CAMILA OLIVEIRA SILVEIRA

INCLUSÃO DE COLESTEROL NA MEMBRANA PLASMÁTICA DE ESPERMATOZOIDES CAPRINOS

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

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APROVADA: 22 de fevereiro de 2013.

Eduardo Paulino da Costa
(Coorientador)

Ciro Alexandre Alves Torres

Rogério Oliveira Pinho

José Domingos Guimarães
(Orientador)

"Nunca pense que uma meta não foi feita para si... pense apenas nas forças que precisa mobilizar para atingi-la." (Roberto Shinyashiki)

Dedico esta obra a Deus e minha família, minha mãe ***Ercy Silveira****, meu pai* ***José de Oliveira*** *e meu irmão* ***Ronaldo Silveira****, meus avos, tios, tias, primos e primas.*

Ao meu amado namorado ***Jurandy Mauro Penitente Filho*** *e aos meus amigos.*

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BIOGRAFIA

Camila Oliveira Silveira, filha de José Eustáquio de Oliveira e Ercy Silveira Machado Oliveira, nasceu em Boa Vista-RR em 04 de dezembro de 1984 e residiu em Boa Vista-RR até seus 19 anos.

Em 2004 ingressou no curso de Medicina Veterinária no Centro Universitário Nilton Lins, Manaus-AM, graduando-se em fevereiro de 2009.

Em março de 2011 ingressou no programa de pós-graduação em Medicina Veterinária na Universidade Federal de Viçosa, Viçosa-MG, na área de Reprodução Animal, tendo apresentado sua dissertação de Mestrado em fevereiro de 2013.

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RESUMO

SILVEIRA, Camila Oliveira, M.Sc., Universidade Federal de Viçosa, fevereiro de 2013. **Inclusão de colesterol na membrana plasmática de espermatozoides caprinos.** Orientador: José Domingos Guimarães. Coorientadores: Eduardo Paulino da Costa, Maria Eliana Lopes Ribeiro de Queiroz e Sérgio Antônio Fernandes.

O objetivo do presente estudo foi avaliar a capacidade fecundante, integridade acrossomal, além de qualificar e quantificar por técnicas cromatográficas a incorporação do colesterol à membrana plasmática do espermatozoide pela ciclodextrina em diferentes diluentes na criopreservação de espermatozoides caprinos. Foram utilizados quatro machos caprinos das raças Saanen (2) e Parda Alpina (2) seguindo um delineamento inteiramente casualizado, dividido nos seguintes tratamentos: TG- controle negativo para o diluente a base de Tris-glicerol; TGCCC- Complexo ciclodextrina-colesterol (CCC) + diluente Tris – glicerol; TG15CCC- CCC diluído em solução isosmótica ao sêmen (soro fisiológico) com 15 minutos de incubação antes da adição do diluente Tris – glicerol; EE- controle negativo para o diluente a base de Gema de ovo + etilenoglicol; EECCC- CCC + diluente Gema de ovo + etilenoglicol; EE15CCC- CCC diluído em solução isosmótica ao sêmen (soro fisiológico) com 15 minutos de incubação antes da adição do diluente Gema de ovo + etilenoglicol. O sêmen fresco, após realização de sua análise física foi submetido ao processo de criopreservação e estocado em botijão de nitrogênio por 10 dias. Após o descongelamento realizou-se análise da integridade acrossomal, capacidade fecundante do espermatozoide por meio do teste de ligação à membrana perivitelina da gema do ovo de galinha (MPGV) e as análises de motilidade progressiva e vigor espermático. Além destes testes foi realizada a avaliação da incorporação do colesterol à membrana plasmática dos espermatozoides pelas técnicas de cromatografia gasosa e de camada delgada. Para verificação da normalidade e homogeneidade dos dados foi empregado, respectivamente, o teste de Lilliefors e Cochran e Bartlett. As características que atenderam as premissas destes testes foram submetidas à ANOVA e as médias foram comparadas pelo teste de Duncan com 5% de probabilidade de erro. Quando as distribuições não atenderam as premissas de normalidade e homogeneidade, as médias foram comparadas pelo teste de Kruskal-Wallis. Realizou-se a correlação simples de Pearson entre todas as características. A adição do complexo ciclodextrina-colesterol não aumentou a ligação dos espermatozoides a MPGV ($P>0,05$). O tratamento

