (36.2)	2 (1.0)	90 (42.9) [f]

Values with different superscripts in the same column **(abc, de, fg)** differ significantly [Fisher's Exact Test (8, $N = 632$) = 15.682, $p < 0.05$] (p = 0.039).
GV: germinal vesicle, GVBD: germinal vesicle breakdown, MI: metaphase I, MII: metaphase II, Others: unidentified chromatin or degenerated.
Group 1: TCM 199; Group 2: TCM 199 with 5.0% of ACP-318®; Group 3: TCM 199 with 10.0% of ACP-318®

6. DISCUSSÃO E CONSIDERAÇÕES FINAIS

A competência na maturação *in vitro* (citoplasmática e nuclear) de ovócitos de caninos ainda permanece até o presente momento bastante limitada. Muito esforço tem sido feito por parte dos pesquisadores no sentido de desenvolver meios de cultura adequados e que suportem a maturação *in vitro* dos oócitos caninos, mas a taxa destes oócitos que atingem o estádio de metáfase II (MII) raramente excede 20% (OTOI *et al.*, 1999; FARSTAD, 2000; LUVONI *et al.*, 2001; RODRIGUES e RODRIGUES, 2003; KIM *et al.*, 2004; DE LOS REYES *et al.*, 2005; VANNUCCHI *et al.*, 2006).

A ocorrência de degeneração de COCs caninos é bastante elevada em ensaios *in vitro*. Dados obtidos de experimentos anteriores (SONGSASEN *et al.*, 2002) e recentes (RODRIGUES *et al.*, 2009) mostram que a degeneração dos COCs provavelmente pode estar relacionada a fatores prejudiciais desencadeados pela suplementação do meio de maturação com soro, como por exemplo o soro fetal bovino. Porém, o mecanismo pelo qual a presença de soro no meio resulta em elevação do percentual de degeneração *in vitro* dos oócitos ainda não está elucidado. É sabido que o soro provoca reações adversas na estrutura mitocondrial, por via do acúmulo de lipídios no citoplasma em oócitos bovinos (ABE e HOSHI, 2003) e ovinos (GARDNER, 1994; THOMPSON *et al.*, 1995). Devido ao fato de o oócito canino possuir grandes quantidades de gotas lipídicas, o que lhe confere a aparência escura e homogênea (GURAYA, 1965), ele pode assim ser mais susceptível à peroxidação lipídica. Desta forma, o acúmulo de lipídios induzido pela presença do soro no meio de maturação pode tornar o oócito ainda mais susceptível à peroxidação lipídica. É interessante observar que, conforme constatado pelos resultados do experimento realizado pelos presentes autores, a presença de soro no meio de maturação também foi responsável pela ocorrência de altos níveis de apoptose nas células do cumulus dos oócitos caninos (SILVA *et al.*, 2009b). A avaliação de apoptose nas células do cumulus tem sido usada como indicador de qualidade dos oócitos, visto que estas células circundam e estão interligadas aos oócitos tanto durante o desenvolvimento folicular quanto após a ovulação (RUVOLO *et al.*, 2007). A conexão entre células do cumulus e os oócitos em caninos persiste *in vivo* durante o desenvolvimento embrionário até o estabelecimento do estádio de mórula (RENTON *et al.*, 1991), sugerindo assim que a sua função é importante durante as etapas iniciais do desenvolvimento embrionário nessa espécie (NICKSON *et al.*, 1993). Assim, a ocorrência de apoptose nas células do cumulus, além de afetar diretamente a

capacidade dos oócitos de retomar a meiose, pode levar o oócito ao processo de degeneração.

A presença das células do cumulus em experimentos de maturação *in vitro* (MIV) é necessária à maioria dos ovócitos para que eles expressem sua competência (SIRARD *et al.*, 1988; TANGHE *et al.*, 2002). O metabolismo das células do cumulus controla o trânsito de metabólitos até o ovócito (CETICA *et al.*, 2002). O papel positivo das células do cumulus durante a maturação é atribuído à sua capacidade metabólica e está associado à inerente habilidade na estabilização da progressão da meiose, assim como na sua retomada. As células do cumulus suportam a maturação citoplasmática e reduzem a tensão gasosa de oxigênio nas imediações do ovócito (RODRIGUES, 2003).

O metabolismo da glicose é também um fator importante durante a maturação *in vitro* de oócitos caninos, especialmente porque a glicose é o principal substrato energético utilizado pelos oócitos caninos (SONGSASEN *et al.*, 2005). Desta forma, é recomendável a suplementação de glicose ao meio de maturação em altas concentrações, visto que o requerimento de glicose aumenta durante o período de maturação *in vitro* (SUTTON *et al.*, 2003). Entretanto, a presença de altas concentrações de glicose pode ser prejudicial ao desenvolvimento embrionário em algumas espécies, tais como em bovinos, especialmente nos estádios mais jovens (PINYOPUMMINTR e BAVISTER, 1991; HASHIMOTO *et al.*, 2000b), e em hamster (SCHINI e BAVISTER, 1988).

