ALBERTO YUKIO CHAYA

PRODUÇÃO IN VITRO DE EMBRIÕES CAPRINOS PRÉ-PÚBERES: EFEITO DA MELATONINA NOS MEIOS DE MATURAÇÃO IN VITRO E CULTIVO IN VITRO EMBRIONÁRIO

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Medicina Veterinária, para obtenção do título de Doctor Scientiae.

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LISTA DE ABREVIATURAS

µg	Microgram
µm	Micrometer
AI	Artificial Insemination
AI´	Anaphase I
ATP	Adenosine Triphosphate
CM	Conventional Medium
COCs	Cumulus–Oocyte Complexes
cAMP	Cyclic Adenosine Monophosphate
DMSO	Dimethylsulphoxide
EG	ethylene glycol
ES	Equilibrium Solution
EGF	Epidermal Growth Factor
FCS	Fetal Calf Serum
FF	Folicular Fluid
FSH	Follicle Stimulating Hormone
GDF9	Growth-Differentiation Factor 9
GH	Growth Hormone
GnRH	Gonadotrophin-Releasing Hormone
GPx	peroxidase glutationa
GV	Germinal Vesicle
GVBD	Germinal Vesicle Breakdown
HIOMT	hydroxyindole-O-methyl transferase
HM	Holding Medium
ICM	Inner Cell Mass
IGF-I	Insulin-Like Growth Factor-I
IP3	1,4.5-trisphosphate
IVC	In vitro Culture
IVEP	in vitro Embryo Production

IVF	In vitro Fertilization
IVM	In vitro Maturation
IVP	In vitro Production
LH	Luteinizing Hormone
LOPU	Laparoscopic Ovum Pick-Up
MI	Metaphase I
MII	Metaphase II
mL	Milliliter
mm	Millimeter
mM	Millimolar
MOET	Multiple Ovulation and Embryo Transfer
MPF	Maturation Promoting Factor
NAT	serotonin-N-acetyl transferase
ng	Nanogram
PA	Post - Actiovation
PN	Pronuclei
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
SOF	synthetic oviduct fluid
TALP	Tyrode’s Albumin Lactate Pyruvate
TE	Trophectoderm
TGFβ	Transforming Growth Factor β
TI	Telophase I
VS	Vitrification Solution
ZP	Zona Pellucida
6-DMAP	6-Dimethylaminopurine

RESUMO

CHAYA, Alberto Yukio, D.Sc., Universidade Federal de Viçosa, julho de 2016. **Produção in vitro de embriões caprinos pré-púberes: efeito da melatonina nos meios de maturação in vitro e cultivo in vitro embrionário.** Orientador: José Domingos Guimarães. Coorientador(es): Luiz Sérgio de Almeida Camargo e Eduardo Paulino da Costa.

A produção in vitro de embriões caprinos ainda não é considerada uma biotecnologia rotineiramente efetiva. Um dos fatores mais importante e que dificulta a evolução no processo de todo o processo da PIV é o estresse oxidativo. A proposta adotada neste estudo foi avaliar os efeitos da adição de melatonina em meios de maturação e cultivo in vitro no desenvolvimento de embriões caprinos e na qualidade dos blastocistos oriundos de oocitos de cabras pré-púberes. O objetivo do primeiro estudo foi avaliar o efeito da melatonina no meio de cultura in vitro de embriões partenogenéticos através da taxa de desenvolvimento de blastocistos e vitrificação. Foi utilizado um meio de maturação e de cultivo in vitro e convencional com e sem melatonina (10^{-9} mol L^{-1}). Os oocitos com células do Cumulus oophorus foram maturados in vitro naqueles meios de maturação por 27 h e então ativados. Estas células ativadas foram cultivadas nos respectivos meios de cultivo por oito dias e então aferidos as taxas de blastocistos. O método de vitrificação foi utilizado de acordo com Mermillod et al., 1998. A adição da melatonina a 10^{-9} mol L^{-1} aos meios de maturação e de cultivo in vitro não apresentou melhora na taxa de blastocistos produzidos. Na segunda parte deste estudo objetivou-se avaliar o efeito da melatonina no meio de cultura in vitro, porém em embriões fertilizados artificialmente, por meio da taxa de desenvolvimento de blastocistos. Utilizou-se um meio convencional para MIV com ou sem adição de melatonina a 10^{-9} mol L^{-1}. Após a MIV, os oocitos maturados foram inseminados com semen a fresco, e os espermatozoides foram capacitados em meio de capacitação contendo heparina. Os possíveis zigotos foram separados e uma parte foi cultivado em meio de cultura padrão com adição da melatonina

a 10^{-9} mol L^{-1}. Ambos foram cultivados durante oito dias e a taxa de blastocisto foi aferida. Os resultados desta segunda etapa mostraram que ao utilizar melatonina a 10^{-9} mol L^{-1} adicionada ao meio de maturação in vitro convencional melhoraram quanto ao número de células fertilizadas (34,57% vs. 17,39%, $p<0,05$), embora, não houve melhora na taxa de clivagem e no número de blastocistos produzidos. A conclusão mostra que a melatonina a 10^{-9} mol L^{-1} adicionada tanto em MIV, quanto em CIV, não melhora o desenvolvimento e a produção dos embriões in vitro, embora possa aumentar a taxa fertilização in vitro.

ABSTRACT

CHAYA, Alberto Yukio, D.Sc., Universidade Federal de Viçosa, july, 2016. **In vitro production of prepubertal goats embryos: effect of melatonin in in vitro maturation and culture media.** Advisor: José Domingos Guimarães. Co-advisors: Luiz Sérgio de Almeida Camargo and Eduardo Paulino da Costa.

The in vitro production of goat embryos is not considered a routinely effective biotechnology. There are a number of factors that hinder the progress in the process of maturation, fertilization, cultivation and freezing. One of the most important factors that cause deleterious effects throughout the IVP process is the oxidative stress. The proposal of this study in order to prevent and to control that stress condition was to supplement the in vitro maturation (IVM) and in vitro culture media (IVC) with melatonin evaluating it effects on the development of goat embryos and quality of embryos derived from prepubertal goats oocytes. The aim of the first experiment was to evaluate the effect of melatonin supplementation in the in vitro culture on development of parthenogenetic embryos. It was used two in vitro maturation media [conventional maturation media (CM) and CM + 10^{-9} M melatonin] and two in vitro culture media [conventional cultivation media (CC) and CC + 10^{-9} M melatonin. The oocytes with cumulus cells were randomly distributed in the IVM and then activated by ionomycin method followed by 6-DMAP. Afterwards, presumptive zygotes were randomly distributed in the IVC media and cultured for eight days. Supplementation of 10^{-9} M melatonin to the IVM and IVC media showed no improvement in the parthenogenetic blastocyst rate. The blastocyst rate diminished when melatonin was added in vitro culture medium. The second study was also evaluate the effect of melatonin in vitro culture medium, but using in vitro fertilization over the blastocyst development rate. We used a conventional medium (CM) to in vitro maturation media and other CM was supplemented with melatonin at 10^{-9} M. After IVM, the matured oocytes were inseminated with fresh semen, and then, that

spermatozoa were capacitated and cultured for eight days. There was an in vitro culture media used and other one added with melatonin (10^{-9} M). The results of this second study demonstrated that the use of melatonin at 10^{-9} M added to the in vitro conventional maturation medium presented a beneficial effect on the number of fertilized cells (34.57 % vs. 17.39%, $p<0.05$). However, this addition there was no improvement in the cleavage rate nor the blastocyst rate. We concluded that melatonin at 10^{-9} M added in both IVM medium and IVC medium do not improve the development of embryos. However, it can be a gain in the in vitro fertilization rate when melatonin is added in the IVM medium.

INTRODUÇÃO

A caprinocultura é uma atividade agropecuária importante no cenário mundial, principalmente em países em desenvolvimento, pertencentes a Asia e Africa, que necessitam desta atividade como uma fonte geradora de renda através da comercialização de animais, carne, pele e leite, e como fonte nutricional, pelo consumo de proteína de alto valor biológico e do leite. O leite de cabra se dá por possuir alta digestibilidade, maior alcalinidade natural e elevado valor nutritivo. Para a saúde pública, o leite de cabra é uma tendência para substituir o leite de vaca em casos de alergia ou intolerância digestiva, principalmente em crianças. Além de ser o terceiro leite mais consumido mundialmente, atrás do de vaca e de búfala (Walter et al., 1965; Park, 1994; Haenlein et al., 2004; FAO, 2010; FAO, 2012).

O cenário brasileiro frente aos grandes produtores de pequenos ruminantes no mundo é ainda pouco expressivo, ocupa a 11ª posição do ranking mundial, possui um rebanho de 8,7 milhões de cabeças, e representa 1,3% do rebanho mundial de cabras (FAO, 2006; IBGE, 2013). Historicamente, a região nordeste é a mais tradicional, portanto, predomina a forma extensiva e de baixa tecnologia, porém possui o maior rebanho e produção de caprinos do Brasil. Por outro lado, é na região Sudeste, onde há a maior produtividade de leite de cabra, com sistema de produção tecnificado e um rebanho de alta seleção genética (Borges, 2003).

Diante deste fato, a disseminação de material genético de animais de raças especializadas é um fator crucial para o melhor aproveitamento da caprinocultura. Um programa inserido e que facilitará seu discernimento são as biotecnologias da reprodução, como o congelamento de sêmen, Inseminação Artificial (IA), Transferência de Embriões (TE), Produção de embriões in vitro (PIV), transgênese e clonagem.

Outro programa de melhoramento animal que promoverá a dispersão de um ganho genético e encurtará o intervalo de gerações é a utilização de fêmeas pré-púberes. Muitos pesquisadores são contra o uso de fêmeas jovens como doadoras de oocitos para fertilização in vitro. Defendem que a colheita dos oocitos devem ser em fêmeas com idades próximas à puberdade, pois acreditam que com o passar da idade favorecerá o desenvolvimento e crescimento das células oocitárias in vitro. Para o crescimento total dos oocitos in vivo é necessário a liberação de LH, armazenamento de proteínas e acúmulo de RNA, aumento da atividade de MPF e MAPK e a redistribuição das organelas celulares. Assim, o diâmetro dos folículos ovariano estão correlacionados positivamente com diâmetro de oocitos em vacas e búfalas (Fair et al., 1995; Raghu et al., 2002). E em cabras adultas, esta relação de folículos com diâmetros maiores (>5mm) também está envolvida com melhor desenvolvimento embrionário (Crozet et al., 1995). Já para as pré-púberes, pesquisadores constataram uma maior quantidade de folículos e oocitos nos ovários, e até resultados semelhantes em maturação in vitro e fertilização in vitro. Entretanto, outras pesquisas confirmam taxas menores de FIV e de blastocistos em bovinos, ovinos, suinos e caprinos (Armstrong, 2001; Romanguera et al., 2011).

A produção in vitro de embriões caprinos ainda não é uma biotecnologia rotineiramente efetiva devido a uma série de fatores que dificultam a evolução no processo de maturação, fertilização, cultivo e congelamento. Utilizando fêmeas pré-púberes torna-se um fator a mais que dificulta a evolução deste processo. Outro fator deletério durante todo o processo da PIV é o estresse oxidativo que ocorre em células de cultura in vitro. Este ocasiona um desequilíbrio na quantidade de espécies reativas de oxigênio (ERO) e de agentes antioxidantes, o que provoca danos ao DNA de ambas as células da linha germinativa. As consequências destes levam a alterações mitocondriais, bloqueio do desenvolvimento embrionário e apoptose (Wang et al., 2002).

A adição da melatonina (N-acetyl-5-methoxytryptamine) em meios básicos utilizados na PIV tem como finalidade a de se obter a sua função protetora contra danos oxidativos e sua capacidade de eliminar os radicais hidroxilas. Ela atua também como substratos, mediadores de transferência de elétrons e reações de radicais livres (Poeggeler et al.,1993; Reiter et al., 1994). Estas propriedades a tornam uma antagonista oxidativa em condições in vitro e farmacológicas o que contribuem para proteção fisiológica nas células (Hardeland & Pandi-Perumal, 2005).

Esta proposta tem como finalidade produzir embriões in vitro de cabras pré-púberes, na tentativa de oferecer futuramente a opção de se utilizar a melatonina em meio de cultura para multiplicação acelerada de fêmeas de alta produção comprovada.

REVISÃO BIBLIOGRÁFICA

A produção in vitro de embriões (PIV) é uma a biotecnologia aplicada em animais de produção com o objetivo de não somente obter embriões de animais selecionados geneticamente ou de aqueles que estão em risco de extinção, mas também de produzi-los em laboratórios para escala comercial. Outra alternativa da PIV seria a de evitar que fêmeas de alto valor econômico e genético sejam descartadas precocemente. A PIV está fundamentada em três fatores essenciais e contínuos: maturação dos oocitos colhidos de doadoras; a fertilização destes oocitos maturados e o cultivo destes embriões produzidos até o estágio de blastocisto.

1. Maturação in vitro

A maturação oocitária é definida como a reinicialização da primeira meiose e sua completa divisão, caracterizado pelo estágio de vesícula germinal até a metáfase II, acompanhada da maturação citoplasmática necessária para fertilização e para o desenvolvimento embrionário.

A obtenção de um oocito competente apto para ser ovulado e fecundado depende de dois fatores essenciais: maturação nuclear e maturação citoplasmática. O desenvolvimento normal de um oocito é caracterizado pela extrusão do primeiro corpúsculo polar (GVBD, germinal vesicle breakdown), com o restabelecimento da meiose, os cromossomos progridem da metáfase I para telófase I, completando a primeira divisão meiótica. Há a segunda divisão meiótica, de início rápido após a primeira divisão meiótica, com o surgimento do oocito na fase de metáfase II para posterior ovulação (Cha and Chian, 1998).

1.1. Maturação nuclear ou meiotica

O inicio da meiose no oocito se dá desde a vida fetal quando há o desenvolvimento dos ovários e se mantem estacionado na fase de diplóteno com a formação da vesícula germinal (VG). Através da vida reprodutiva de uma fêmea, os oocitos adquirem a habilidade de iniciar os processos de inversão meiótica dos oocitos em prófase I com o rompimento da vesícula germinativa (RVG) e consequentemente, o oocito, em seguida, irá ao estágio de diacinese, metáfase I (MI), anáfase I (AI´) e telófase I (TI), encerrando a primeira divisão meiótica. Logo, os oocitos progridem para a metáfase II da segunda divisão meiótica antes da ovulação (Cha and Chian, 1998).