empregando o complexo ciclodextrina-colesterol (CCC) diluído em solução isosmótica ao sêmen com 15 minutos de incubação antes da adição do diluente a base gema de ovo + etilenoglicol (EE15CCC) foi superior aos valores médios do tratamento EECCC na manutenção da integridade acrossomal, porém não diferiu dos valores do tratamento controle para este diluente (EE; P >0,05). No diluente Tris-Glicerol, os valores observados no tratamento controle foi superior (P<0,05) aos valores médios dos demais tratamentos (TGCCC e TG15CCC) na manutenção da integridade acrossomal. Houve correlação negativa entre o teste de ligação e a integridade do acrossoma (r= -0,25) e positiva entre o teste de ligação e a motilidade espermática progressiva (r= 0,20). A motilidade espermática progressiva e a integridade do acrossoma apresentaram correlação negativa (r= -0,26). Na avaliação quantitativa e qualitativa do colesterol pelas técnicas cromatográficas (gasosa e camada delgada) não se verificou diferença entre as amostras do sêmen nos diferentes tratamentos (P>0,05). Ambas as técnicas demonstraram que não houve incorporação do colesterol aos espermatozoides. Conclui-se que o complexo ciclodextrina-colesterol no meio diluidor não melhorou os aspectos físicos do sêmen caprino pós-descongelamento e a capacidade de ligação à membrana perivitelina da gema do ovo de galinha. A pré-incubação do sêmen com o CCC por 15 minutos antes da adição do diluente a base de Etilenoglicol+ gema de ovo (EE15CCC) não proporcionou um aumento na integridade do acrossoma a ponto de diferir dos valores do tratamento controle (EE). A cromatografia gasosa e de camada delgada demonstraram, respectivamente, um eficiente método quantitativo e qualitativopara determinar o colesterol presente no espermatozoide caprino criopreservados. A concentração de 1 mg do complexo ciclodextrina-colesterol adicionada ao sêmen caprino não foi eficaz em aumentar a concentração de colesterol presente no espermatozoide.

ABSTRACT

SILVEIRA, Camila Oliveira, M.Sc., Universidade Federal de Viçosa, February, 2013. **Inclusion of cholesterol to goat sperm membrane.** Advisor: José DomingosGuimarães. Co-advisors: Eduardo Paulino da Costa, Maria Eliana Lopes Ribeiro de Queiroz and Sérgio Antônio Fernandes.

The aim of this study was to evaluate the fertilizing capacity, acrosomal integrity, and qualify and quantify by chromatographic techniques the incorporation of cholesterol to the sperm membrane by cyclodextrin in different diluents on cryopreservation of goat sperm. Four males, Saanen (2) and Parda Alpine (2) breeds were used. It was performed a completely randomized design, with each semen sample divided into the following treatments: TG - Tris-glycerol diluent; TGCCC - Cyclodextrin-cholesterol complex (CCC) + Tris-glycerol diluent; TG15CCC - CCC diluted in isosmotic solution (saline) to semen with 15 minutes of incubation before addition of the Tris-glycerol diluent; EE - egg yolk + ethylene glycol diluent; EECCC - CCC + egg yolk + ethylene glycol diluent; EE15CCC - CCC diluted in isosmotic solution (saline) to semen with 15 minutes of incubation before addition of the egg yolk + ethylene glycol diluent. Fresh semen, after its examination, was submitted to the cryopreservation process and stored in a cryogenic cylinder for 10 days. After thawing, the following analysis were performed: acrosomal integrity, sperm fertilizing capacity through perivitelline membrane of hen egg yolk binding test (MPEY) and analysis of sperm motility and vigor. Besides, techniques of gas and thin layer chromatography were conducted to evaluate the incorporation of cholesterol to the sperm membrane. Data were submitted to Lilliefors and Cochran and Bartlett tests to verify normality and homogeneity of variances, respectively. The characteristics that met the assumptions of these tests were submitted to ANOVA and means were compared by Duncan's test at 5% of probability. When data did not meet the assumptions of normality and homogeneity of variances, the means were compared by Kruskal-Wallis test. Pearson's correlation coefficient was calculated in all features.Addition of cyclodextrin-cholesterol complex did not increase binding of sperm to MPEY ($P > 0.05$). The EE15CCC treatment was superior to EECCC treatment in maintaining the acrosomal integrity but did not differ from control (EE; $P > 0.05$). In Tris-glycerol diluent, the values observed in the control treatment were higher ($P < 0.05$) than values of the other treatments (TGCCC and TG15CCC) in