Por outro lado, tem sido reportado que oócitos bovinos cultivados em altas concentrações de glicose no meio podem desenvolver ate o estádio de MII, desde que os mesmos sejam cultivados sob baixa tensão de oxigênio (5%) (HASHIMOTO *et al.*, 2000a). Nossos resultados (SILVA *et al.*, artigo 2, em processo de submissão) mostraram que altas concentrações de glicose no meio suportam a maturação *in vitro* dos oócitos caninos ate o estádio de MI nas duas tensões de oxigênio testadas (20% e 5%), não havendo portanto diferenças significativas entre os dois níveis de oxigênio. Nossos achados corroboram os dados previamente reportados por Songsasen *et al.* (2001), confirmando que a tensão de oxigênio não é essencial para a progressão da meiose de oócitos caninos. É interessante destacar que, em um estudo recente sobre interações entre tensão de oxigênio e concentração de glicose, foi mostrado que o desenvolvimento de oócitos bovinos não foi afetado pela tensão de oxigênio quando estes foram maturados sob altas concentrações de glicose (20mM), ao passo que com

baixas concentrações de glicose (5,6 mM), os oócitos se desenvolveram melhor sob alta tensão de oxigênio (DE CASTRO e HANSEN, 2007).

Sob baixa tensão de oxigênio o oócito experimenta um déficit na disponibilidade de oxigênio (DE CASTRO e HANSEN, 2007). Exposição de células a baixas tensões de oxigênio causa um decréscimo de adenosina trifosfato (ATP) via metabolismo oxidativo mitocondrial (CZYZYK-KRZESKA, 1997; WENGER e GASSMANN, 1997). Tal decréscimo é compensado pelo aumento no metabolismo da glicose, o que pode ser comprovado pelo aumento na expressão do transportador de glicose 1 (GLUT1) (BASHAN *et al.*, 1992; EBERT *et al.*, 1995) e enzimas glicolíticas (FIRTH *et al.*, 1994; SEMENZA *et al.*, 1994). Como a glicólise é menos eficiente que a fosforilação oxidativa na produção de ATP, é provável que a produção de ATP pelo oócito se torne insuficiente em ambientes com restrição de oxigênio (DE CASTRO e HANSEN, 2007). Desta forma, altas concentrações de glicose no meio de maturação parecem fornecer suficiente glicose através do aumento do metabolismo da glicose induzido pela baixa tensão de oxigênio.

É importante ressaltar também que, apesar do fato de que os resultados obtidos em nossos experimentos indicarem que a tensão de oxigênio não exerceu influência na maturação nuclear dos oócitos caninos (SILVA *et al.*, artigo 2, em processo de submissão), ela se mostrou eficiente no sentido de reduzir a ocorrência de apoptose nas CCs (SILVA *et al.*, 2009b). Diante da importância e necessidade da presença das CCs para o processo de maturação e metabolismo dos oócitos (SIRARD *et al.*, 1988; TANGHE *et al.*, 2002), posterior fecundação e clivagem (NICKSON *et al.*, 1993), a redução da tensão de oxigênio se faz uma estratégia interessante no sentido de reduzir a ocorrência de apoptose nas CCs visando preservar a viabilidade do oócito nas etapas subseqüentes a maturação.

Com relação ao uso de água de coco em pó (ACP-318®) adicionada ao meio de maturação, nossos resultados mostraram que o meio contendo ACP-318® é capaz de suportar a maturação *in vitro* de oócitos caninos (SILVA *et al.*, 2009a). Os efeitos positivos da água de coco em pó (ACP) observados no presente estudo podem ser atribuídos a alta concentração de ácido ascórbico presente na mesma como reportado por Leong e Shui (2002). O acido ascórbico é considerado como um dos mais importantes seqüestradores de oxigênio intracelular (LEVINE, 1986; BUETTNER, 1993; ROSE e BODE, 1993) protegendo os oócitos contra o estresse oxidativo (WANG

et al., 2002) caracterizado pelo aumento significativo das espécies reativas ao oxigênio (ROS) *in vitro* (LUVONI *et al.*, 1996).