De acordo com CHA & CHIAN (1998) uma das formas para alcançar a competência dos oocitos para se obter o rompimento da VG seria a liberação de LH pela onda pré-ovulatória e a degeneração dos folículos atrésicos. Pois, estes fatores são responsáveis pelo rompimento da vesícula germinal em animais in vivo durante o crescimento folicular. Porém, segundo os mesmos, para se conseguir a competência dos oocitos e desta forma o rompimento da VG, depende de certas características interligadas que se iniciam desde a formação dos folículos a fatores relacionados às células da granulosa, como os esteroides, Ca^{2+}, inositol 1,4.5-trisphosphate (IP3), cAMP, purinas e o hormônio LH. Além de existir também o papel das proteínas no RVG, tais como as citoplasmáticas, maturation promoting factor (MPF) e fator citostático.

Estudos como de Samake et al. (2000) e Bormann et al. (2003) demonstraram que sob estas condições citadas e por uma seleção qualitativa dos oocitos, a maturação nuclear pode ter resultados de 70-90% destes em MII. Diminui-se este número para 72% quando os oocitos foram adquiridos de folículos maior de 3 mm de cabras pré-púberes (Martino et al., 1994).

1.2. Maturação citoplasmática

Compreende a mesma fase do oocito em maturação nuclear, período do oocito que envolve desde seu estágio em VG até o progresso da metáfase I até a metáfase II, caracterizando-a em alterações estruturais citoplasmáticas, tais como a migração e organização dos grânulos corticais à região cortical do ooplasma (Mermillod et al., 2006). No período de crescimento e maturação dos oocitos, há a produção e o armazenamento de proteínas e de RNA no citoplasma, essenciais para a recomposição genômica, ativação e envios de mensagens indispensáveis para regulamentação da embriogênese. Desta forma, o metabolismo do oocito é caracterizado por ativar a transcrição e tradução de proteínas e RNA durante o período pré-ovulatório para serem utilizados pelos oocitos e blastocistos iniciais durante seus desenvolvimentos (Wassarman & Kinloch, 1992; Cha & Chian, 1998).

Os oocitos dos mamíferos são envolvidos por camadas das células do cumulus durante a foliculogeneses. A importância destas células para a maturação citoplasmática é sua sensibilidade às gonadotrofinas. Com isto, elas passam a produzir e secretar várias substâncias, como: polipeptídeos e hormônios esteroides, prostaglandinas, progesterona e AMP cíclico, todos relacionados ao promover o reinício da meiose (Thibault, 1977; Cha & Chian, 1998).

Sumariamente, para obter resultados positivos na produção de embriões, a qualidade de oocitos está totalmente correlacionada. Pois, através da maturidade do ooplasma, o citoplasma está apto para fornecer todo o aporte (RNA e proteínas) que a fertilização e o desenvolvimento embrionário necessitam (Cha & Chian, 1998).

Apesar de estes estágios serem concluídos com êxito com relação à competência oocitária, é de se esperar que alguns oocitos não sejam capazes de serem fertilizados e se

desenvolverem a embriões. Existem alguns fatores que estejam relacionados ao crescimento do mesmo, no folículo. Segundo Mermillod (1998), a progressão do número de oocitos competentes em uma população de folículos em crescimento pode ser o resultado daqueles que foram capazes de acumularem fatores como: RNA e proteínas, e que obtiveram maior aporte de hormônios e uma melhor regulação interfolicular.

No estágio da competência meiótica, como já foi citado em Mermillod (2008), oocitos maduros, fertilizados e que alcançam ao estágio de blastocisto in vitro não significa necessariamente que houve competência desenvolvida. Isto se torna uma forma de investigar uma situação fisio-patológica que ocorre na competência dos oocitos. Existem outros critérios mais específicos que poderão avaliar a qualidade dos embriões através das expressões genéticas, metabolismo do embrião ou taxa de sobrevivência in vitro dos embriões após a vitrificação.

1.3. Importância do cumulus oophorus na maturação oocitária

A morfologia do folículo, parâmetros foliculares e a qualidade do oocito devem estar associados ao êxito na MIV, na FIV e na produção de embriões in vitro. O complexo do cumulus oocito (CCO), principal estrutura adjunta e responsável pela morfologia do oocito, tem sua classificação correlacionando-o às quantidades de camadas pelas quais o oocito possui (Blondin & Sirard, 1995).

A associação entre oocito e células do cumulus se dá metabolicamente pela ligação gap junctions, permitindo a troca e o fornecimento de vários produtos metabólicos ao ooplasma. Esta comunicação no folículo é essencial para o desenvolvimento do oocito e consequentemente sua fertilidade. Dentre este metabolismo inclui a dos carboidratos, tanto de glicose, piruvato e lactato, como de aminoácidos (glutamina) e nucleotídeos para a produção de energia ao oocito. Além disto, é através do gap juctions que há passagens

de cAMP, purinas, citoquinas, peptídeos, hormônios esteroides, eletrólitos, e outras proteínas entre o fluido folicular (FF) e as células dos cumulus (Eppig and Downs, 1984). As células do cumulus possuem também uma função de auto-ajuda. Há produção de ácido hialurônico no meio extracelular que os permitem a se dispersarem e se expandirem, completando seu desenvolvimento (Salustri et al., 1989). Esta comunicação também se dá do modo inverso, do oocito às células do cumulus. O oocito secreta alguns fatores de crescimento como o growth differentiation factor 9 (GDF-9) e GDF-9B (conhecido também como bone morphogenic protein 15 (BMP-15), importantes para a diferenciação e controle das linhagens das células da granulosa (Sutton et al., 2003).

Um dos critérios mais seguidos pelos embriologistas na seleção de oocitos aptos à MIV é justamente a morfologia das células do cumulus oophurus. Através da definição de expansão do cumulus, permite-se presumir qual folículo esteja provavelmente em atresia (Hinrichs & Williams, 1997). A compactação seleciona-se aqueles que possuem maior número de camadas e de maior grau. Há uma correlação positiva entre o aumento das células do cumulus e a competência desenvolvida (Hashimoto et al., 1998). Assim, conclui-se que oocitos desnudos (os que não possuem células do cumulus ao seu redor) não apresentam habilidade em incorporar glicose e dependem da glutamina e do piruvato para a síntese de ATP (Shioya et al., 1988; Madison et al., 1992).

1.4. Fatores que afetam a qualidade oocitária

A qualidade embrionária está diretamente relacionada e é totalmente dependente da qualidade dos oocitos adquiridos pela fêmea. Os oocitos por sua vez, dependem da foliculogênese, do complexo cumulus-oocito selecionado no laboratório, do tamanho do folículo e do oocito adquirido para seleção, da temperatura e do pH adquiridos os oocitos, da idade da doadora e do meio de maturação utilizado para seu crescimento e maturação.

Composição do fluido folicular

A composição do fluido folicular (FF) é composta durante a foliculogenese e está diretamente relacionada ao estado nutricional da fêmea. Em ruminantes, a nutrição está correlacionada à fertilidade devido ao fornecimento de nutrientes específicos e essenciais aos animais, influenciando positivamente na fisiologia reprodutiva das fêmeas, sendo possível o desenvolvimento do folículo, ovulação, maturação oocitária, fertilização, sobrevivência embrionária e o estabelecimento da gestação (Robinson et al., 2006).

O principal efeito da nutrição sobre o desenvolvimento do oocito é o estresse celular. Este sob qualquer efeito adverso ocasionado por um desequilíbrio no balanço energético levará a um déficit no metabolismo das células da granulosa alterando a composição do fluido folicular, impedindo seu crescimento (Rooke et al., 2004).

O fluido folicular é constituído por proteínas, fatores de crescimento/citoquinas e outros hormônios peptídeos, esteroides (progesterona e 17β-oestradiol), metabólitos energéticos, eletrólitos, lipídeos (ácido lineico), outros fatores indefinidos e esta relacionado com a fisiologia ovariana, pois esta constituição não é constante e varia de acordo com o crescimento e o desenvolvimento de cada folículo (Sutton, 2003).

Efeito do folículo

A relação entre o tamanho do folículo e a competência oocitaria é muito evidente e conhecida. Trabalho como de Lonergan et al. (1994) permitiu, em bovinos, ampliar as características para seleção de oocitos e conseguir aumentar as chances de produzir embriões in vitro e uma melhora em sua morfologia. Em caprinos, foi Crozet et al. (1995) que obteve resultados semelhantes.

De acordo com Sirad et al. (1992), o que permitiu esta relação foi o fato de folículos maiores possuírem a capacidade de produzirem e armazenarem uma forma muito estável de RNA, possibilitando um microambiente folicular favorável.

Porém, em bovinos, a taxa de produção de embriões in vitro provindos de folículos grandes e médios é igual quando se trata de qualidade biológica. Já em caprinos, oocitos de folículos antrais médios (3,1-5 mm) tiveram uma capacidade oocitária prejudicada e se refletiram na baixa taxa de blastocisto (Crozet et al., 1995).

Segundo De Smedt et al. (1994), a competência meiótica dos oocitos de cabras progressivamente melhora durante o crescimento folicular (foliculogênese). Folículos de 2 mm estão ainda sintetizando RNA para quando alcançarem os 3 mm estejam no período de atividade de trascripcional baixa e sua morfologia nucleolar completa. Folículos menores que 0,5 mm de diâmetro, os oocitos permanecem no estagio de VG; folículos entre 0,5-0,8 mm, os oocitos retomam a meiose e permanecem na pro-metáfase I; folículos entre 1-1,8 mm entram na metáfase I; e aos 3 mm ou mais, há condensação nucleolar dos oocitos e alcançam à metáfase II, completando sua maturação.

O tamanho do folículo foi relacionado a composição do FF e ao desenvolvimento do Complexo Cumulus Oocito (CCO), e consequentemente, está correlacionado à quantidade de energia metabólica produzida. O CCO utiliza a glicose como sua principal fonte de energia, produzindo substratos como piruvato e lactato, principais nutrientes consumidos pelo oocito. A atividade glicolítica além de produzir energia em forma de ATP, fornece o piruvato e reduz os equivalentes utilizados para uma futura oxidação (Sutton et al., 2003). O metabolismo do piruvato pelo oocito ocorre durante as 12h iniciais da maturação. Já para os oocitos desnudos, estes possuem baixa capacidade de utilizar glicose e são dependentes da síntese de ATP pelo metabolismo da glutamina e do piruvato (Zuelke & Brackett, 1992).

Efeito reprodutor

Existem parâmetros extremamente críticos que estão correlacionados para o sucesso da fertilização in vitro. O macho reprodutor é um parâmetro que pode comprometer completamente a PIV de embriões. Espermatozoides de mamíferos não são imediatamente capazes de fertilizarem os oocitos, eles passam por um período de preparação que in vivo ocorre no órgão genital feminino (Rogers, 1978; Parrish et al., 1988). É necessário um processo que possibilite aos espermatozóides recém coletados se capacitem para ocorrer a a união desses dois gametas e iniciar o desenvolvimento embrionário. Este meio utilizado é uma alternativa para a capacitação espermática e fertilização, pode ser de tais tipos como: meio TALP, modified Defined Medium e TCM199. Proteínas como o soro-albumina, o cálcio e o bicarbonato são geralmente adicionadas em meios de capacitação também. Porém, o suplemento mais importante para a capacitação dos espermatozoides foi a heparina (Younis et al., 1991; Parrish et al., 2014).

Heparina é um polissacarídeo pertencente ao grupo das glicosaminoglicanas (GAG), é secretada pelo epitélio do aparelho reprodutivo feminino, principalmente na tuba uterina, e atua na capacitação espermática, influencia na motilidade e melhora a habilidade de fertilização dos espermatozoides em muitas espécies, como: bovinos, caprinos, suinos, ratos, ovinos (Handrow et al., 1982; Cognie et al., 1996; Dora et al., 2006; Borg and Holland, 2008; Towhidi et al., 2009). A heparina estimula a capacitação espermática através das mudanças que ocorrem na membrana do plasma dos espermatozoides, tais como: redistribuição dos lipídeos na bicamada lipídica, o influxo de cálcio, fosforilação de proteínas específicas e outras mudanças que ocorrem no metabolismo intracelular do espermatozoide (Bergqvist et al., 2006).

Outra condição importante para o sucesso da FIV é a concentração espermática. A quantidade de espermatozoides nos quais serão incubados pode afetar a eficiência da capacitação. Assim também as condições dos incubadores. O tempo de incubação e o contato entre espermatozoide-oocito-meio capacitante também é muito importante a considerar (Rogers, 1978). Segundo Izquierdo et al. (1998), em caprinos, alguns fatores são responsáveis pelo aumento da taxa de penetração espermatozoide-oocito, como: a utilização de ejaculados de semen a fresco de alta qualidade, uso da concentração de 4 x 10^9 espermatozoides \ mL, seleção do semen com motilidade de no mínimo de 70%, e tempo de incubação com heparina de 45 min, a temperatura de 38.5°C.

Idade das doadoras

O objetivo de adotar fêmeas pré-púberes como doadoras de oocitos está relacionado ao programa de melhoramento genético. Antigos programas como inseminação artificial e teste de progênie conseguiram durante os últimos 20 anos aumentar a intensidade e acurácia na seleção genética das raças de gado bovino. Atuais programas, tais como: transferência de embrião, aspiração folicular guiada por ultrassom, MIV, FIV e CIV pretendem utilizar estas fêmeas pré-púberes e diminuir o intervalo de gerações e aumentar o ganho genético (Duby et al., 1996).

Outra vantagem para o uso de fêmeas pré-púberes é a maior quantidade de folículos e oocitos que estas possuem em relação a uma vaca adulta. Os folículos vesiculares surgem no ovário de bovinos durante a gestação, entre 220-240 dias. E, o ovário neonatal possuem entre 75.000 a 300.000 oocitos aos 5 dias pós-nascimento (Erickson, 1966).