maintaining the acrosomal integrity. There was a negative correlation between the binding assay and acrosomal integrity ($r = -0.25$) and positive correlation between the binding assay and sperm motility ($r = 0.20$). The sperm motility and acrosomal integrity were negatively correlated ($r = -0.26$). In quantitative and qualitative evaluation of cholesterol by chromatographic techniques (gas and thin layer) there was no difference between the samples of semen in different treatments ($P > 0.05$). Both techniques showed no incorporation of cholesterol to spermatozoa. It was concluding that addition of cholesterol-cyclodextrin complex to the medium did not improve the physical aspects of goat semen, or the sperm binding capacity. Pre-incubation of semen with CCC for 15 minutes before addition of ethylene + egg yolk diluent (EE15CCC) did not enhance the integrity of the acrosome in relation to control (EE). The gas and thin layer chromatography showed, respectively, an efficient method for quantitatively and qualitatively determining the cholesterol present in the cryopreserved goat sperm. The concentration of 1 mg of cholesterol-cyclodextrin complex added to the goat semen was not effective for increasing the concentration of cholesterol in sperm.

INTRODUÇÃO GERAL

A inseminação artificial assume um papel importante na criação de caprinos, pois facilita o controle reprodutivo e auxilia na realização de testes de progênie de forma precisa em curto intervalo de tempo (LEBOEUF et al., 2000). Essa técnica associada ao congelamento de sêmen tem como justificativa o melhoramento genético dos rebanhos, possibilitando um grande número de descendentes por macho em lugares distintos (LEBOEUF et al., 1998).

No entanto, o processo de congelamento causa diversas lesões à estrutura espermática, como a desestabilização das membranas celulares (HOLT, 2000). Segundo Hopkins & Evans (1991) a sensibilidade dos espermatozoides frente às mudanças de temperatura ocorre devido à ação protetora realizada pelo plasma seminal e a integridade da membrana espermática, estando relacionado tanto com sua composição lipidio-protéica como de colesterol e fosfolipídios (DARIN-BENETT & WHITE, 1977). Portanto são necessários estudos objetivando a formulação de diluentes que possam diminuir as lesões que acometem a estrutura espermática, para que desta forma o espermatozoide possa manter sua motilidade e vigor mesmo após o processo de congelamento.

O colesterol, juntamente com os fosfolipídios, são os componentes de membrana responsáveis pela sua fluidez e, espécies que possuem um maior teor de colesterol em sua membrana sofrem menores danos causados pelo processo de congelamento. Este estudo tem como objetivo empregar a inclusão de colesterol carreado pela ciclodextrina em diluentes comerciais com o intuito de avaliar a capacidade de ligação de espermatozoides à membrana perivitelina da gema do ovo, predizendo a fertilidade dos espermatozoides e verificar se o uso do colesterol melhora a motilidade espermática pós-descongelamento e, utilizar as técnicas de cromatografia de camada delgada e gasosa para avaliar quantitativamente e qualitativamente a inclusão do colesterol na membrana do espermatozoide.

REVISÃO DE LITERATURA

Espermatozoide

Os espermatozoides são o produto final do processo espermatogênico, que ocorre nos túbulos seminíferos. A espermatogênese é um processo sincronizado e regulado de divisão e diferenciação celular que pode ser dividida em duas fases, mitótica e meiótica. Durante a fase mitótica, a progênie de células germinativas é submetida a uma série de divisões visando expandir a população espermatogonial. A fase meiótica culmina em duas divisões que ocorrem de forma rápida e sem replicação de DNA, produzindo espermátideshaplóides (KNOBIL & NEILL, 2006).

O espermatozóide ganha sua forma característica na fase pós-meiótica (espermiogênese), sendo esta fase caracterizada pelo extensivo remodelamento das espermátides, com formação do acrossoma, condensação nuclear, desenvolvimento do flagelo e perda de grande parte do citoplasma. Como resultado destes eventos, há uma célula altamente diferenciada em estrutura e função, com capacidade de se combinar com o ovócito para iniciar o processo de uma nova geração (KNOBIL & NEILL, 2006).