Apesar do fato de que não houve diferenças significativas na progressão de meiose até o estádio de MII entre os oócitos maturados em diferentes concentrações de ACP-318® (5% e 10%), surpreendentemente, a menor concentração de ACP (5%) no meio de maturação proporcionou um percentual discretamente mais elevado de MII do que a concentração de 10% de ACP. Apesar da pequena diferença em termos de meiose entre os grupos com diferentes concentrações de ACP, aparentemente os resultados inferiores em termos de maturação até estádio de MII e configuração de cromatina no grupo com 10% de ACP pode ter sido resultante da maior concentração de água de coco em pó neste grupo, levando a uma característica curva de dose-resposta em forma de sino. De fato, a adição de quantidades excessivas de acido ascórbico no meio de cultivo tem sido visto como indutor do processo de degeneração em oócitos de búfalos maturados *in vitro* (ULLAH *et al.*, 2006) e em embriões de camundongo criopreservados (LANE *et al.*, 2002). A aparente redução na retomada de meiose ocorrida no grupo com 10% de ACP pode também ser resultante de interações negativas entre a auxina presente na água de coco, o ácido 3-indol-acético (IAA), fatores nutricionais e alta concentração de glicose no meio de maturação. Conforme já relatado em literatura, em ambientes com altas concentrações de glicose, o desenvolvimento celular promovido pelas auxinas é reduzido de maneira inversamente proporcional à concentração de glicose (NANDA e JAIN, 1971). Nossos resultados podem confirmar estas observações, no entanto requerem mais investigações. Desta forma, mais estudos e avaliações se fazem necessárias no sentido de confirmar nossas observações e identificar as variáveis até então ainda desconhecidas e que afetam os resultados obtidos nos experimentos com oócitos caninos, bem como aprimorar o entendimento sobre o processo de maturação *in vitro* de oócitos caninos.

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ANEXOS

ANEXO A: THE INFLUENCE OF OXYGEN TENSION ON CUMULUS CELLS VIABILITY OF CANINE COCs MATURED IN HIGH-GLUCOSE MEDIUM

AEF Silva, P Rodriguez, LF Cavalcante, F Ongaratto, BA Rodrigues, JL Rodrigues

(Resumo publicado nos anais do 6th International Symposium on Canine and Feline Reproduction, & 6th Biannual European Veterinary Society for Small Animal Reproduction Congress. Vienna, Austria, pp. 226-228, July 2008)

Introduction - The low rate of meiotic maturation of canine oocytes cultured in vitro is a main obstacle to the in vitro production of canine embryos. It has been suggested that the addition of serum to maturation medium can increase the rate of degenerated oocytes [2] [3], as well as to increase the incidence of apoptosis [1]. Cumulus cells surround and intercommunicate with oocytes during follicular development and after ovulation, suggesting that the incidence of apoptosis in cumulus cells could influence the developmental capacity of the oocyte. It is known that high oxygen tensions in vitro are associated with high levels of reactive oxygen species (ROS), which are implicated in the occurrence of apoptosis [5].

Objectives - The objective of this study was to determine the influence of oxygen tension on cumulus cells viability from canine oocytes matured in high-glucose medium.

Materials and Methods - Canine ovaries were obtained after ovariohysterectomy (OVH) surgery from 9 bitches at unknown reproductive stages, and with ages ranging from 1 to 12 years. The ovaries were transported to the laboratory at room temperature in phosphate buffered saline (PBS) and were processed within 2h of collection. The ovarian cortex was sliced and washed in PBS supplemented with 1% of foetal calf serum (FCS) to release cumulus-oocyte complexes (COCs). COCs were placed in TCM199 with 25 mM HEPES (M-2520; Sigma), and COCs with a multilayered compact cumulus-oophorus and dark cytoplasm (Grade 1) were selected to in vitro maturation (IVM). The oocytes were then distributed into three groups and two oxygen

tensions (5% or 20%). The control group (CG) was matured in TCM199 with 2.2 mg/ml sodium bicarbonate (11150; Gibco) and supplemented with 10%
(v/v) of foetal calf serum (FCS) (Nutricell), 50 μg/ml gentamicin, 22 μg/ml pyruvic acid, 20 μg/ml oestradiol (E-8875; Sigma), 0.5 μg/ml follicle-stimulating hormone (FSH) (Folltropin-V; Vetepharm Inc), 0.03 IU/ml human gonadotropin (hCG) (Chorulon®; Intervet), and 1.0 μg/ml human somatotropin (hST) (Humatrope, Lilly). The two others groups, which were designed as T5 and T20, were matured in a high-glucose medium, constituted by TCM199 with 2.2 mg/ml sodium bicarbonate (11150; Gibco), and supplemented with 0.1% Polyvinyl Alcohol (PVA) (P-8136; Sigma), 0.991 mg/ml glucose (108337; Merck), 50 μg/ml gentamicin, 22 μg/ml pyruvic acid, 20 μg/ml oestradiol (E-8875; Sigma), 0.5 μg/ml folliclestimulating hormone (FSH) (Folltropin-V; Vetepharm Inca), 0.03 IU/ml human gonadotropin (hCG) (Chorulon®; Intervet), under 5% or 20% oxygen tension, respectively. After 48h of IVM the cumulus-cells were removed by mechanical cell displacement using a smalldiameter glass micropipette and stained with propidium iodide (1 mg/ml) in PBS. Viability in cumulus cells was evaluated by assessing cytoplasmic features and integrity in nuclear morphology, as previously reported [4]. Incidence of apoptosis (marginated chromatin, pyknotic appearance, multiple nuclear fragments, and apoptotic bodies) in cumulus cells was assessed as percentages. Counts were performed at various randomly selected fields of the cumulus mass pipetted onto a microscope slide and mounted with coverslip sealed with incolor nail polish.