Quanto a competência oocitária, Wang et al. (2002) utilizando oocitos de cabras pré-púberes e de adultas obtidos por LOPU, obtiveram resultados semelhantes tanto para

MIV, quanto para a FIV. A conclusão para estes autores está relacionada quanto a qualidade dos oocitos e não a idade dos oocitos coletados, ou seja, eles devem estar envoltos por várias camadas de células do cumulus, possuírem um citoplasma homogêneo e terem um diâmetro grande.

Armstrong et al. (1992) também utilizando oocitos de vacas pré-púberes e de adultas obtidos por LOPU e por abatedouro, obtiveram maiores taxas de mórula e de blastocisto em novilhas. Apesar de ser baseados em um número pequeno de amostragem, como o mesmos ressaltaram, a proporção na taxa de clivagem não se diferiu entre novilhas e vacas.

Contudo, Armstrong (2001) afirma que a idade da doadora é um fator significativo por influenciar a competência oocitária e consequentemente a eficiência na produção in vitro de embriões de vacas, ovelhas e porcas. Uma das explicações cabíveis é o fato de a competência ser sequencial ao crescimento e desenvolvimento do folículo. Isto significa que para alcançar o estágio final do folículo pré-ovulatório, o folículo deve sofrer a influência dos hormônios gonadotróficos (FSH e LH) e ter acúmulo de cAMP (Bagg et al., 2006). Necessariamente, para ocorrer este fenômeno, a fêmea deve estar na fase pré-púbere, próximo de sua fase adulta.

Para Revel et al. (1995), vacas adultas além de possuírem uma taxa de blastocisto maior que em novilhas, mostraram também uma taxa de prenhez melhor. Apenas 4% das novilhas conseguiram levar a prenhez. Desta forma, mostra-se a importância da formação de um microambiente folicular adequado antes da puberdade. Não somente pelo estabelecimento pulsátil da secreção de LH, mas também pelo amadurecimento gonadal, quando há o aumento do número específico de receptores gonadotróficos.

1.5. Meios e suplementos utilizados para MIV

Os componentes adicionados ao meio de cultura são fundamentais para o desenvolvimento dos oocitos imaturos utilizados para a produção de embriões in vitro. Através destes, serão possíveis criar condições favoráveis e úteis para optimizar a maturação e o desenvolvimento destas células imaturas. São adicionados em diferentes estágios de crescimento, de acordo com a necessidade fisiológica nos quais se encontram estas células (Cognié et al., 2003).

Os suplementos mais comuns adicionados ao meio de cultura são: MEM, Waymouth, Ham-F12. Entretanto, os componentes mais utilizados são: meio TCM 199, bicarbonate buffered, minerais, carbono e fontes de energia (glicose, glutamina), vitaminas, aminoácidos, L-glutamine, pyruvate, FSH, LH, estradiol-17ß, fluidos (soro fetal bovino, soro de cabra), EGF e diferentes componentes thiol, tais como: cystine, cysteine, cysteamine, glutathione, ß-mercaepthanol e protetores celulares antioxidativos (Cognié et al., 2003, Paramio, 2010).

Utiliza-se também o fluido folicular (FF) adquirido de folículos maiores (>4mm) e não atrésicos, através deste, verifica-se uma influência positiva na maturação oocitária quanto a presença de fatores de crescimento (IGF-I e EGF), hormônios esteroides, e peptídeos intra-ovariano. Há receptores específicos nas células da granulosa promovendo este efeito positivo na maturação in vitro. Além, de estarem relacionados à extrusão do primeiro corpúsculo polar na meiose II e na recuperação dos folículos atrésicos (Ainsworth et al., 1980; Guler et al., 2000; Cognié et al., 2003).

Melatonina

A melatonina (N-acetyl-5-methoxytryptamine) é o principal hormônio da glândula pineal. Porém, há a produção em outros órgãos como a retina e o trato gastrointestinal. É sintetizado do aminoácido triptofano e secretado como serotonina. Para se tornar em melatonina, são necessários duas enzimas, serotonin-N-acetyl transferase (NAT) e hydroxyindole-O-methyl transferase (HIOMT). A característica primordial destas enzimas é que são expressadas de acordo com a quantidade de luz captada pela retina do animal (fotoperiodo) (Claustrat et al., 2005; Tamura, 2009).

A propriedade principal da melatonina fisiologicamente é a hormonal. Por ser altamente solúvel tanto em água como em lipídios, atravessam facilmente as membranas celulares e alcançam vários fluidos, tecidos e compartimentos celulares, como: saliva, urina, fluidos cerebrospinal, folículos pré-ovulatórios, semen, fluidos amniótico e o leite. A melatonina está relacionada ao ritmo circadiano e a reprodução em animais considerados de dia curto, como ovelhas e cabras. Desta forma, a variação na produção e na secreção deste hormônio, depende da flutuação anual do tempo e da duração do dia. A atividade ovariana de grande parte das cabras é nula durante alguns meses do ano, como o outono e o inverno na região sul do país (Claustrat et al., 2005). Desta forma, existem maneiras artificiais para imitar os efeitos dos dias curtos nestes animais, elevando o nível de melatonina circulante. Administrar melatonina exógena de forma contínua e lenta com este intuito, pode ser obtida com a aplicação de implantes, como dispositivos subcutâneos (Chemineau et al., 1996).

Alguns dos papéis mais importantes da melatonina na reprodução se devem a existência de seus receptores no hipotálamo, na hipófise e no ovário, e também de possuírem a capacidade anti-oxidativa, em ambos os níveis fisiológico e farmacológico,

com a ação contra radicais livres (Woo et al., 2001; Dubocovich et al., 2003. Retire et al., 2005).

Melatonina na performance reprodutiva

A optimização da performance reprodutiva quando se utiliza implantes com melatonina reflete na produtividade e na fertilidade em cabras (Zarazaga et al., 2012). Diferentes concentrações de melatonina podem afetar algumas funções reprodutivas, principalmente, no ovário, onde foram encontrados receptores específicos para melatonina em ratos, camundongos e humanos (Niles et al., 1999; Lee et al., 2001; Soares et al., 2003). Há maior produção nas células da granulosa, na esteroidogenese e aumento nos folículos de galinhas e humanos (Webley & Luck, 1986; Ayre & Pang, 1994; Tamura et al., 2012).

Melatonina e sua ação oxidante

Melatonina também é considerada uma eficiente e poderosa protetora contra danos oxidativos pela sua capacidade de eliminar os radicais hidroxilas. Ela atua como substratos e mediadores de transferência de elétrons e reações de radicais livres (Poeggeler et al.,1993; Reiter et al., 1994). Esta propriedade a torna uma antagonista oxidativa em condições in vitro e farmacológicas o que a contribui para proteção fisiológica nas células (Hardeland & Pandi-Perumal, 2005).

Outras ações da melatonina como proteção antioxidante, acrescenta-se a de regular outras enzimas antioxidantes. Ela é capaz de estimular a glutathione peroxidase (GPx), uma enzima responsável pela redução de lipídios e peroxidos de hidrogênio o que diminui danos oxidativos nos tecidos (Arthur, 2000).

A melatonina também atua na cadeia respiratória e seu efeito está por diminuir a formação de radicais livres. Diferentemente do efeito antioxidante, ela elimina radicais já formados (Hardeland & Pandi-Perumal, 2005). Tal efeito já citado por Poeggeler (2004), o qual a melatonina foi a responsável por reduzir a formação H_2O_2 mitocondrial.

Melatonina na produção in vitro de embriões

Para maximizar o potencial de fêmeas geneticamente superiores, é importante o desenvolvimento de métodos que permitam a produção de maior número de descendentes em menor intervalo de tempo e impedir o descarte precoce de fêmeas geneticamente privilegiadas portadoras de alterações adquiridas que impedem que a reprodução ocorra de forma natural ou até mesmo pela transferência de embriões (Gonçalves - Bulnes et al., 2002). A produção in vitro de embriões (PIV) é um exemplo, envolvendo as etapas de colheita, maturação e fecundação de oócitos, bem como o cultivo e co-cultivo de zigotos e estruturas embrionárias.

Apesar da PIV estar bem estudada em diferentes espécies, é considerada ainda uma técnica que possui uma baixa eficiência. Em bovinos, a taxa de prenhez de embriões produzidos in vivo supera os produzidos in vitro (Pontes et al., 2009). Uma das causas para diminuir esta eficiência se dá pelas condições aeróbias que as células in vitro são submetidas. O oxigênio é indispensável para o crescimento e o desenvolvimento destas células. É através deste elemento que as reações bioquímicas são realizadas para suas atividades biológicas (Sugino, 2004; Devine et al., 2011; Tamura et al., 2012).

As espécies reativas de oxigênio (do inglês Reactive Oxigen Species - ROS) tais como: radical superóxido (O^{2-}), radical hidroxila (OH^-) e peróxido de hidrogênio (H_2O_2) são provenientes do oxigênio, e considerados os mais importantes. ROS são formados durante a fosforilação oxidativa e na produção de ATP quando há liberação de elétrons

da membrana mitocondrial interna (Devine et al., 2012). Eles interagem com proteínas, lipídios e ácidos nucleicos, ocasionando danos no DNA, peroxidação lipídica e principalmente, afetam a estrutura e a função da membrana celular (Sugino, 2005).

Entretanto, para evitar esta tamanha destruição celular provocada pela ROS, as células sob condições aeróbicas produzem antioxidantes específicos. Há enzimas nos quais eliminam os radicais superóxido, como o cobre-zinco superoxido desmutase (SOD), e o manganês superoxido, ambos localizados na mitocôndria. Existem também as catalases, convertem os peroxidos em água e oxigênio. E a peroxidase glutationa (GPx), que degradam os peroxidos lipídicos e o hidrogênio (Sugino, 2005). ROS e antioxidantes estão em constante equilíbrio nas células dos mamíferos, caso haja uma produção excessiva de ROS, desencadea-se o estresse oxidativo (Agarwal et al., 2005).

ROS são produzidos nos ovários, especificamente dentro dos folículos, durante a ovulação. Nas células esteroidogenicas também são fonte de ROS. Nas mitocôndrias, retículo endoplasmático, membrana nuclear e plasmática também o são (Sugino, 2005). A excessiva produção destes metabólitos de oxigênio danificam estruturas celulares e moleculares contribuindo para enfermidades que comprometem a reprodução e fertilidade (Agarwal et al., 2005).

Um dos principais objetivos para se obter um excelente crescimento e desenvolvimento embrionário é protegê-lo do estresse oxidativo que as condições de cultura in vitro o fornecem. Uma das formas cabíveis de evitar a toxicidade provocada pela ROS é a de adicionar antioxidantes nos meios embrionários (Takahashi, 2012).

Há dois tipos de antioxidante utilizados em meio de cultivo embrionário in vitro, o enzimático\proteico e o não-proteico. Os antioxidantes enzimáticos são conhecidos como naturais. São compostos de superóxido desmutase, catalase, glutathione peroxidase e glutathione reductase, os quais reduzem o peróxido de hidrogênio em água e álcool. Os antioxidantes não enzimáticos são conhecidos como sintéticos. São as vitaminas e os

minerais como a vitamina C, vitamina E, selênio, zinco, taurina, hipotaurina, glutationa, beta-caroteno e caroteno.

A utilização de antioxidantes no cultivo embrionário in vitro foram capazes de melhorar o desenvolvimento embrionário. A melatonina, um exemplo de antioxidante enzimático, foi efetiva em meios de cultura in vitro de camundongos, bovinos, suínos, búfalos, ratos e murinos (Ishizuka et al., 2000; Papis et al., 2007; Rodriguez-Osorio et al., 2007; Manjunatha et al., 2009; Tian et al., 2010; Wang et al, 2013). Apesar de seu elevado peso molecular, sua funcionalidade seria como de radicais livres, nos quais retiram o ROS extracelular gerado para fora dos meios dos embriões (Takahashi, 2012). Outra função, seria a de estimular a expressão de genes antioxidantes e de genes anti-apoptóticos, o que levaria a uma melhoria na qualidade dos blastocistos (Wang et al., 2013).

2. Fertilização in vitro

A FIV é uma biotecnologia já muito bem estabelecida que tem uma papel na ciência da reprodução como uma técnica básica e aplicada a produção de embriões. Desde 1878, seu objetivo continua sendo uma alternativa para infertilidade em humanos e animais, aumentar a produtividade de carnes e conservação genética de animais (Bavister, 2002).

É um procedimento minucioso que para obter resultados satisfatórios deve ter cautela aos protocolos propostos na maturação oocitária, seleção espermática, capacitação espermática e meios adotados. Em cabras, a utilização de sêmen a fresco são frequentemente mais utilizados (Cox et al., 1994; Keskintepe et al., 1994; Crozet et al., 1995; Mogas et al., 1997a; Izquierdo et al., 1998; Anguita et al., 2007; Romaguera et al., 2011). Já para sêmen congelados não são tão comuns (Keskintepe et al., 1998; Rho et al., 2001; Bormann et al., 2003).

Tendo em vista os avanços nas tecnologias adotadas na FIV, o foco voltado para a manipulação do espermatozoide, principalmente centralizado para capacitá-lo, permitiu ser possível a fertilização in vitro em humanos em 1969 (Bavister, 2002). Para estas condições, é necessário preparar os espermatozoides para estes serem capazes de fertilizar um oocito maduro. Desta forma, o primeiro passo para a manipulação dos espermatozoides começa separando os componentes viáveis dos inviáveis do sêmen. Os componentes viáveis são constituídos pelos espermatozoides vivos e potencialmente fertilizáveis, e os inviáveis são os espermatozoides imóveis ou mortos, debris, microrganismos e plasma seminal.

No macho caprino, utiliza-se dois métodos de separação, o swim-up (Keskintepe et al., 1994; Izquierdo et al., 1998; Katska-Ksiazkiewicz et al., 2004; Jiménez-Macedo et al., 2005; Anguita et al., 2007; Romaguera et al., 2010b) e o de centrifugação em gradiente de densidade descontínuo (Pawshe et al., 1996; Rho et al., 2001; Wang et al., 2002a; Bormann et al., 2003). A diferença entre estes dois métodos se vê quando se compara somente na quantidade de espermatozoides móveis (melhor para o swim-up), pois para a taxa penetração do espermatozoide no oocito e a taxa de clivagem não resultam em diferença entre eles (Palomo et al., 1999).