A espermatogênese e maturação espermática são processos típicos de diferenciação celular. Essas modificações reestruturam os componentes celulares e transformam a célula imóvel em uma espermátide oval e, em seguida, em um espermatozóide com motilidade vigorosa (YAFFE, 1997).

Duas partes funcionais e morfologicamente distintas formam os espermatozoides: a cabeça e a cauda, ligadas pelo colo, sendo esta uma peça de conexão. Na primeira estrutura encontra-se o núcleo, o acrossoma e estruturas de citoesqueleto. A cauda é composta pelas peças intermediária, principal e terminal. A parte central da peça intermediária, em conjunto com o comprimento total da cauda, forma o axonema. Este é composto por nove pares de microtúbulos dispostos radialmente ao redor de dois filamentos centrais. Na peça intermediária, o padrão 9+2 de microtúbulos está circundado por nove fibras densas. O axonema e as fibras associadas são recobertos perifericamente por numerosas mitocôndrias, responsáveis pelo fornecimento de energia para motilidade espermática (HAFEZ & HAFEZ, 2004; KNOBIL & NEIL, 2006).

Membrana espermática

A membrana plasmática é formada por uma bicamada lipídica, com proteínas integrais e periféricas, glicoproteínas de superfície e glicolipídios organizados em um mosaico fluido. As proteínas integrais e periféricas estão entremeadas ao longo da bicamada lipídica. As membranas são impermeáveis a grande parte dos solutos polares, porém são permeáveis a substâncias apolares (LEHNINGER et al., 2005).

Os lipídios são responsáveis pela integridade estrutural, enquanto as proteínas são as principais responsáveis pela ocorrência da vários processos dinâmicos e, os carboidratos tem papel importante na interação entre as células (AMANN & GRAHAM, 1993). Os fosfolipídios formam uma bicamada em que as regiões apolares das moléculas lipídicas de cada camada interagem entre si na parte interior da bicamada e, as regiões polares, interagem na fase aquosa, ou seja, na fase externa (LEHNINGER et al., 2005). Essa barreira hidrofóbica previne a entrada de água e outras moléculas. Em temperatura corporal, a membrana plasmática encontra-se em estado fluído e o arranjo lamelar permite a movimentação dos fosfolipídios ao longo da bicamada (AMANN & GRAHAM, 1993).

A composição lipídica da membrana plasmática varia entre as espécies de mamíferos, porém, em geral, possuem cerca de 70% de fosfolipídios, 25% de lipídios neutros e 5% de glicolipídios, estando distribuídos assimetricamente entre os dois folhetos da bicamada. O colesterol é o componente da membrana plasmática mais variável, possuindo relação direta com a capacitação espermática (HARRISON & GADELLA, 2005). A relação entre colesterol e fosfolipídio é que determina a fluidez da membrana. Regiões de membrana com elevado teor de colesterol possuem menor fluidez. Segundo Darin-Bennet et al. (1977) espécies que possuem maior concentração de colesterol apresentam menor danos causados a membrana, durante o processo de resfriamento. A relação entre colesterol e fosfolipídios na espécie ovina é de 0,85 (HOLT & NORTH, 1985); 0,83 na espécie humana (MACK et al., 1986); 0,36 na espécie eqüina (AMANN & GRAHAM, 1993); 0,20 em suínos (PARKS & GRAHAM, 1992) e, em bovinos, oscila entre 0,51 e 0,53 (PARKS& GRAHAM, 1992).

Quando a relação entre o colesterol e fosfolipídio é alterada, ou seja, a relação encontra-se menor que 1:2, o processo de resfriamento causa um rearranjo devido à fase de transição do estado líquido para o cristalino (AMANN & PICKETT, 1987). Essa

mudança nas membranas celulares ocorre quando a temperatura alcança certo ponto de transição, portanto, para se adquirir uma boa taxa de sobrevivência dos espermatozoides após o congelamento, deve-se estabelecer velocidades de resfriamento e reaquecimento adequadas (MAZUR, 1984).

Injúrias a membrana plasmática

Durante os processos de congelamento-descongelamento, os espermatozoides são submetidos a condições desfavoráveis (PARKS & GRAHAM, 1992) e, consequentemente, pode ocorrer danos à membrana plasmática e acrossoma (WATSON, 1995).