Statistical Analysis - Statistical analysis was performed using the data analysis software SPSS, version 13.0. Chi-square test with adjusted residual was used to compare differences among apoptotic groups.

Results - Cumulus cells were removed from a total of 405 COCs: i) CG (n = 136); ii) T5 (n = 135); T20 (n = 134). A total of 2700 cumulus cells were counted, being 900 for each group. Rates of apoptosis in cumulus cells were statistical different between groups, with 57.9% (521/900), 38.9% (350/900), and 54.4% (490/900) for CG, T5 and T20, respectively ($P<0.001$). Predominant features in the 1361 counted apoptotic cells were those containing multiple nuclear fragments, and observed with 94.0% (1280/1361) incidence.

Conclusions - Cumulus cells of canine COCs cultured in high-glucose medium presented significant less apoptosis than those cultured in medium with FCS. The FCS might be responsible for the high level of apoptosis observed in the control group. Low level of oxygen tension was efficient to reduce the occurrence of apoptosis in canine cumulus cells.

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ANEXO B : PRELIMINARY STUDY IN IMMATURE CANINE OOCYTES STAINED WITH BRILLIANT CRESYL BLUE (BCB) AND OBTAINED FROM BITCHES WITH LOW AND HIGH PROGESTERONE SERUM PROFILES

BA Rodrigues, P Rodriguez, AEF Silva, LF Cavalcante, C Feltrin, JL Rodrigues

(Resumo publicado nos anais do 6th International Symposium on Canine and Feline Reproduction, & 6th Biannual European Veterinary Society for Small Animal Reproduction Congress. Vienna, Austria, pp. 199-200, July 2008)

Introduction - Lack of developmental competence and degeneration of canine oocytes in vitro matured endorse the necessity of using more specific criteria to assess quality and integrity of gametes destined to in vitro maturation (IVM). Appropriate oocyte selection may in consequence enhance positively in vitro meiosis in the bitch. By far, morphological appearence of immature cumulus oocyte complexes (COCs), as determined by features such cumulus thickness, homogeneity of the ooplasm, and size [2] [3] has been routinely used to select dog oocytes subjected to IVM. In other species like bovine, swine, caprine, and more recently in the mice [5], the brilliant cresyl blue (BCB) test has been incorporated prior to culture to the selection methods as a non invasive technique for identifying fully-grown oocytes, with ability to mature to MII stage. The BCB test is based on the capability of glucose-6-phosphate dehydrogenase (G6PDH) to convert the dye from blue to colorless. Oocytes that have finished their growth show decreased G6PDH activity and will exhibit blue coloration (BCB+), whereas growing oocytes contain G6PDH and reduce the dye to a colourless solution (BCB-) [1].

Objective - The aim of the present study was to observe the features and levels of blue color impregnation in high quality (grade 1) immature canine oocytes stained with the BCB dye, as an indirect quality and integrity indicators of nuclear chromatin configuration in COCs selected to IVM. Moreover, because lack of developmental competence in oocytes has been attributed to follicular atresia, depending on ovarian donor reproductive status [4], we observed as well the influence of serum progesterone concentrations from ovary donors on BCB staining of immature oocytes.