Uma vez separado os espermatozoides viáveis, é necessário capacitá-los. A capacitação é definida como modificações estruturais que ocorrem na membrana do espermatozoide após a reação acrossômica. É ativada pela liberação de enzimas proteolíticas produzidas no órgão genital feminino e causam alterações no acromosso, expondo os receptores de membrana, tornando-o apto a ligar aos receptores específicos da zona pelúcida no oocito. Em caprinos, utiliza-se uma serie de agentes, tais como: estro de ovelhas inativado por calor (De Smedt et al., 1992), soro de cabra (Koeman et al., 2003; Katska-Ksiazkiewicz et al., 2004), heparina (Izquierdo et al., 1998; Jiménez-

Macedo et al., 2005), heparina e ionomicina (Wang et al., 2002a; Urdaneta et al., 2004), inóforo de cálcio (Pereira et al., 2000), e heparina e cafeína (Younis et al., 1991).

O meio de fertilização utilizado para caprinos, incluem: modified Defined Media (mDM) (Crozet et al., 1995), meio Tyrode's Albumin Lactate Pyruvate (TALP) (Parrish et al., 1986) suplementado com hypotaurine (Mogas et al., 1997b; Izquierdo et al., 1998), e meio Synthetic Oviductal Fluid (SOF) (Rho et al., 2001). O meio seletivo para a capacitação dos espermatozoides selecionados com mDM com heparina, e meio TALP com hypotaurine para fertilização dos oocitos forneceram altas taxas de oocitos penetrados (Izquierdo et al., 1998).

3. Cultivo in vitro (CIV)

A CIV é o último evento para a produção de embriões in vitro. É neste período de cultura pós-fertilização onde se há o maior impacto para a qualidade dos blastocistos produzidos (Rizos et al., 2002a). Existem vários eventos que necessitam ocorrer para o melhor desenvolvimento do embrião, como: primeira divisão da clivagem, ativação do genoma embrionário, compactação da mórula e a formação do blastocisto. No blastocisto, há a diferenciação de dois tipos celulares: a massa celular interna (ICM), que originará o feto e o trofoderma (TE), que formará a placenta (Watson, 1992).

Um fator crucial para que o embrião se desenvolva bem é a concentração de oxigênio que há nos incubadores. Na maioria dos mamíferos, a tensão de oxigênio varia entre 3,5 a 8% no órgão genital feminino (Fischer and Bavister, 1993). Apesar do controle que há neste equipamento, o que preocupa é o acúmulo de ROS que é produzido no desenvolvimento embrionário.

Outro fator importante a acrescentar nos meios de culturas é o suporte de energia metabólica que os embriões necessitam em seus crescimentos. O fornecimento do soro

constituído de aminoácidos, vitaminas e fatores de crescimento, possibilitaram o melhor desenvolvimento aos embriões, porém também causaram um certo tipo de toxicidade. Este efeito tóxico se deve ao metabolismo oxidativo que ocorre no desenvolvimento dos blastocistos. Entretanto, a ação benéfica do soro é devido a sua propriedade antioxidativa. Desta maneira, diminui o estresse oxidativo e provoca menor fragmentação de células ocasionadas pela cultura de embriões in vitro (Bavister, 1995).

O meio de cultura muito utilizado para o crescimento e desenvolvimento de embriões de pequeno ruminantes é o TCM 199, porém foi pelo meio SOF (synthetic oviduct fluid) que obteve sucesso de se conseguir o estádio de blastocisto na cultura de zigotos de ruminantes. A partir disto, laboratórios rotineiramente suplementam o meio SOF com o soro. O soro, além de possuir as características citadas anteriormente, tem o poder de inibir a divisão inicial de clivagem. Isto pode acelerar o efeito tardio de desenvolvimento, resultando em blastocistos iniciais (Lonergan et al., 1999; Gutiérrez-Adán et al., 2001).

Visando o melhorar o meio de cultura, pesquisadores adicionam também fatores de crescimento, como epidermal growth factor (EGF), insulin-like growth factors (IGF), pela influência que estes possuem no metabolismo e desenvolvimento embrionário, resultando em aumento na taxa de blastocistos e no número de células dos blastômeros (Bavister, 1995; Głabowski et al., 2005).

4. Vitrificação de embriões

É uma biotecnologia aplicada nas técnicas reprodutivas assistidas com o intuito de preservar o material genético em forma de oocitos ou embriões de animais de alta produtividade e de animais com risco de extinção. A aplicabilidade desta técnica permitiria a formação de um banco de germoplasma para o uso no futuro. Existiria

benefícios práticos e vantagens econômicas. Porém, os procedimentos de vitrificação de embriões não são totalmente seguros e não existe um protocolo padrão espécie-específica (Gibbons et al., 2011).

O procedimento de vitrificar é considerado difícil de criopreservar embriões especialmente pelo fato de estes serem extremamente sensíveis à formação de injurias, como cristais de gelo ao ato de congelar (Leibo et al., 1996). Desta forma, os embriões são expostos a diferentes concentrações de crioprotetores, aumentando sua viscosidade, sendo capaz de eliminar completamente a formação de cristais de gelo até os resfriarem a baixas temperaturas. Este procedimento necessita-se de alta concentrações de crioprotetores, o que pode ser tóxico para os embriões. Uma alternativa para diminuir esta toxicidade, utilizada em embriões de pequenos ruminantes, foi o etileno glicol, uma substancia que possui uma alta taxa de penetração celular e baixa toxicidade. Geralmente, associado com o glicerol, um crioprotetante já bem conhecido na literatura e que apresenta boas taxas de sobrevivência de embriões e de prenhez (Martinez et al., 2002; Guignot et al., 2006). Outra alternativa recomendada é vitrificar pequenos volumes (0.6–2 µl), assim, evitará a osmolaridade nos embriões durante a criopreservação. Existem diferentes metodologias aplicadas em diferentes espécies, como por palhetas (Brail et al., 2001), grades de microscopia eletrônica (Martino et al., 1996), capilares finos (Vajta et al., 1997), cryo-tops (Kuwayama and Kato, 2000), cryo-loops (Lane et al., 1999), ou pontas de micropipetas (Cremades et al., 2004).

Dentre os vários métodos de congelamento de embriões in vitro, a vitrificação não requer uma série de equipamentos podendo ser adotada rotineiramente (Cognie et al., 2003). Portanto, um método para mensurar a criopreservação de embriões produzidos in vitro é a habilidade de sobrevivência dos embriões em cultura após o processo congelamento-descongelamento (Cognie et al., 2003).

5. Partenogênese

A produção de descendentes por uma fêmea sem a contribuição genética de um macho e sem a redução meiotica dos cromossomos é um fenômeno reprodutivo denominado de parthenogenesis, do grego “nascimento virgem”. É um processo comum entre os insetos, principalmente nas abelhas, mosquitos e formigas. Também pode ocorrer em alguns vertebrados, como as cobras, pássaros e anfíbios. Em mamíferos, só é possível verificá-la em estágios iniciais do desenvolvimento embrionário, através de alguns estímulos in vitro no oocito de ratos, cabras, vacas, macacos e humanos (Hipp & Atala, 2004; Kharche & Birade, 2013).

6. Ativação partenogenética

A ativação partenogenética de oocitos de mamíferos fornece uma ferramenta de estudo que permite investigar separadamente o papel do genoma paterno e materno no controle do inicio do desenvolvimento embrionário, contribui para o melhor entendimento do mecanismo de fertilização, e permite compreender os princípios gerais do sistema de sinalização da célula (Susko-Parrish et al., 1994; Lan et al., 2005; Malik et al.; 2014).

A ionomicina associada sequencialmente com 6-dimethylaminopurine (6-DMAP) é o método mais utilizado para se obter a ativação de oocitos e é considerado semelhante para os de FIV quanto ao desenvolvimento de blastocistos (Susko-Parish et al., 1994; Rho et al., 1998; Ongeri et al., 2001). Neste tratamento, permite o aumento intracelular de Ca^{2+} pela liberação de cálcio das reservas citoplasmáticas (ionomicina), similar ao fenômeno observado após a penetração dos espermatozoides no oocito, enquanto a proteína 6-DMAP acelera a formação pronuclear e o desenvolvimento partenogenético

de oocitos em metafase II de camundongos e bovinos por inibir as funções da proteína quinase e promover a mitose (Szollosi et al., 1993; Susko-Parish et al., 1994). O 6-DMAP também pode melhorar a inativação do maturation promoting factor (MPF) e o mitogen-activated protein kinase (MAPK). Ambos se aumentarem, iniciará a quebra da vesícula germinal e a progressão da metáfase durante a maturação oocitária. Após a FIV ou a ativação partenogenética, a MPF é inativada em oocitos que estão na fase de transição da MII e, a atividade da MAPK diminui quando inicia a formação pró-nuclear (Liu et al., 1998).

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OBJETIVOS

Objetivos da proposta

Os objetivos propostos são:

✓ Avaliar o uso de melatonina a 10^{-9} M em meio de maturação in vitro no desenvolvimento e na qualidade embrionária de oocitos de cabras pré-púberes.

✓ Avaliar o uso de melatonina a 10^{-9} M em meio de cultivo in vitro no desenvolvimento e na qualidade embrionária de oocitos de cabras pré-púberes.

✓ Investigar o efeito da melatonina no meio de MIV convencional sobre o aumento da taxa de blastocistos partenogeneticos;

✓ Investigar o efeito da melatonina no meio de CIV convencional sobre o aumento da taxa de blastocistos partenogeneticos;

✓ Avaliar o uso de suplementar melatonina a 10^{-9} M em meio de maturação in vitro no desenvolvimento de embriões partenogeneticos vitrificados.

✓ Avaliar o uso de suplementar melatonina a 10^{-9} M em meio de cultivo in vitro no desenvolvimento de embriões partenogeneticos vitrificados.

CAPÍTULO 1 - EFFECT OF MELATONIN ON IN VITRO DEVELOPMENT OF PARTHENOGENETIC EMBRYOS FROM PREPUBERTAL GOATS

Scientific paper in preparation

Abstract

The objective of this experiment was to test the effect of melatonin at 10^{-9} M supplementation during in vitro maturation (IVM) and in vitro culture (IVC) of small oocytes from pre-pubertal goat on the parthenogenetic blastocyst yield and quality. We used two types of IVM media: (i) basic medium supplemented with 10 μg/mL follicle stimulating hormone (FSH), 10 μg/mL LH, 1 μg/mL and 17 β-oestradiol (conventional IVM medium, CM); (ii) basic medium supplemented with 10 μg/mL follicle stimulating hormone (FSH), 10 μg/mL LH, 1 μg/mL, 17 β-oestradiol (conventional IVM medium, CM) and melatonin (10^{-9} M). After IVM, oocytes were activated by the method ionomycin associated sequentially with 6-DMAP. We also used two types of culture medium: (i) base medium supplemented with 10% fetal cow serum (FCS); (ii) base medium supplemented with 10% fetal cow serum (FCS) and melatonin (10^{-9} M). After cultured for 8 days, the blastocysts quality was assessed by the survival following vitrification/thawed and the mean cell number. The different maturation media were combined, the blastocyst rate did not improved. Following the results of IVM, the different culture media were combined, the blastocyst rate did not improved, on the contrary, the blastocyst rate were worst when added melatonin on IVC medium. In conclusion, adding melatonin at 10^{-9} M on IVM media and/or IVC media will not improve the quality of in vitro blastocysts.
Key words: caprine, melatonin, embryo, parthenogenetic, ionomycin.

1. Introduction

The in vitro embryo production (IVP) of goats is an important method for reproductive and genetic biotechnologies goats, but it has not yet become a routinely effective biotechnology. The results of embryos produced by IVP are low and there are still a number of factors that hinder the progress in the maturation process, fertilization, cultivation and freezing. One of the factors which causes deleterious effects throughout the IVP process is the oxidative stress. This causes an imbalance in the amount of reactive oxygen species (ROS) and antioxidants in the cell medium in vitro, which causes damage to the DNA of cells. The consequences of this lead to mitochondrial alterations, blocking embryonic development and apoptosis (Wang et al., 2002).

The addition of melatonin (N-acetyl-5-methoxytryptamine) in basic media used in IVP aims to achieve its efficient and powerful protective function against oxidative damage and its capacity to eliminate hydroxyl radicals. It also acts as substrates, electron transfer mediators and reactions of free radicals (Poeggeler et al., 1993; Reiter et al., 1994). These properties make it an oxidative antagonist in vitro and pharmacological conditions which contribute to the physiological protection cells (Hardeland and Pandi-Perumal, 2005).

The functionality of melatonin in culture media for in vitro production of embryos is theoretically very evident, especially as antioxidant action. What that is still unfounded is the exact amount, physiologically and pharmacologically, to its action in the cells. Few references are known about the concentration of toxic potential. Studies using exogenous doses of melatonin in rats showed that the toxicity in acute or chronic is extremely low (Jahnke et al., 1999). The use of different melatonin concentration (10-3 to 10-12 M) in the culture media intensified the in vitro embryo development as in mice, bovine, porcine, mouse and murine (Ishizuka et al., 2000; Papis et al., 2007; Rodriguez-Osorio et al., 2007; Shi et al., 2009; Tian et al., 2010; Gao et al., 2012; Takada et al., 2012; Wang et al., 2013; Wang et al., 2014).

The melatonin concentration that achieved best results in both IVM as the IVC was 10^{-9} M in porcine, mouse and bovine (Rodriguez-Osorio et al., 2007; Shi et al., 2009; Tian et al., 2010; Wang et al., 2014). A very important feature of this concentration is because it is very close to the physiological level of some species as human and rabbit (Adriaens et al., 2006; Mehaisen and Saeed, 2015). However, few of them have related some difficulty and problem on development of embryos, mainly in caprine and using prepubertal female donors.