Essas injúrias ocorrem porque na temperatura de 37ºC, os lipídios encontram-se dispostos de forma aleatória e em estado de fluidez. Quando ocorre diminuição na temperatura do meio onde essas células estão contidas, há um alongamento das cadeias de ácidos graxos, resultando em aumento na rigidez da membrana. Isto ocorre porque os lipídios que possuem estruturas semelhantes se agrupam, originando estruturas cristalinas com forma hexagonal. Esse arranjo favorece o deslocamento das proteínas para esses locais, que se fundem e formam agregados protéicos que resultam em um aumento na permeabilidade da membrana e diminuição do metabolismo celular, sendo tal efeito denominado choque térmico (AMANN & GRAHAM, 1993). Essa desestabilização das membranas devido ao resfriamento tem como consequência a reação acrossômica e a capacitação desordenada, pois as mudanças ocorridas na fluidez da membrana são semelhantes àquelas ocorridas durante a capacitação (WATSON, 1995).

O inicio do estresse sofrido pelos espermatozoides ocorre durante o resfriamento a 5ºC, esta fase caracteriza-se pela transição da membrana do estado líquido cristalino, para o estado de gel. Com o objetivo de amenizar o choque térmico, a taxa de resfriamento deve ser controlada e lipídios ou lipoproteínas devem ser adicionados ao diluente, além da utilização da taxa de resfriamento de forma lenta (-0,05ºC/min; MEDEIROS et al., 2002).

As alterações da fase lipídica e/ou aumento na peroxidação lipídica da membrana plasmática tem como consequências a redução na velocidade e na porcentagem de espermatozoides móveis, além de alterações no volume da água intracelular. Como resultado dessas modificações, ocorre o estresse mecânico na

membrana (NOILES ET al., 1995), devido à desestabilização de sua bicamada lipídica e alterações dos componentes da membrana celular, tais como: desnaturação das proteínas da membrana, alteração no metabolismo energético celular, da osmolaridade e pH (WATSON, 2000). A peroxidação ocorre porque os espermatozoides possuem em suas membranas grande quantidade de ácidos graxos poli-insaturados e baixas concentrações de enzimas antioxidantes, como consequência, os danos peroxidativos induzem a formação de espécies reativas ao oxigênio (ROS), levando a uma redução da viabilidade e fertilidade dos espermatozoides (HSU et al., 1998).

Visando a proteção do efeito da formação excessiva de ROS, a célula possui um sistema de defesa antioxidante no plasma seminal, porém para a criopreservação, há a necessidade da prévia diluição do sêmen e, consequentemente, diminuição de antioxidantes fisiológicos (BALL, 2001).

Tanto o congelamento como o descongelamento do sêmen promovem alterações no espermatozoide, devido à cristalização da água e a mudanças osmóticas no meio extracelular, ocorre aumento da permeabilidade da membrana, reduzindo a atividade metabólica, havendo danos no acrossoma e em outras estruturas, além de alterações nas concentrações de eletrólitos intracelulares. Como consequência destas alterações ocorre perda da fertilidade dos espermatozoides (HOFMO & ALMLID, 1990).

A cristalização do meio extracelular ocorre de acordo com a velocidade de congelamento e dos crioprotetores utilizados. Durante esta cristalização, as células ficam expostas a soluções hiperosmóticas, ocorrendo uma saída de agua do seu interior e o influxo de íons. No descongelamento, o efeito ocorre de forma reversa, com o influxo de água para o meio intracelular, podendo levar ao rompimento da membrana plasmática (HOLT, 2000).

Membranas plasmáticas que contenham em sua composição uma concentração de colesterol maior (humanos 0,99 e coelhos 0,88) possui menor susceptibilidade ao choque térmico, pois a fluidez da membrana é dependente da relação do colesterol e fosfolipídios (AMORIM, 2008).

Ciclodextrina

O colesterol é capaz de reduzir a fluidez da membrana acima da temperatura de transição, porém aumenta esta fluidez quando a membrana é resfriada para uma

temperatura abaixo da temperatura de transição. Portanto a molécula de colesterol é capaz de proteger a célula durante o resfriamento e, sua adição ao espermatozoide na criopreservação pode minimizar ou eliminar a fase de transição (GRAHAM et al., 1987).

Moore et al. (2005) demonstraram que a adição de ciclodextrinas carreadas com colesterol durante o processo de criopreservação, possibilita as células manterem um elevado teor de colesterol, impedindo assim, a pré maturação para capacitação das células, aumentando assim sua viabilidade.