Materials and methods - It was used the protocol proposed by El Shourbagy et al. [1] with slight modifications. Immediately after morphological selection, grade 1 oocytes were distributed in groups of maximum 5-7 and incubated in 80µl drops of 26µM BCB (Sigma, B-5388) diluted in modified PBS with 1.090mg/ml glucose, 35.2mg/ml pyruvate acid, 0.4% (w/v) BSA fraction V (Sigma A-9647) at +37 °C, in humidified atmosphere with 5% CO2 in air for 60 min. After the incubation time, the oocytes were examined under fluorescence microscopy and classified according to: (i) dark blue cytoplasmic staining (BCB+), (ii) fainty blue cytoplasmic staining (BCB±), and (iii) colourless cytoplasm (BCB–). Bitches were distributed in two groups according the serum progesterone levels as following: (i) bitches with serum progesterone varying from 0-2.5ng/ml (n = 5); (ii) bitches with serum progesterone varying from 2.6ng/ml to 16.7ng/ml(n = 4). Serum concentrations of progesterone were measured by chimoluminescence. The synchronization of BCB coloration between the ooplasm and cumulus cells was qualitatively assessed, but not included in the statiscal analysis.

Statistical analysis - Data were analyzed using Chi-square analysis with adjusted residual to compare the effect of oocyte´s morphology on BCB staining. ANOVA with repeated measures and two factors was performed to analyze the influence of serum progesterone concentrations of ovary donors on oocytes BCB staining, and ANOVA with repeated measures was used to to determine the differences on mean numbers (mean ± SD) of BCB stained oocytes from 09 routines. The values were considered statistically significant when $P<0.05$.

Results - From 138 morphologically high quality COCs recovered from ovaries following slicing, the mean number of oocytes classified as the BCB+ was 9.9± 5.9, while the BCB± and the BCB- were 4.9± 2.9, and 0.6±1.7, respectively. Mean number of oocytes classified as grade 1 and stained BCB+ were statiscally different from the BCB- ($P= 0.010$). Also, mean numbers of oocytes BCB± were different from those of BCB- ($P= 0.014$). In this experiment, the percentage of germinal vesicle (GV) in BCB+ stained immature oocytes was 67.4% (60/89) and much higher than the percentages observed in BCB± (52.2%) and BCB- (20%) stained oocytes. The rates of germinal vesicle break down (GVBD) stage also differ between the BCB groups with higher rates been observed at BCB± and BCB- stained oocytes ($P=0.023$). There was no effect of

serum progesterone concentrations on the mean numbers of oocytes stained by the BCB dye (P= 0.680).

Discussion and Conclusion - In this experiment, the majority of BCB+ stained oocytes were observed at the germinal vesicle (GV) stage, demonstrating that in the dog this is the most probably feature to be expected in grade 1 oocytes previously selected by visual morphological appearence. Despite that, also we observed that resumption of meiosis, as identified by oocytes at the GVBD stage, was presented in 12.3% BCB+ oocytes, and this configuration may be vinculated to oocytes obtained from atretic follicles, as was previously proposed [2]. The GVBD pattern was found to be higher in BCB± and BCB- stained oocytes, and therefore the dye could possibly be used to separate oocytes from healthy follicles with developmental competence from those presumably derived from the atretic ones and therefore uncapable to achieve meiosis. One important factor was the inconsistency in BCB impregnation between the cumulus cells and the ooplasm in various from the observed oocytes. As reported by Wu et al. [5] for the mice, assyncrony in BCB coloration in COCs might suggest a disruption of metabolic coupling between the oocyte and its cumulus cells. This criterion might be additionally useful in predicting canine oocyte competence in vitro. Therefore, the preliminary findings on BCB staining of dog oocytes seem worthy of further investigation.

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ANEXO C: CUMULUS CELLS VIABILITY AND THE RELATIONSHIP WITH NUCLEAR MORPHOLOGY IN OOCYTES FROM PRE-PUBERTAL AND ADULT BITCHES AT 0, 24, 48 AND 72 HOURS AFTER IN VITRO MATURATION

P Rodriguez, N Arruda, LF Cavalcante, AEF Silva, BA Rodrigues, JL Rodrigues

(Resumo publicado nos anais do 6th International Symposium on Canine and Feline Reproduction, & 6th Biannual European Veterinary Society for Small Animal Reproduction Congress. Vienna, Austria, pp. 201-202, July 2008)

Introduction - In vitro maturation of oocytes in canine species is a complex process. Progression of meiosis is essential for the achievement of fertilization in vitro. At the present time both processes have been observed with low success in the bitch. Initial morphology of cumulus oocyte complexes (COCs), before in vitro culture is one important parameter inside these processes, and has been viewed as a predictor of oocyte competence.

Objective - The objective of this experiment was to observe the relationship of viabilitybetween the components of COCS, which was established through cumulus cells and oocytenuclear morphology analysis. Individual COCs evaluations were done at different moments ofin vitro culture (0, 24, 48, and 72 h). Furthermore, comparisons of above mentionedparameters were established between COCs from pre-pubertal and adult bitches.