The IVF process requires methodologies for oocyte maturation, sperm preparation, sperm-oocyte fertilization and embryos culture. However, in vitro embryo production continue demonstrating low efficiency due to many factors involving each step of this process. So, the elimination of two that those factors, as sperm preparation and fertilization and add parthenogenetic activation of mammalian oocytes, it will facilitate separately the knowledge about the role of paternal and maternal genome in the control of early embryonic development, contribute to better understanding of fertilization mechanism, and allows to understand the general principles of the system signaling cell (Susko-Parrish et al., 1994; Lan et al., 2005; Malik et al., 2015). Furthermore, the goat IVF process has not been improved and developed until now to get to the optimal level and produce as enough number of percentage of blastocyst development. The low percentage of blastocysts developing from goat IVF oocytes could be attributed to the polyspermy that it observed in goat IVF. In addition, Ongeri et al. (2001) have gotten better results to the blastocyst development than IVF control. Therefore, the use of ionomycin associated sequentially with 6-DMAP is the most method used to obtain oocytes activation (Susko-Parrish et al., 1994; Rho et al., 1998; Ongeri et al., 2001). This treatment allows elevating intracellular concentration of $Ca2+$ by release cytoplasmic calcium reserves (ionomycin), while 6-DMAP protein accelerates parthenogenetic pronuclear formation and promotes early development of mouse, bovine and goat oocytes (Szollosi et al., 1993; Susko-Parrish et al., 1994; Ongeri et al., 2001).

Oocytes from prepubertal females have been used in in vitro embryo production in several studies for many years and most of them have reported low oocyte competence and, consequently, smaller blastocyst percentage stage when it were compared with adults oocytes (Armstrong, 2001). The genetic breeding program using prepubertal female is a way to reduce the interval between generations of families and obtain a rapid genetic gain, sometimes they do not happen in practice (Duby et al., 1996). Although, the use of prepubertal female has the increase amount of oocytes than an adult female, the oocytes from prepubertal are smaller than 125 µm being unable to development into blastocyst stage. The age of the donor is a significant factor to influence oocyte competence and consequently the efficiency of in vitro production of cattle embryos, sheep, sows and caprine (Armstrong, 2001; Romaguera et al., 2011). Thus, it suggests that the difficult of embryos development was due to culture conditions provided in in vitro embryo production.

The aim of this study was to increase the efficiency of in vitro embryos production from prepubertal goats oocytes. The proposed objectives were: (i) to investigate the activation

of prepubertal goats oocytes using ionomycin subsequently combined with 6-DMAP and subjected to addition of melatonin in in vitro maturation medium; (ii) to investigate the effect of adding melatonin in conventional IVM medium in order to increase the parthenogenetic blastocysts rate; (iii) to investigate the effect of adding melatonin in conventional IVC medium in order to increase the parthenogenetic blastocysts rate; (iv) to evaluate the effectiveness of parthenogenetic embryos vitrified.

2. Material and Methods

2.1. Reagents

All chemical reagents used in processing media were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Oocytes collection, its classification and IVM

Ovaries of pre-pubertal goat (1-2 months old) were obtained from a slaughterhouse located in Castelbisball, Barcelona - Spain. After collection, these ovaries were transported to the Reproduction Laboratory Small Animal Veterinary School, UAB and it maintained at 38 °C in PBS to be processed for a period of two hours.

The samples were washed in PBS and maintained at 38 °C to be subsequently recovered by the slicing technique. The selection of oocytes for IVM was a priority for the choice of cumulus-oocyte complex (COC), those with two or more layers of compact cumulus cells and homogeneous cytoplasm were used.

The COCs were washed in wash dishes containing three drops of maturation medium. Groups of 25-30 COCs were transferred into 100 μL drops of IVM medium covered with mineral oil and incubated at 38.5 °C and 5% de CO_2 in humidified air. The basic medium adopted for IVM was TCM-199 supplemented with 275 μg/mL sodium pyruvate, 146 μg/mL L-glutamine, 50 μg/ml gentamycin, 10% (v/v) donor bovine serum (CanSera, Otario, Canada) and 100 μM cysteamine. There was used a basic medium supplemented with 10 μg/mL follicle stimulating hormone (FSH), 10 μg/mL LH, 1 μg/mL e 17 β-oestradiol and basic medium supplemented with 10 μg/mL FSH, 10 μg/mL LH, 1 μg/mL, 17 β-oestradiol (conventional IVM medium, CM) e 10^{-9} M melatonin (M-5250, Sigma, St. Louis, MO, USA).

2.3. Parthenogenetic activation of matured oocytes

At the end of IVM, it were denuded by pipetting. Ionomicyn and 6-DMAP were previously diluted in DMSO, both diluted in CR1aa and the last supplemented with 5% FCS before using it (Rosenkrans et al., 1993). Oocytes were exposed to an ionomycin concentration for 4 min and then washed in PBS and incubated in duplicates in 6-DMAP (Sigma) to 38.5 ºC and 5% CO_2 in humid air for 3 hours.

2.4. In vitro embryo culture (IVC)

After 4h, the oocytes were washed to remove remaining cumulus cells and 6-DMAP. Groups of 18-25 presumptive embryos were transferred to microdrops of 10 μL of synthetic oviductal fluid medium (SOF) (Takahashi and First, 1992). Two types of culture medium were used: (i) base medium supplemented with 10% fetal cow serum (FCS); (ii) base medium supplemented with 10 % FCS and melatonin (10^{-9} M); both covered with mineral oil in a humidified atmosphere of 5 % CO_2, 5 % O_2 and 90 % N_2 for 8 days. Cleavage rate was evaluated at 48 h after activation, and at the end of the culture period, the number of total cells of parthenogenetic embryos was assessed by Hoechst 33342 staining method (Sigma, USA) on fluorescent microscope. Total embryos rate (embryos with two or more blastomeres), morula (16 or more embryos with cells and without blastocele) and blastocyst (embryo with 60 or more cells and formed blastocele) were measured.

2.5. Vitrification of parthenogenetic blastocysts and warming

The vitrification method used was described by (Mermillod et al., 1998). For thawing method was described by (Morató et al., 2010). As vitrification as thawing the basic medium used was holding medium (HM) consisting of Hepes buffered TCM 199 with 20% FCS. Survival rate were calculated from the extent of re-expansion of the blastocyst after 3 and 24h post-recovery hours.

2.6. Experimental design

Effectiveness of melatonin added in the culture medium during IVM and IVC and its effects on the parthenogenetic embryo development of oocytes goat prepubertal. The effect of melatonin concentration of 10^{-9} M added in IVM medium was evaluated in cleavage, in blastocyst and in embryonic quality rates. Oocytes were selected and added in conventional IVM medium and in conventional IVM medium supplemented with melatonin (10^{-9} M). After IVM, COCs were activated using ionomycin and 6-DMAP and rates of cleavage and blastocyst were observed on days 2 and 8 pa (post - activation). On day 9 pa, blastocysts were fixed and stained to determine the total number of cells. To analyze the effect of melatonin in embryonic development, presumptive zygote were divided in group of 10 / 10 µl of culture medium containing concentrations of 0 and 10^{-9} M melatonin. To analyze the effect of melatonin in the IVM medium and in the IVC medium, it were divided in four treatments: (1) Treatment A: IVM medium without melatonin and IVC medium without melatonin; (2) Treatment B: IVM medium without melatonin and IVC medium added with melatonin; (3) Treatment C: IVM medium added with melatonin and IVC medium without melatonin; and (4) Treatment D: melatonin added in IVM medium and IVC medium. The total oocytes collected after slicing technique were divided in a half and put them on in two plates, one plate with maturation medium without melatonin added, and other one with melatonin added. All those oocytes after matured, they were activated by parthenogenetic technique and those oocytes were divided equally in other two culture plates. One plate of IVC medium without melatonin and other one, IVC medium with melatonin added. After 24h PA, it was calculated the cleavage rate. Then, after eight days PA, it was calculated the blastocyst rate.

Effectiveness of the culture medium during IVM on the tolerance to parthenogenetic blastocysts vitrification. The objective of this study was to analyze the effect of melatonin (10^{-9} M) in embryonic development during IVM measured by survival rate of embryos subjected to the method of vitrification / devitrification. The early blastocysts were vitrified on day 8 pa and cultivated for 24 hours post-devitrification for analysis of viability in vitro, as previously mentioned.

Effectiveness of the culture medium during IVC on the tolerance to parthenogenetic blastocysts vitrification. The objective of this study was also to analyze the effect of melatonin (10^{-9} M) in embryonic development during IVC measured by survival rate of embryos subjected to the method of vitrification / devitrification. The blastocysts were

vitrified on day 8 pa and cultivated for 24 hours post-devitrification for analysis of viability in vitro, as previously mentioned.

2.7. Statistics

For data analyses the statistical analysis system was used (SAS, 2002). Percentages of the cleavage, total blastocyst, early, expanded, hatched and expanding blastocyst were submitted to logistic regression (Logistic procedure) using total of oocytes and total of cleavage as trials. Comparisons were performed using the Chi square test. Significant differences were adopted as $P<0.05$.

3. Results

Effectiveness of melatonin added in the culture medium during IVM and IVC and its effects on the parthenogenetic embryo development of oocytes goat prepubertal.

The results prepubertal goats oocytes activation using ionomycin subsequently combined with 6-DMAP subjected to addition of melatonin in the maturation medium in vitro are presented in Table 1. After oocytes were exposed to a concentration ionomycin for 4 min and it washed in PBS and incubated in duplicates in 6-DMAP (Sigma) to 38.5º C and 5% CO_2 in humid air for 3 hours, a total of 824 oocytes were used in four replicates to evaluate the effects of activation. It demonstrated that oocytes were perfectly activated using ionomycin/6-DMAP for all treatment.

The effect of adding melatonin in conventional IVM medium in order to increase the rate parthenogenetic blastocysts did not exist between control and melatonin, as showed on Table 1. There were not difference ($p>0.05$) between group control and group melatonin in relation on cleavage rate.

Embryo development rates of oocytes cultured in the culture medium without and with melatonin (10^{-9} M) supplemented according to number of total oocytes can be seen on Table 1. There was no difference for the cleavage rate ($p>0.05$). However, there was evidence of a difference in total blastocyst rate, melatonin added in the culture medium got worse in relation of total blastocyst and expanded rates than those without melatonin in the medium.

The effect of melatonin (10^{-9} M) in the maturation medium and culture medium on prepubertal goats embryo development according number of total oocytes is presented on

Table 1. The cleavage rate for all four treatment were: 92.54 %, 94.55 %, 94.50 % and 90.95 %. It that means no difference among all treatments ($p>0.05$).

In the case of total blastocyst and expanded blastocyst, on Table 1, can be observed that the treatment A had a bigger number of total blastocyst and expanded blastocyst (47.26 %, 21.39 %; $p < 0.05$) than treatment B (26.73 %, 10.89 %) and treatment D (24.89 %, 5.43 %) but there was no difference in the treatment C (40.50 %, 18.00 %). The treatment B had the same number of blastocyst and expanded blastocyst than treatment D, but it was less than A and C. The treatment C, as the treatment A, had more blastocysts and expanded than treatment B and D. Finally, the treatment D had the result for blastocyst and expanded blastocyst as treatment B and presented less number of blastocyst than treatment A and C. When is analyzed the Table 1 is possible to see that some numbers of early and expanding blastocysts, both presented the same number and thus, it did not have difference ($p>0.05$).

Table 2 shows the effect of melatonin (10^{-9} M) in the maturation medium and cultive medium on pre-pubertal goats embryo development according cleavage rate. Similar results in the number of total blastocyst were observed between the treatment A (51.08 %) and C (42.86 %); and between the treatment B (28.27 %) and D (27.36 %). An interesting result was seen for expanded blastocysts. The worst result was when added melatonin in convencional medium of IVM and in the conventional medium of IVC (treatment D) 5.97 % of expanded blastocyst. This number was less than all treatments. The second worst result was for the treatment B (11.52 %), particularly when only add melatonin in the conventional medium of IVC. The best results were for both treatment A and C, 23.12 % and 19.05 %, respectively. It that means better results when no add any supplemented in the conventional medium of IVM, and doesn´t matter if add or no melatonin in the conventional medium of IVC. And there were no difference ($p>0.05$) for early and expanding blastocysts to the all treatments.

Effectiveness of the culture medium during IVM on the tolerance to parthenogenetic blastocysts vitrification.

The viability of the goat early blastocysts vitrified/warming is summarized in Table 3. At 2 hours after warming, there was no difference ($p>0.05$) observed in the survival rates among the viable early blastocysts of all 4 groups. At 24 hours later after warming also there was no difference among 4 groups.

Table 1. Effect of melatonin (10^{-9} M) in the maturation medium and culture medium on prepubertal goats embryo development according number of total oocytes.

Parameters	Treatments			
	A	B	C	D
Oocytes (n)	201	202	200	221
Cleavage (%)	92.54 [a]	94.55 [a]	94.50 [a]	90.95 [a]
Blastocyst (%)	47.26 [a]	26.73 [b]	40.50 [a]	24.89 [b]
Expanded (%)	21.39 [a]	10.89 [b]	18.00 [a]	5.43 [c]
Hatched (%)	4.98	0.00	2.50	0.00
Early (%)	8.96 [a]	9.90 [a]	12.50 [a]	8.14 [a]
Expanding(%)	11.94 [a]	5.94 [a]	7.50 [a]	11.31 [a]

Within a row different letters indicate significance ($P < 0.05$). Four repetitions were made for each Treatment. Treatment A: IVM medium without melatonin and IVC medium without melatonin; Treatment B: IVM medium without melatonin and IVC medium added with melatonin; Treatment C: IVM medium added with melatonin and IVC medium without melatonin; and Treatment D: melatonin added in IVM medium and IVC medium; n: number.

Table 2. Effect of melatonin (10^{-9} M) in the maturation medium and cultive medium on pre-pubertal goats embryo development according cleavage rate.

Parameters	Treatments			
	A	B	C	D
Cleavage (n)	186	191	189	201
Blastocyst (%)	51.08 [a]	28.27 [b]	42.86 [a]	27.36 [b]
Expanded (%)	23.12 [a]	11.52 [b]	19.05 [a]	5.97 [c]
Hatched [*] (%)	5.38	0.00	2.65	0.00
Early (%)	9.68 [a]	10.47 [a]	13.23 [a]	8.96 [a]
Expanding (%)	12.90 [a]	6.28 [a]	7.94 [a]	12.44 [a]

Within a row different letters indicate significance ($P < 0.05$). [*] Significant association ($P < 0.05$) by Freeman-Halton test. Four repetitions were made for each Treatment. Treatment A: IVM medium without melatonin and IVC medium without melatonin; Treatment B: IVM medium without melatonin and IVC medium added with melatonin; Treatment C: IVM medium added with melatonin and IVC medium without melatonin; and Treatment D: melatonin added in IVM medium and IVC medium; n: number of total cleavage.

Table 3. Cryotolerance assessed by blastocoele re-expansion at 24 h post-warming of pre-pubertal goat blastocysts according to IVM and IVC treatment with melatonin.

Treatment	Cryotolerance		
	% Re-expansion after warming		
	Early Blastocysts, n	3h	24h
A	27	0	2
B	25	1	0
C	20	0	1
D	22	0	0

($P > 0.05$); n: number of early blastocysts.

4. Discussion

This study have presented none beneficial effects of melatonin on in vitro prepubertal goat embryonic development. It demonstrate that melatonin did not improve the in vitro development of parthenogenetic embryo as much adding or not in vitro maturation medium. An important and interesting result brought from our study was that using oocytes from prepubertal goat did not influence directly in the parthenogenetic activated embryo development. Moreover, in this study was observed that prepubertal oocytes irrespective of their follicle size origin represented optimal embryo development. Our results showed that using prepubertal oocytes was capable to complete meiotic maturation and get a cleavage average rate of 90% demonstrating that the development of these oocytes were equivalent or better than adult oocytes and the parthenogenetic activation with ionomycin associated sequentially with 6-dimethylaminopurine (6-DMAP) was a method considered similar to FIV for the development of blastocysts (Susko-Parish et al. 1994; Rho et al, 1998; Ongeri et al., 2000; Ongeri et al, 2001; Lan et al., 2005). In common, assisted reproductive technologies (ART) have been used mainly for oocytes from adults females that represent normal reproductive age. It is implicated that female fertility get better with increasing age until reach the sexual maturation which include normality of hypothalamic-pituitary-gonadal axis. Armstrong (2001) demonstrated in his review that the age of oocyte donor is a limiting factor influencing development competence of the oocyte. It can be involved with meiotic incompetence or inability to complete meiotic maturation that results in oocytes impossible of fertilization, meiosis errors that can be fertilized but occurs genetic abnormalities that affect embryo viability and cytoplasmic incomplete developmental. Although these results and studies using IVF with oocyte of adult females and our be of oocytes from prepubertal goats and parthenogenetic activated this was not considered distinct when compared with the rates in the production of embryos in vitro.

Our results demonstrated that prepubertal oocytes is possible to be activated by parthenogenetic activation as a model of study to embryonic research but using melatonin as a antioxidant provider for in vitro culture medium this did not happen. Adding melatonin (10^{-9} M) on in vitro maturation medium did not interfere directly in the cleavage rate, nor in production of total blastocysts. Although, (Papis et al., 2007), in their study, adopted another concentration of melatonin (10^{-4} M), they did not find also a direct effect of melatonin on embryo cleavage rate.

Otherwise, it was observed in ours study that using melatonin on in vitro culture medium has a negative effect on parthenogenetic embryo growing, diminishing number of total blastocyst, expanded and hatched blastocysts. Differently to others studies, as (Tian et al., 2010) working with mouse two-cell embryonic, their work had an addition of concentration of melatonin from 10^{-13} to 10^{-5} M in the culture medium and consequently, they got an increase in the rates of blastocyst formation, hatching/hatched blastocysts and blastomere number per blastocyst. (Takahashi et al., 2002) had the same results than (Tian et al., 2010) in relation to increase the rate of blastocyst by supplementing with antioxidant ß-mercaptoethanol in the culture medium in bovine IVF embryos.

(Papis et al., 2007) suggested that melatonin administered for a short period, as 2 days of treatment, it can be able to reverse possible toxic effects correlated to high oxygen levels. Similarly, (Lim et al., 1999) using ß-mercaptoethanol also obtained a decrease the blastocyst rate under low (5%) oxygen tension in the culture. Probably, in the current study happened exactly the same negative effect like both studies cited above. For a longer period than IVM (1 day), IVC (5 days) can contribute to elevate a consumption of oxygen by the embryos and then causing an toxic effect. Even, existing a function of antioxidant for both substances, melatonin and ß-mercaptoethanol.

Melatonin is considered an efficient and powerful protective against oxidative damage by have an ability to remove hydroxyl radicals and acts as substrates and electron transfer mediators and reactions of free radicals (Poeggeler et al., 1993; Reiter et al., 1994). This property makes it an oxidative antagonist in vitro and pharmacological conditions which contributes to the physiological protection cells (Hardeland and Pandi-Perumal, 2005). Other melatonin actions as antioxidant protection, adds up to regulate other antioxidant enzymes. It is capable of stimulating glutathione peroxidase (GPX), an enzyme responsible for reducing hydrogen peroxide and lipids which decreases oxidative tissue damage (Arthur, 2001). Unfortunately, all those features for melatonin were not applicable in this present study. It reinforces to ourselves the idea that it is necessary to increase knowledge about the correct dose of melatonin applied in culture media to be useful to the growth of cells animals in vitro. In spite of 10^{-9} M be a low concentration for some studies, it not means that is for others. Adding melatonin in the culture medium of different species with different concentrations, it can influence a different answer in the embryo development of different specie. Emphasizing this event as (Rodriguez-Osorio et al., 2007), they suggested that melatonin in the culture medium could have depleted or due to the fact that the concentration employed have been too low that was not able to protect the embryo against free radicals. This event could activate anti-apoptic activity

and contribute to decreasing the total blastocyst rate. Occasionally, which may have occurred to the low rate of blastocysts formed during IVC was the melatonin concentration adopted in this study. This concentration may have been high for embryo development, causing a toxic effect on these cells, causing cell injury and reducing the blastocyst formation rate, as in high concentrations (10^{-3} M), the melatonin retards embryo development (Tian et al., 2010; Wang et al., 2014), increase apoptosis, stop progression from G1 to S phase (Choi et al., 2008) and inhibit cell division (Büyükavcı et al., 2006). Usually, the addition of any antioxidants to the culture medium is specially to decrease apoptosis in embryo growing and prevent oxidant damage (Liu et al., 2003). As (Reiter et al., 2009) considered in their research, it is not possible conclude wether the melatonin is a physiologically applicable antioxidant. To confirm this function, it would be necessary to measure whether there is enough melatonin molecules within the cells able to act directly in other free radical scavengers. Because its functional role as a molecular antioxidant is effective only under extreme conditions the production of free radicals produced is overly (Reiter et al., 2008).

The reason for choosing the melatonin concentration 10^{-9} M was due to recommendation of other studies which obtained from the melatonin it anti-oxidative function as an free radical scavenging potency and anti-apoptotic ability (Rodriguez-Osorio et al., 2007). The same reason was chosen by this concentration of melatonin be close to physiological levels of the some species, as human (Adriaens et al., 2006) and rabbit (Mehaisen and Saeed, 2015). (Tian et al., 2010) also confirm some benefit the in vitro embryonic development from two-cell mouse embryos making addition of melatonin at 10^{-9} M in the culture medium. (Shi et al., 2009) supplemented melatonin into both IVM and IVC medium and obtained increase in the cleavage and blastocysts of porcine rates. Their suggestion was supplement melatonin in the both progress of IVM and embryo development, instead of adding melatonin in IVM medium or in IVC. It was the first successful report that recommended the use of melatonin in the entire progress.

(Hardeland et al., 1993) concluded in their study that melatonin had a powerful pharmacologically action with unique properties such as non-toxicity, effectively radical scavenger and protector of neurodegeneration against mutagenic and carcinogenic action of hydroxyl radicals. The same conclusion reported by (Reiter et al., 1994), which pharmacologically found that melatonin is relevant, and it is apparently of low toxicity, considering the ease of melatonin to be absorbed by cells. (Jahnke et al., 1999) evaluated the maternal and developmental toxicity of melatonin administered exogenous (0, 50,

100, or 200 mg/kg/day) to the rats and concluded that the melatonin did not affect embryo/fetal growth, viability, or morphological development.

In the last part of the present study, early blastocysts treated with melatonin were used as an attempt to cryopreserve goats embryos allowing better storage of vitrified cells. The method used still contribute positively to the oocytes and also to embryo survival (Mermillod et al., 2008). To our study, it was not represented. Our survival rate was lower than others as (Hochi et al., 2004) in rabbit, (Hong et al., 2007) in goat, (Mermillod et al., 2008) in human/bovine, (Abdalla et al., 2010) in bovine, (Wu et al., 2016) in porcine.

Considering the results of our study that the treatments A and C represented higher blastocyst rate, it was expected to these a higher survival rate pos-warming. However, some factors may affect the process of cryopreservation in embryos of domestic animals by directly changing the physiological and morphological characteristics of those cells. The stage of embryonic development becomes a critical factor for the success of cryopreservation of embryos. Including embryos of sheep, cow and pigs are more sensitive at early stages of development compared with those at advantage stages. However, in ovine, the pregnancy and embryo survival rates showed similar for different stages as morale and blastocysts (Gibbons et al., 2011).

On the other hand, Gibbons et al. (2011) did not find the pregnancy rate for vitrified /thawed morulae in the goat, but got a high rate of pregnancy and embryo survival for blastocysts vitrified / thawed. It that means the rate of embryo survival tends to increase as progressing stages of development, since morula to blastocyst (Garcia-Garcia et al., 2006). It becomes more difficult to cryopreserve embryos at the morula stage because there was more intrinsic sensitivity of morulae to freezing (Massip, 2001), fewer number and the bigger size of cells (Széll and Shelton, 1986), appears limited the link of intercellular features (Ziomek and Johnson, 1980), and the permeability of cryoprotectant could be lower in these early stages (Mazur et al., 1976).

According to (Li et al., 2012), one of the important parameters to have a great result for vitrified embryos is the age of the embryos subjected to cryopreservation. To parthenogenetic porcine embryos, the best time to vitrification is the day 4 post activation. Days 5 and 6 after activation, the results for survival and expansion rates were reduced significantly. Following the results of these researchers, the present study also could show us that the age of the embryos is important and predominant at the time of choosing for vitrified. Day 8 post activation proposed in our study was beyond the (Li et al., 2012) and confirm with (Hasler et al., 1995), if the development of the embryos become delayed to

reach the Day 8 or at most a Day 9 of culture, these are considered poor quality and whatever its utility will be affected.

For these reasons, we had not sufficient numbers of embryos post devitrification to conclude whether or not melatonin improved the survival rate of vitrified embryos. This work supports the idea of others researchers that the age of embryos is a crucial factor for the establishment of the vitrification method. The method of cooling become cells membrane more vulnerable to damage due to exchange of cryoprotectant and water between the intracellular and extracellular medium. And the method of thawing results in osmotic stress (Nedambale et al., 2006). It was expected that melatonin could protect embryos development against the elevated levels of ROS induced by process of vitrification. ROS production is involved in developmental arrests, fragmented embryos and as a result of programmed cell death (Jurisicova et al., 1996; Yang et al., 1998).

The detoxification effect with melatonin could be an important factor in embryo survival post vitrification. Several studies have been successfully applied to determine the effect of melatonin in embryonic samples using vitrification (Gao et al., 2012; Succu et al., 2014).

5. Conclusion

The results of this study present that supplemental melatonin during IVM do not improve the oocyte maturation, neither the development of parthenogenetic embryo. At the same occurs during IVC when adding melatonin in the culture medium. The melatonin did not improve the embryo cleavage and blastocyst formation rates. It was the opposite, supplementation of melatonin in the culture medium impaired the development of parthenogenetic embryos. The effect of melatonin in parthenogenetic embryos using vitrification method was not possible measured due to low number of lives samples acquired after post thawed.

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CAPÍTULO 2 - MELATONIN IMPROVES IN VITRO FERTILIZATION BUT DOES THE DEVELOPMENT OF PREPUBERTAL GOAT EMBRYOS

Scientific paper in preparation

Abstract

This study evaluates the effect of melatonin at concentration of 10^{-9} M in the in vitro maturation medium in development and embryo quality of pre-pubertal goats oocytes as well evaluates the efficacy and selectivity of supplemental melatonin 10^{-9} M in the in vitro cultivation in embryonic development and quality of prepubertal goats oocytes. Oocytes were recovered from slaughterhouse, it were matured. We used two types of IVM media: (i) basic medium supplemented with 10 μg/mL follicle stimulating hormone (FSH), 10 μg/mL LH, 1 μg/mL and 17 β-oestradiol (conventional IVM medium, CM); (ii) basic medium supplemented with 10 μg/mL follicle stimulating hormone (FSH), 10 μg/mL LH, 1 μg/mL, 17 β-oestradiol (conventional IVM medium, CM) and melatonin (10^{-9} M). After IVM, oocytes were inseminated with fresh semen, after capacitated semen and thus it cultivated. At 17 h post insemination, a sample of assumed embryos was performed acetic orcein staining technique. We also used two types of culture medium: (i) base medium supplemented with 10% fetal cow serum (FCS); (ii) base medium supplemented with 10% fetal cow serum (FCS) and melatonin (10^{-9} M). After cultured for 8 days, the blastocysts quality was assessed. The results obtained in this study indicated that using 10^{-9} M of melatonin in the IVM medium presented higher fertilization rate (34.57% vs. 17.39%, $p<0.05$) and better oocytes development. However, did not present better results for cleavage and blastocyst rates. In conclusion, the addition of 10^{-9} M of melatonin to the maturation medium enhances fertilization but do not improve embryo development from prepubertal goat oocytes.

Key words: goats, melatonin, blastocyst, fresh semen, prepubertal

1. Introduction

The goat production is presenting a global growth cycle. In Brazil, this production has been attractive to new consumers, mainly in the form of liquid milk and cheese (Fonseca et al., 2005). Historically, the Northeast is the most traditional and where has the largest herd of goats in Brazil. On the other hand, it is in the Southeast, where there is increased production of goat milk, 21% of the total produced in the country, and where there is an intensive production system and has a herd of high genetic selection (Borges, 2003).

Given this fact, the spread of genetic material of breeds specialized animals is a crucial factor for the better use of the goat production. The in vitro embryo production (IVP) is a biotechnology applied to animal production in order to not only get embryos from selected animal models genetically or those who are at risk of extinction, but also to produce them in laboratories in commercial scale. This production can be regarded as functional by the alternative used to avoid premature disposal of high genetic value females. The PIV is based on three essential and continuous factors: maturation of oocytes collected from donors; fertilization of these matured oocytes with capacitated sperm and cultivation from these embryos produced till the blastocyst stage (Paramio, 2010).