Materials and methods - Ovaries were collected from pre-pubertal bitches (6-8 months) (n = 03), and from adult bitches (2-10 years) (n = 03) at unknown reproductive status, undergoing elective ovariohysterectomy. Oocytes from pre-pubertal bitches (n = 74) and adult bitches (n = 90) were obtained by slicing of ovarian cortex in PBS supplemented with foetal bovine serum (FBS) (Nutricell, São Paulo, Brazil) at 37°C. Oocytes of high quality (grade 1) with more than two compact layers of cumulus cells, dark pigmented and uniform ooplasm, and observed as having the largest diameter among their counterpartners (subjectively assessed) were selected and individually matured in vitro. Evaluation of viability of each oocyte and its respective cumulus cells

was performed after 0, 24, 48 and 72 h of culture, by denudation with strained glass pipettes. Fluorescence microscopy was used to observe the progression of nuclear maturation by using the fluorescent dye Hoechst 33342 as described previously [1]. Viability of cumulus cells (200 cells per COC) was performed by using a differential staining with propidium iodide (PI) (1mg/ml) and Hoechst 33342 (5µg/ml). The total of cumulus cells counted for each COC, were distributed into groups of viability as following: <50%, 50-70%, and >70%.

Statistical analysis - Statistical analyses were performed using the data analysis software SPSS, version 13.0. Data were analyzed using Chi-square analysis with adjusted residual. Fisher´s exact test was used for comparison of nuclear maturation in oocytes. The values were considered statistically significant when $P<0.05$.

Results - The results showed an intimacy of the viability between the cumulus cells and chromatin configuration in COCs, both in adult and in pre-pubertal bitches. In adult bitches this statement was statiscally confirmed by the rates of oocyte nuclear degeneration (20/23; 87%), when incidence of viability in cumulus cells was <50%, and by observation of meiosis progression to the MI stage (4/27; 15%) when incidence of viability in cumulus cells was >70%, while in pre-pubertal females rates of 100% (12/12) of nuclear degeneration in oocytes were observed at cumulus cells viability < 50%, and 9 % (3/32) meiosis progression to the MI stage, when incidence of viability in cumulus cells was >70% ($P < 0.001$). Rates of degeneration, as measured by numbers of PI positive cells, were enhanced in pre-pubertal (20/27; 74%) and different from the rates in adult females (2/40; 5%), when comparison was established between groups with 50-70% of viability in cumulus cells ($P<0.001$). Furthermore, in the group with cumulus cells viability <50%, rates of 12.5% (3/24) of degeneration in COCs from adult bitches at 0h interval time, increased to rates of 22.7% (5/22) at 48h, and 59% (13/22) at 72h of in vitro culture ($P<0.001$).

Conclusions - Viability of cumulus cells has an intimate relationship with nuclear chromatin in immature oocytes. Also, the results of this experiment showed that with the progression of interval time of in vitro culture, oocyte nuclear morphology was associated with the viability features presented by cumulus cells.

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ANEXO D: MENSAGEM DR. RODRIGUEZ-MARTINEZ – aceite artigo periódico Reproduction in Domestic Animals

Reproduction in Domestic Animals - Decision on Manuscript ID RDA-OA-Nov-2008-0318.R3

heriberto.rodriguez@kv.slu.se **Wed, May 13, 2009**
<heriberto.rodriguez@kv.slu.se> **at 1:10 PM**
To: tursilva@gmail.com, tursilva@hotmail.com

13-May-2009

Dear Mr. Silva:

It is a pleasure to accept your manuscript entitled "The influence of powdered coconut water (ACP-318®) in in vitro maturation of canine oocytes" in its current form for publication in the Reproduction in Domestic Animals.

Thank you for your fine contribution. On behalf of the Editors of the Reproduction in Domestic Animals, we look forward to your continued contributions to the Journal.

Sincerely,
Prof. Heriberto Rodriguez-Martinez
Editor-in-Chief, Reproduction in Domestic Animals
heriberto.rodriguez@kv.slu.se

ANEXO E: CONVITE COMO REVISOR CIENTÍFICO – Periódico Reproduction in Domestic Animals

Manuscript Assigned for Review - Reproduction in Domestic Animals

heriberto.rodriguez@kv.slu.se **Thu, May 28, 2009**
<heriberto.rodriguez@kv.slu.se> **at 1:52 PM**
To: tursilva@gmail.com, tursilva@hotmail.com

28-May-2009

Dear Mr. Silva:

Manuscript ID RDA-OA-May-2009-0154 entitled "DNA fragmentation in canine immature grade 1 cumulus-oocyte complexes" has been submitted to the Reproduction in Domestic Animals, where experts act as peer-reviewers. The manuscript has been, owing to your documented expertise in the area, assigned to you for possible peer-reviewing. To access the entire manuscript (for a pre-screening or further reviewing), you may easily do it DIRECTLY by a click on the link below (which will take you right to the score sheet),

http://mc.manuscriptcentral.com/rda?URL_MASK=5TRGdYS4H3SCHCFSx5Yd

In case you can undertake this substantial academic task, please submit your review before 17-Jun-2009. If for some reason you can not do it, please let us know in a reply e-mail as soon as possible, hopefully with a suggestion for a potential replacement reviewer.