Obtaining a competent oocyte able to be ovulated and fertilized depends on two key factors: nuclear maturation and cytoplasmic maturation. The normal development of oocytes is characterized by extrusion of the first polar body (GVBD germinal vesicle breakdown), with the restoration of meiosis, the chromosomes progress from metaphase I to telophase I, completing the first meiotic division. There is the second meiotic division, quick start after the first meiotic division, with the emergence of the oocyte in the metaphase II stage for subsequent ovulation (CHA & CHIAN, 1998).

In vitro fertilization is a detailed procedure that to get a satisfactory results needs to be faithful to the protocols proposed in oocyte maturation, sperm selection, sperm capacitation and medium selection. In goats, using fresh semen are often adopted (Cox et al., 1994; Keskintepe et al., 1994; Crozet et al, 1995; Mogas et al, 1997; Izquierdo et al, 1998;. Anguita et al., 2007, Romaguera et al, 2011). As for frozen semen are not as common (Keskintepe et al, 1998; Rho et al., 2001; Bormann et al., 2003; De Souza et al., 2013).

The in vitro culture is the last event for the production of embryos in vitro. It is in this post-fertilization culture period where there is the greatest impact on the quality of the produced blastocysts (Rizos et al., 2002). There are several events that need to occur for the optimal development of the embryo, such as: first division of cleavage, the activation

embryonic genome, compaction of the morula and blastocyst formation (Watson, 1992). An important factor for the embryo to develop is related to energy support that embryos need in their growth. The use of amino acids in these media, allowed the best development of embryos. This is due to the antioxidant action. Thus, it decreases oxidative stress and cause less fragmentation caused cells in culture in vitro embryos (Bavister, 1995).

Melatonin (N-acetyl-5-methoxytryptamine) known as the main hormone of the pineal gland, and responsible for the circadian rhythm, has this feature that makes it a great choice to be added in a culture medium. It is considered efficient and powerful protective against oxidative damage and has the capability of eliminating hydroxyl radicals produced in the medium. It acts as substrates and electron transfer mediators and reactions of free radicals (Poeggeler et al., 1993; Reiter et al., 1994). This property makes it an oxidative antagonist in vitro and pharmacological conditions which contributes to the physiological protection cells (Hardeland & Pandi-Perumal, 2005).

Therefore, melatonin is capable of promoting the maturation of oocytes and developing embryos of various species, such as: rats, mice, sheep, and pigs (Abecia et al, 2002; Papis et al, 2007; Rodriguez-Osorio et al, 2007; Shi et al .; 2009; Tian et al, 2010). Ishizuka et al. (2000) found that melatonin concentration ranging from 10^{-6} to 10^{-8} M promoted the development of early embryos mice. Tian et al. (2010) using 10^{-9} M melatonin in mice culture medium, got improvement in blastocyst rate, the hatched blastocyst rate and the number of blastocyst cells. In cloned embryos of porcine incubated with melatonin at a concentration of 10^{-9} M and 10^{-12} have also improved in their development (Pang et al., 2007).

This study looks forward to increase the efficiency of in vitro production of embryos derived from pre-pubertal goats oocytes. The proposed objectives are: (i) evaluate the efficacy of supplemental melatonin 10^{-9} M in the in vitro maturation medium in development and embryo quality of pre-pubertal goats oocytes, (ii) evaluate the efficacy of supplemental melatonin 10^{-9} M in the in vitro cultivation in embryonic development and quality of pre-pubertal goats oocytes.

2. Material and Methods

2.1. Reagents

All chemical reagents used in processing media were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Oocytes collection, its classification and IVM

The ovaries were obtained from prepubertal goats (1-2 months) of Gremial Catalunya SL slaughterhouse, located in Castelbisball, Barcelona - Spain. After collection, these ovaries were maintained at 38 ° C in PBS, stored in thermos bottle, to be submitted to the Small Animal Reproduction Laboratory of Veterinary School, UAB, for a period of two hours. The samples were washed in PBS in duplicates and maintained at 38 ° C to be subsequently realized the slicing technique. The selection of oocytes for IVM was a priority for the choice of cumulus-oocyte complex (COC), collecting those with compact cumulus with intact and homogeneous cytoplasm, established by the UAB laboratory, according to their diameters. The COCs were washed on washing plates containing three drops of maturation medium. Groups of 25-30 COCs were made and placed into 100 uL drops of IVM medium covered with mineral oil and incubated at 38 ° C, 5% CO_2 in humid air. Basal medium for maturation was TCM-100 supplemented with 275 μg / ml sodium pyruvate, 146 μg / ml L-glutamine, 50 / ml gentamycin, 10% (v / v) donor bovine serum (Cansera; Sucker, Canada) and 100 uM Cysteamine. There were 2 types of maturation medium used: (i) base medium supplemented with 10 μg/mL follicle stimulating hormone (FSH), 10 μg/mL LH, 1 μg/mL and 17 β-oestradiol (conventional IVM medium, CM); (ii) base medium supplemented with 10 μg/mL follicle stimulating hormone (FSH), 10 μg/mL LH, 1 μg/mL, 17 β-oestradiol (conventional IVM medium, CM) and 10^{-9} M melatonin (M-5250, Sigma, St. Louis, MO, USA).

2.3. In vitro fertilization (IVF)

Sperm capacitation.

It was used the ejaculates of two male adult Murciano-Granadina goats provided of optimal fertility collected by artificial vagina and transported to the laboratory at 37 ° C for a period of 10 min. The appearance, motility and sperm vigor were evaluated in both ejaculated. The ejaculates from two adults males were pooled in equals parts to get a volume of 1 ml. The mobile sperm fraction was acquired by Percoll density gradient for the separation of semen sperm fresh goat, according to Parrish et al. (1995). An isotonic Percoll® solution 90% was prepared by adding 10 ml to the medium 10 x sp-TALP 90 mL de Percoll® and supplemented with 80 mM NaCl, 3.1 mM KCl, 0.29 mM NaH_2PO4, 1.97 mM $CaCl_2$, 0.39 mM $MgCl_2$, 10 mM HEPES, 26 mM lactic acid and 25 mM

$NaHCO_3$. Percoll® 90% and sp-TALP Medium (1: 1, v / v) is mixture to prepare a solution of Percoll® 45%. The Percoll® density gradient is made by layering 2 ml of 45% of Percoll® solution and 2 mL of 90% Percoll® solution in a 15 mL tube FALCON®. At the top of the gradient is deposited 200 µl of fresh semen and then the tubes were centrifuged at 300G x for 25 min. The pellet is resuspended with 4 ml of sp-TALP and centrifuged at 300G x for 5 min. The sperm pellet formed was resuspended in 200 µl of sp-TALP and resuspended in a 1: 1 with mDM containing heparin (Sigma, USA) and incubated for 30 min in humidity and air atmosphere (5% CO_2) at 38.5° C.

IVF.

After IVM, 25-30 oocytes were transferred onto plates containing microdroplets of 100 µl of media modified Tyrodes (TALP), described by Parish et al. (1996), supplemented with 1 µg/ml of Hipotaurina (Sigma, USA) covered with mineral oil. Oocytes were co-cultured with capacitated spermatozoa in a final concentration of 4 x 10^6 mobile spermatozoa / ml, 38.5 ° C, 5% CO_2 in humid air according Romanguera et al. (2010).

Evaluation of oocytes.

At 17 hours post-insemination (pi), oocytes were washed to remove died cells, sperm and capacitation media. It were taken between 10 and 15 assumed zygotes to be performed the assessment of nuclear maturation stage, using the acetic orcein staining technique. The cumulus cells were removed from oocytes by vortex and oocytes were washed three times in PBS. Oocytes were fixed in 3: 1 (ethanol: acetic acid, v/v) for 24h, 4° C. The oocytes were put on microscope slides with vaseline, covered by a glass coverslip and stained with 1% aceto-orcein stain. The stage of nuclear maturation assessed by phase-contrast microscopy (magnification, x40).

The classification of oocytes after IVF was adopted in accordance with these groups:

a) Asyncrony: Head of condensed sperm and female pronucleus visible in the cytoplasm

b) Monospermy : two pronuclei (male and female) and a sperm tail visible in the cytoplasm

c) Polyspermy : more than two condensed tails or heads of sperm decondensed in the cytoplasm

d) Unfertilized : no sperm pronuclei and no tail in the cytoplasm

2.4. In vitro embryo culture (IVC)

At 24 h post-insemination, presumptive zygotes were washed in order to remove cumulus cells, sperm cells and sperm. Groups of 18-25 embryos of similar diameters were placed in microdrops of 10 μL of SOF (Synthetic oviductal fluid medium), Takahashi & First (1992) supplemented with 10% fetal cow serum (FCS) covered with mineral oil under an atmosphere humidified at 5% CO_2, 5% O_2 and 90% N_2 for 8 days. At 48 h after insemination, the cleavage rate was assessed. The rate of total embryos, morula and blastocyst were measured.

2.5. Experimental design

Effectiveness of melatonin added in the maturation medium during IVM and its effect on embryo development of oocytes goat prepubertal. It analyzed the effect of adding the hormone melatonin in base medium used for oocyte maturation to assess embryonic development. The effect of the concentration of melatonin at 10^{-9} M added in IVM medium on nuclear maturation was evaluated in cleavage rate, blastocyst embryo quality compared with a control group without melatonin. The COCs were selected and added into two treatment groups: (i) IVM medium without melatonin; (ii) IVM medium with melatonin at 10^{-9} M. After IVM , COCs were fertilized and the cleavage and blastocyst rate were observed on day 2 , 7 and 8 PI (post - insemination). On day 9 PI the blastocysts were fixed and stained to determine the total number of cells.

Effectiveness of melatonin medium during the IVC and its effect on embryo development of oocytes goat prepubertal. To analyze the effect of melatonin in embryonic development, it were used 15-20 zygotes of two cells derived from the treatment in IVM. The cultured were selected and maintained in their respective groups (i and ii), and then, the group (i) was separated into two groups: (a) IVC with melatonin at 10^{-9} M and (b) IVC without melatonin. After the IVC, the cells were observed on day 2, 7 and 8 PI (post - insemination).

Effectiveness of melatonin added in the culture medium during IVM and IVC and its effects on the embryo development of oocytes goat prepubertal. To analyze the effect of melatonin in the IVM medium and in the IVC medium, it were divided in four treatments: (1) Treatment A: IVM medium without melatonin and IVC medium without melatonin;

(2) Treatment B: IVM medium without melatonin and IVC medium added with melatonin; (3) Treatment C: IVM medium added with melatonin and IVC medium without melatonin; and (4) Treatment D: melatonin added in IVM medium and IVC medium. The total oocytes collected after slicing technique were divided in a half and put them on in two plates, one plate with maturation medium without melatonin added, and other one with melatonin added. All those oocytes after matured, they were fertilized and those oocytes were divided equally in other two culture plates. One plate of IVC medium without melatonin and other one, IVC medium with melatonin added. After 24h post-fertilizated, it was calculated the cleavage rate. After eight days post fertilizated, it was calculated the blastocyst rate.

2.6. Statistics

For data analyses the statistical analysis system was used (SAS 2002). Percentages of the one and two pro-nucleus, polyspermy, asymmetry, metaphase-I and II, and anaphase-telophase, were submitted to logistic regression (Logistic procedure). Significant level adopted was 5%. Percentages of the cleavage, total blastocyst, early, expanded, hatched and expanding blastocyst were submitted to logistic regression (Logistic procedure) using total of oocytes and total of cleavage as trials. When $n < 20$, the association test of Freeman-Halton was used. Comparisons were performed using the Chi square test. Significant differences were adopted as $P<0.05$.

3. Results

Experiment 1: Effectiveness of melatonin added in the maturation medium during IVM and its effect on embryo development of oocytes goat prepubertal.

The evaluation of oocyte nuclear maturation stage are showed in Table 1. After 17 h of maturation, presumptives zygotes were stained by orcein and the classification of those were determined in accordance with oocyte penetrated by sperm (monospermy, polyspermy and asymmetry) and no penetrated (Female Pro-nucleus, MII, A-TI and MI). The penetration rate of oocytes matured with melatonin added in the IVM medium was bigger than those matured without melatonin in the maturation medium (53.09 % vs 31.88 %). About that rate, In Table 1 can be observed that group Melatonin presented more percentage of monospermy than Control (34.57 % vs. 17.39 %, $p < 0.05$) but others parameter as Polispermy and Asymmetry did not show difference between these Groups.

The No-Penetrated rate of Group Control and Melatonin did not present difference. However, in Group Control can be seen more percentage of oocytes in MII than Group Melatonin (52.17 % vs. 32.10 %, $p < 0.05$). Others parameters, as Female pro-nucleus, MII, Anaphase-Telophase I and Metaphase I did not present statistical difference between Control and Melatonin Group. Table 2 shows in vitro fertilization rate between Control and Melatonin Group. It means that Melatonin Group presents more oocytes fertilized by a spermatozoon than Control Group ($p < 0.05$). However, in both Groups Unfertilized oocytes were bigger than fertilized oocytes. Table 2 shows also cleavage rate and embryo development to blastocyst stage of pre-pubertal goat oocytes in relation to add melatonin at concentration of 10^{-9} M on conventional medium, according to number of total oocytes. As cleavage rate as blastocyst rate did not present different between those two groups, Control and Melatonin. Table 3 shows embryo development to blastocyst stage of pre-pubertal goat oocytes in relation to add a concentration of melatonin 10^{-9} M on conventional maturation medium, but in this is according to cleavage rate. There were statistical difference to percentage of blastocyst, Melatonin group presented more percentage than group control (66.67 % vs. 37.50 %). However, hatched, early and expanding blastocysts did not show differences between control group and melatonin.

Experiment 2: Effectiveness of melatonin medium during the IVC and its effect on embryo development of oocytes goat prepubertal.

Adding melatonin (10^{-9} M) on culture medium did not improve cleavage rate, nor embryo development to blastocyst stage of pre-pubertal goat oocytes in relation to a conventional culture medium, as showed on Table 2. The culture medium was supplemented with 10^{-9} M melatonin concentration, and the results from seven replicates for this group were recorded. No significant difference in the cleavage rate in the presence or absence of melatonin was seen according to number of total oocytes ($p > 0.05$). Even comparing to number of cleavage in relation to embryo development to blastocyst stage, it did not show an improvement using melatonin (10^{-9} M) on culture medium.

Experiment 3. Effectiveness of melatonin added in the culture medium during IVM and IVC and its effects on the embryo development of oocytes goat prepubertal.

Table 4 is about the effect of melatonin (10^{-9} M) in the maturation medium and culture medium on prepubertal goats embryo development according number of total oocytes. All Treatments (A, B, C and D) did not present statistical difference to cleavage rate (8.59 %, 13.94 %, 7.98 % and 5.65 %, respectively). In the case of total blastocyst, expanded, hatched, early and expanding blastocysts for all Treatment presented the close percentage and thus, it did not have significant difference. Table 5 is the effect of melatonin (10^{-9} M)

in the maturation medium and culture medium on prepubertal goats embryo development according to cleavage rate. The total blastocyst, expanded, hatched, early and expanding blastocysts for all Treatment (A, B, C and D) presented the close percentage and thus, it did not have significant difference also.

Table 1. The evaluation of oocyte nuclear maturation stage after 17 h of maturation in IVM medium with and without melatonin added. Presumptive zygotes were stained by orcein, and the classification of those were determined in accordance with oocyte penetrated by sperm.

Parameters	Group	
	Control	Melatonin
N	69	81
Penetrated (%)	31.88[b]	53.09[a]
Monospermy (%)	17.39[b]	34.57[a]
Polispermy (%)	10.14[a]	8.64[a]
Asymmetry (%)	4.35[a]	9.88[a]
No-Penetrated (%)	68.12[b]	46.92[a]
Female Pro-Nucleus (%)	7.25[a]	4.94[a]
Metaphase II (%)	52.17[a]	32.10[b]
Anaphase-Telo (%)	5.80[a]	7.41[a]
Metaphase I (%)	2.90[a]	2.47[a]

Within a row different letters indicate significance ($P < 0.05$). Seven repetitions were made for each treatment; N: number of total oocytes.

Table 2. Effect of melatonin (10^{-9} M) in the maturation medium on the embryo development according to number of total oocytes.

Parameters	Group	
	Control	Melatonin
Oocytes (n)	363	390
Cleavage (%)	11.02	6.92
Blastocyst (%)	4.13	4.62
Expanded (%)	0.55	1.03
Hatched (%) *	0.28	0.00
Early blastocyst (%)	3.31	3.59

P >0.05; Seven repetitions were made for each Treatment; n: number of total oocytes; * By Fisher's exact test.

Table 3. Effect of melatonin (10^{-9} M) in the maturation medium on the pre-pubertal goats embryo development according to cleavage.

Parameters	Group	
	Control	Melatonin
Cleavage (n)	40	27
Blastocyst (%)	37.50 [b]	66.67 [a]
Expanded (%)	5.00 [a]	14.81 [a]
Hatched (%) *	2.50 [a]	0.00 [a]
Early blastocyst (%)	30.00 [a]	51.85 [a]

Within row different letters indicate significance (P < 0.05); Seven repetitions were made for each Treatment; n: number of total cleavage; * By Fisher's exact test.

Table 4. Effect of melatonin (10^{-9} M) in the maturation medium and the culture medium on pre-pubertal goats embryo development according to number of total oocytes.

Parameters	Treatments			
	A	B	C	D
Oocytes (n)	198	165	213	177
Cleavage (%)	8.59	13.94	7.98	5.65
Total Blastocyst (%)	4.55	3.64	5.16	3.95
Expanded (%)	0.00	1.21	1.41	0.56
Hatched (%)	0.51	0.00	0.00	0.00
Early (%)	4.04	2.42	3.76	3.39

Within a row different letters indicate significance ($P < 0.05$). * Significant association ($P < 0.05$) by Freeman-Halton test. Four repetitions were made for each Treatment. n: number of total oocytes. Treatment A: IVM medium without melatonin and IVC medium without melatonin; Treatment B: IVM medium without melatonin and IVC medium added with melatonin; Treatment C: IVM medium added with melatonin and IVC medium without melatonin; and Treatment D: melatonin added in IVM medium and IVC medium.

Table 5. Effect of melatonin (10^{-9} M) in the maturation medium and culture medium on pre-pubertal goats embryo development according to number of cleavage.

Parameters	Treatments			
	A	B	C	D
Cleavage (n)	17	23	17	10
Total Blastocyst (%)	52.94	26.09	64.71	70.00
Expanded (%)	0.00	8.70	17.65	10.00
Hatched (%)	5.88	0.00	0.00	0.00
Early (%)	47.06	17.39	47.06	60.00

P > 0.05; Seven repetitions were made for each Treatment; n: number of total cleavage.

4. Discussion

The results of this study demonstrated that melatonin added in the IVM medium increase synchronous pronuclear formation and enhance the penetration rate between oocyte - spermatozoon, IVF and the in vitro development of prepubertal goat oocytes (Table 1 and 2). In the preliminary studies, there was the supplementation of melatonin during maturation and/or embryo culture. The oocytes treated with melatonin in the IVM medium improve male pronucleus (MPN) formation and enhance both nuclear and cytoplasmic maturation. As also it was found by (Kang et al., 2009) in porcine oocyte media included with and without melatonin. Their results showed similarity with our results by the greater proportion of MII oocytes in the melatonin treatment but none influence in cleavage and blastocyst rates. It suggest that melatonin supplementation in vitro is correlated to reduction in oxidative stress and improved oocyte maturation. Although, in our study, it was not made any oxidative analysis, (Tamura et al., 2012) exposed oocytes from immature mice to H_2O_2 with differences concentrations of melatonin and they found a bigger number of mature oocytes associated to increasing amount of melatonin. (Shi et al., 2009) also confirm that melatonin promotes the maturation of oocytes in mouse and improves its in vitro fertilization. (Ishizuka et al., 2000) conclude that naturally melatonin is found in the follicular fluid of various

mammals, thus, it may have a physiological role in the fertilization and early embryo development.

In the present study, the formation of both male and female pronuclei within 17 h from the beginning of insemination significantly was higher to melatonin group than control group. It corroborated to (Ishizuka et al., 2000) and (Shi et al., 2009) that melatonin improve in vitro fertilization. Besides, melatonin may stimulate and support fertilization through its ROS scavenging action (Ishizuka et al., 2000). According to (Reiter et al., 2009), the principal function of melatonin or it metabolites is that could protect the male sperm from oxidative damage and other free radical. In human spermatozoa, there is a series of cells capable of generating ROS, especially when they are incubated under aerobic conditions. This production of ROS by sperm is considered a normal physiological process, because are needed to capacitation, hyperactivation, acrossome reaction and fertilization, but when output exceeds the basal limit, becomes an association with infertility in males. Consequently with exceed of ROS, the sperm has an abnormal flagella movement, loss of motility, diminished capacity for sperm-oocyte fusion and loss of fertility (Mortimer et al., 1986; Sharma and Agarwal, 1996).

ROS is also related as the main agent of the lipid peroxidation of fatty acids unsaturated of human sperm plasma membrane. This peroxidation is one of the responsible for the abnormal acrosome reaction and penetration of the oocyte. Probably this phenomenon in the membrane causes a fluidity by changing the link key/enzyme and the ionic channels (Kothari et al., 2010; Sikka et al., 1995). DNA damage is also associated with decrease in vitro fertilization rates and early embryo death. This occurs because the oxidative stress induced DNA fragmentation, such as: oxidation of bases, strand breaks, cross-linking, deletions, frame shifts and rearrangement of chromosomes (Aitken and Krausz, 2001; Sikka et al., 1995). This process accelerates germ cell apoptosis declining the sperm counts (Agarwal et al., 2003; Kothari et al., 2010). In relation to apoptosis, ROS could be a stimulus to activate the mitochondria to release the signaling molecule cytochrom. This one is responsible to initiate a cascade of event that involve sperm apoptosis (Agarwal et al., 2008; Agarwal et al., 2004). When it is about assisted reproductive techniques, which require methods of manipulation of gametes such as centrifugation and prolonged incubation, inevitably, these cells are exposed to additional oxidative stress, making it necessary to use an exogenous protection (Agarwal et al., 2004; Taylor, 2001).

Biochemically, there is also in embryos of domestic mammals high concentrations of lipids, it become a source of energy during fertilization and early embryonic development, but make those embryos more susceptible to the negative effects of oxidative stress

mainly during in vitro culture (Sturmey et al., 2009). In these conditions, there is increased production of ROS in the culture by the embryonic cells leaving the medium more susceptible to oxidative stress conditions, making it harmful to embryos. The ROS interact with cellular lipid molecules belonged to celular membrane, resulting in loss of membrane integrity, structural or until functional when appears alteration in the chain of proteins or damage to nucleic acids (Choi et al., 2008; Tamura et al., 2012). When the antioxidant activity diminished and consequently, increase ROS levels in oocytes occurs a problem in the development of embryos, as apoptosis, DNA fragmentation and embryonic arrest (Khalil et al., 2013; Kitagawa et al., 2004). According to (Ali et al., 2003), to have an appropriate fusion between the oocyte and spermatozoa is necessary to be a small portion of concentration of ROS in the medium to have a successful IVF.

This interpretation may explain why adding melatonin on the goat oocytes culture have promoted to the developing cells an essential factor that can be able to eliminate some factors which are prejudicial to their growth.. The role of antioxidant is one of the main functions of this hormone (Marshall et al., 1996; Reiter et al., 2005; Reiter et al., 2001; Reiter et al., 1994). Melatonin has the capability of detoxifying highly reactive species derived from oxygen and eliminate free radical or radical products, such as: hydroxyl radicals, hydrogen peroxide, peroxyl radicals, singlet oxygen, nitric oxide, peroxynitrite anion, lipid peroxyl radical (Reiter et al., 2001; Vijayalaxmi et al., 2004). Other function relevant and can be related to increase of in vitro fertilization in our study is that melatonin can counteracts the 2-bromopropane (2-BP). It is involved to induce apoptotic damage by activate caspase 3 (Kothari et al., 2010).

Although melatonin really have done their role as an antioxidant and improved in vitro fertilization of our study, adding melatonin (10^{-9} M) on culture medium did not present a better cleavage rate, neither embryo development to blastocyst stage of pre-pubertal goat oocytes in relation to a conventional culture medium. The results of our study showed that melatonin had no significant effect on the in vitro development of 2-4 cells embryos recovered at 20 h post-insemination. It is in agreement with others authors who did not find effects for melatonin on in vitro development of mouse zygotes (McElhinny et al., 1996), bovine zygotes (Papis et al., 2007; Takada et al., 2012; Tsantarliotou et al., 2007), rabbit zygotes (Mehaisen and Saeed, 2015), porcine zygotes (Pang et al., 2013). In contrast, there were others authors that found some beneficial effects on the in vitro development of embryos of different species with some concentrations of melatonin, as (Ishizuka et al., 2000) in mice (10^{-6} and 10^{-4} M), (Rodriguez-Osorio et al., 2007) in porcine (10^{-9} M), (Manjunatha et al., 2009) in buffalo (10 µM- 50 µM), (Shi et al., 2009) in

porcine (10^{-9} M), (Tian et al., 2010) in mouse (10^{-4} -10^{-8} M), (Asgari et al., 2012) in also mouse (10^{-7} and 10^{-8} M), (Wang et al., 2013) in murine (10^{-7} M), (Bahadori et al., 2013) in mice (10^{-7} and 10^{-8} M), (Wang et al., 2014) in bovine (10^{-11} to 10^{-5} M). This implies that for each species there is an optimal concentration, it means, the effect of melatonin during the growth and development of the embryo depends on the species and concentration (Takada et al., 2012).

The supplementation of culture medium with melatonin at 10^{-9} M did not present enhance on blastocyst rate. It corroborated to (Takada et al., 2012). In their study, it was used the same concentration of melatonin in bovine and they did not find influence in vitro embryo development. Who also did not show influences on the embryos development was (Papis et al., 2007), in bovine, using melatonin at concentration of 10^{-4} M. (Tsantarliotou et al., 2007) also in their work had no beneficial in blastocysts development neither toxic effects in bovine embryos, raging of concentrations from 10^{-6} to 10^{-4} M. To (Rodriguez-Osorio et al., 2007) occurred the same in blastocyst rate to porcine at concentration of melatonin, 10^{-12} to 10^{-6} M, probably because melatonin was impoverished a long of period of culture. One reasonable explanation could be related to the level of melatonin concentration in growth medium in order to have the desired effect on the cells. The concentration used was perhaps not able to effectively protect the embryos from free radicals produced in the culture medium (Rodriguez-Osorio et al., 2007).

There are many researchers using melatonin to improve efficiency in the development of embryos in vitro of many species. To be, undoubtedly, a highly potent antioxidant, adopting a concentration ranging from 10^{-13} to 10^{-4} M there was an increased number of blastocysts of mouse (Asgari et al., 2012; Tian et al., 2010), rabbit (Mehaisen and Saeed, 2015), bovine (Papis et al., 2007; Wang et al., 2014), mice (Ishizuka et al., 2000), murine (Wang et al., 2013), porcine (Kang et al., 2009), buffalo (Manjunatha et al., 2009).

Therefore, supplementation of embryos in vitro in culture medium containing melatonin has a significant result in the development and quality of embryos, especially in medium and low concentrations appear to be more beneficial than high (Fernando and Rombauts, 2014). However, it would be useful to clarify which concentration of melatonin is more effective for each stage of development of embryos of different species.

5. Conclusion

Based on the results of our study, the indicated concentration of melatonin to culture system can be beneficial for the goat oocyte in vitro maturation and matured oocyte in vitro fertilization.

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CONCLUSÃO GERAL

A partir dos resultados desta tese de doutorado permitiu nos concluir que:

1. A adição de melatonina a concentração de 10^{-9} M em meio de MIV não seria útil na maturação dos oocitos de cabras pré-púberes e tampouco seria um antioxidante de escolha para os blastocistos produzidos in vitro;
2. A concentração de 10^{-9} M de melatonina adicionados em meio de cultura apresentaram um certo grau de toxicidade que prejudicaram o desenvolvimento de embriões partonogenéticos;
3. Melatonina a 10^{-9} M suplementada em um meio de MIV foi benéfica para taxa de penetração oocito-espermatozoide durante a fertilização in vitro de oocitos de cabras pré-púberes;
4. A concentração de 10^{-9} M de melatonina adicionados em meio de MIV e de CIV não resultou no melhoramento no desenvolvimentos dos blastocistos.

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