As mentioned before, to access the manuscript, you may either do it DIRECTLY by a click on the link below (which will take you right to the score sheet),

http://mc.manuscriptcentral.com/rda?URL_MASK=qxjBRhqCZZ97N8J6FcB9

or, by login to the Reproduction in Domestic Animals - Manuscript Central site at http://mc.manuscriptcentral.com/rda . Your case-sensitive USER ID is tursilva@gmail.com.

Once you are logged in, the Main Menu will be displayed. Please click on the Reviewer Center, where you will find the manuscript listed under "Awaiting Reviewer Scores." You can click on the manuscript title from this point or you can click on the "View Details" button to begin reviewing the manuscript.

Due to an increase in the number of papers being submitted to Reproduction in Domestic Animals, we now receive many more manuscripts than we are able to print within an acceptable timeframe. Therefore, we encourage you as a peer referee to consider whether the manuscript is:

- within the scope of the journal.

- of sound scientific value, making a novel contribution in the field of reproduction

- of technical nature, in which case manuscripts should only be recommended for acceptance if they are important
developments that will have a significant impact in the area.

In your review, please answer all questions. On the review page, there is a space for "Comments to Editor" and a space for "Comments to the Author." Please be sure to put your comments to the author in the appropriate space.

If you wish to view the manuscript and the review form simultaneously, click on the HTML or PDF icons and the manuscript will open in a new window. Leave the new window open, switch back to the main window, and open the score sheet by clicking on the Score Sheet tab. Follow the instructions for reviewers provided in the Manuscript Central site. I strongly encourage you to elaborate on your review in the space provided. Your specific comments will offer valuable feedback to improve future work. It is essential that you click the "Save" button if you wish to exit the review before you submit it to the Editor. Otherwise, none of the information that you have entered will be saved in the system. When you have completed your review and are ready to submit it to the Editor, click on 'Submit'.

All communications regarding this manuscript are privileged. Any conflict of interest, suspicion of duplicate publication, fabrication of data or plagiarism must immediately be reported to me.

Thank you for evaluating this manuscript.

Sincerely,

Prof. Heriberto Rodriguez-Martinez
Editor, Reproduction in Domestic Animals

Artur E F Silva <tursilva@gmail.com> **Thu, May 28, 2009 at 5:37 PM**

To: heriberto.rodriguez@kv.slu.se

Dear Prof. Rodriguez-Martinez

Thank you for your invitation to be peer-reviewing the Manuscript ID RDA-OA-May-2009-0154 entitled "DNA fragmentation in canine immature grade 1 cumulus-oocyte complexes", it is an honor to receive your invitation, I've appreciated it so much. Unfortunately, I can not do it because I am spending full time finishing my PhD's dissertation, I'm so sorry that I'm not able to do it at this time.

Suggestions for potential replacement reviewer.

Berenice de Avila Rodrigues berenice@portoweb.com.br

Gaia Cecilia Luvoni cecilia.luvoni@unimi.it

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Nucharin Songsasen songsasenn@si.edu

I'm looking forward to being able for further contributions to this prestigious Journal in the near future.

Sincerely,
Artur E F Silva

heriberto.rodriguez@kv.slu.se **Fri, May 29, 2009 at**
<heriberto.rodriguez@kv.slu.se> **8:27 AM**
To: tursilva@gmail.com, tursilva@hotmail.com

29-May-2009

Dear Mr. Silva:

Recently, I asked you to review Manuscript ID RDA-OA-May-2009-0154 entitled "DNA fragmentation in canine immature grade 1 cumulus-oocyte complexes." It has since become apparent that you will not be able to review this manuscript at this time. Thank you for suggesting alternates.

I hope you will be able to review other manuscripts in the near future.

Sincerely,
Prof. Heriberto Rodriguez-Martinez
Editor, Reproduction in Domestic Animals
heriberto.rodriguez@kv.slu.se

ANEXO F: FORMULÁRIO PROTOCOLO EXPERIMENTO GLICOSE/ TENSÃO O_2/CCs

Número:...../.....

Experimento Glicose/Tensão O_2/CCs – Maturação *in vitro* de oócitos caninos

Dados da Paciente:

Raça:	Idade:	Peso:	Último ciclo/progest.:
Razão da castração:		Citologia vaginal:	

Dados da Coleta:

Data:	Peso ovariano:.....X.....	
Hora:	Estruturas/aspecto:	
Início do slicing:	Nº total oócitos:	Grau I:
Obs.:		Grau II:
		Outros:

Dados da MIV:

Data início:	Hora:	Término (dia/hora):

Controle	**Expansão**		
1-	1-	2-	3-
2-	1-	2-	3-
3-	1-	2-	3-
4-	1-	2-	3-
T-	**T-**	**T-**	**T-**

ACP-318 (5%)	**Expansão**		
1-	1-	2-	3-
2-	1-	2-	3-
3-	1-	2-	3-
4-	1-	2-	3-
T-	**T-**	**T-**	**T-**

ACP-318 (10%)	**Expansão**		
1-	1-	2-	3-
2-	1-	2-	3-
3-	1-	2-	3-
4-	1-	2-	3-
T-	**T-**	**T-**	**T-**

Experimento Glicose/Tensão O_2/CCs –Viabilidade CCs oócitos caninos

Data Avaliação:

Responsável:

CCs	**Control**	**T20 (20% O_2)**	**T5 (5% O_2)**
Normal			
Fragmentation			
Apoptotic body			
Pyknosis			
Marginated chromatin			
Total			

Observações:

ANEXO G: FORMULÁRIO PROTOCOLO EXPERIMENTO GLICOSE/TENSÃO O_2/MATURAÇÃO NUCLEAR

Número:...../.....

Experimento Glicose/Tensão O_2/Maturação Nuclear – MIV de oócitos caninos

Dados da Paciente:

Raça:	Idade:	Peso:	Último ciclo/progest.:
Razão da castração:		Citologia vaginal:	

Dados da Coleta:

Data:	Peso ovariano:.....X.....	
Hora:	Estruturas/aspecto:	
Início do slicing:	Nº total oócitos:	Grau I:
Obs.:		Grau II:
		Outros:

Dados da MIV:

Data início:	Hora:	Término (dia/hora):

Controle	**Expansão**		
1-	1-	2-	3-
2-	1-	2-	3-
3-	1-	2-	3-
4-	1-	2-	3-
T-	**T-**	**T-**	**T-**

ACP-318 (5%)	**Expansão**		
1-	1-	2-	3-
2-	1-	2-	3-
3-	1-	2-	3-
4-	1-	2-	3-
T-	**T-**	**T-**	**T-**

ACP-318 (10%)	**Expansão**		
1-	1-	2-	3-
2-	1-	2-	3-
3-	1-	2-	3-
4-	1-	2-	3-
T-	**T-**	**T-**	**T-**

Experimento Glicose/Tensão O_2/Maturação Nuclear – MIV de oócitos caninos

Data Coloração:

Data Leitura:

Responsável:

Mat. Nuclear	**Controle**	**T20 (20% O_2)**	**T5 (5% O_2)**
VG			
VGBD			
MI			
AI			
MII			
Others			
Degen.			
Total			

Observações:

ANEXO H: FORMULÁRIO PROTOCOLO EXPERIMENTO ACP-318®

Número:...../.....

Experimento ACP-318 – Maturação *in vitro* de oócitos caninos

Dados da Paciente:

Raça:	Idade:	Peso:	Último ciclo/progest.:
Razão da castração:		Citologia vaginal:	

Dados da Coleta:

Data:	Peso ovariano:.....X.....	
Hora:	Estruturas/aspecto:	
Início do slicing:	Nº total oócitos:	Grau I:
Obs.:		Grau II:
		Outros:

Dados da MIV:

Data início:	Hora:	Término (dia/hora):

Controle	**Expansão**		
1-	1-	2-	3-
2-	1-	2-	3-
3-	1-	2-	3-
4-	1-	2-	3-
T-	**T-**	**T-**	**T-**

ACP-318 (5%)	**Expansão**		
1-	1-	2-	3-
2-	1-	2-	3-
3-	1-	2-	3-
4-	1-	2-	3-
T-	**T-**	**T-**	**T-**

ACP-318 (10%)	**Expansão**		
1-	1-	2-	3-
2-	1-	2-	3-
3-	1-	2-	3-
4-	1-	2-	3-
T-	**T-**	**T-**	**T-**

Experimento ACP-318 – Maturação *in vitro* de oócitos caninos

Data Coloração:

Data Leitura:

Responsável:

Mat. Nuclear	**Controle**	**ACP-318 (5%)**	**ACP-318 (10%)**
VG			
VGBD			
MI			
AI			
MII			
Others			
Degen.			
Total			

Observações:

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