As ciclodextrinas são oligossacarídeos cíclicos capazes de incorporar um lipídio, no caso o colesterol, no centro do círculo que compõe sua estrutura, permitindo a incorporação à membrana plasmática dos espermatozoides. Essas ciclodextrinas possuem em sua composição, uma cavidade hidrofóbica e, mesmo sendo solúveis em água, podem incorporar moléculas hidrofóbicas, sendo a β-ciclodextrina a forma que possui maior afinidade para encapsular os compostos lipídicos, dentre eles, o colesterol. Além desta, existem outras formas, tais como: α e γciclodextrinas (ZIDOVETZKI & LEVITAN, 2007).

Alguns estudos demonstraram que a ciclodextrina incubada com células em cultura, foi capaz de transferir o colesterol abaixo do gradiente de concentração, da membrana plasmática da célula para dentro de sua região hidrofóbica, causando alteração da estrutura e funcionalidade da membrana devido à retirada do colesterol (ATGER et al., 1995; OHVO et al., 1997). Se essa incubação for realizada apenas com a ciclodextrina e os espermatozoides, a primeira realiza a retirada do colesterol, induzindo a capacitação espermática. Porém, se a mesma for carreada com colesterol e incubada com os espermatozoides, transfere o colesterol abaixo do gradiente de concentração para a membrana plasmática, aumentando a concentração de colesterol na membrana plasmática (PURDY & GRAHAM, 2004; MOORE et al., 2005).

Quando ocorre a incubação do complexo ciclodextrina-colesterol (CCC) no sêmen, há uma maior porcentagem de células móveis e viáveis após o processo de criopreservação e descongelamento (AMORIM et al., 2007). Estudos realizados por Parinaudet al. (2000) demonstraram que os espermatozoides humanos que foram incubados com ciclodextrina possuíram maior afinidade de ligação com a zona pelúcida quando comparados aqueles não tratados. Estes autores atribuem este fato ao aumento no número de espermatozoides capacitados devido à saída de colesterol da membrana plasmática.

Cromatografia gasosa e em camada delgada

A cromatografia é um método físico-químico quetem a capacidade de separar componentes de uma amostra qualquer, realizada pela distribuição destescomponentes entre duas fases. Uma das fasesestá fixa, enquanto a outra passa através dela por capilaridade, sendo esta denominada fase móvel. Aamostra é introduzida no equipamento econduzida pela fase móvel (líquida ou gasosa).Durante a passagem da fase móvel através dafase estacionária (líquida ou sólida), oscomponentes da amostra são distribuídos entre asduas fases, de tal forma que, cada um deles éseletivamente retido pela fase estacionária,resultando em uma migração diferencial quepromove a separação (COLLINS, 1988; COLLINS et al., 2006).

A cromatografia pode ser usada na avaliação do sêmen de mamíferos visando identificar sua composição, visto que, essa técnica pode quantificar os ácidos graxos, colesterol e triglicerídeos do sêmen. Rookeet al. (2001) realizaram estudo para verificar os efeitos da alimentação com óleo de atum na composição lipídica e nas características *in vitro* dos espermatozoides suínos. As análises cromatográficas desse estudo foram sensíveis para detectar mudanças na proporção de ácidos graxos e fosfolipídios com melhora na qualidade *in vitro* do sêmen.

Theriénet al. (1999) utilizaram a cromatografia de camada delgada para determinar a quantidade de fosfolipídios presentes nos espermatozoides bovino radiomarcados com o ácido palmítico visando identificar proteínas de ligação que estimulam o efluxo de fosfolipídios de espermatozoides provenientes do epidídimo.

Komarerket al. (1964) descreveram que a técnica de cromatografia de camada delgada possui grande poder de separação de lipídios no sêmen bovino. Os mesmos autores utilizaram esta técnica para quantificação por análise gravimétrica de lipídios do sêmen bovino. Enquanto, Scott e Dawson (1968) utilizaram desta mesma técnica para identificar os fosfolipídios presentes no plasma seminal de carneiros e, Leβiget al. (2004) analisaram a composição lipídica do sêmen humano e de varrões. Já Sarada e Ramasastri (1983), empregaram a cromatografia de camada delgada para identificação de colina no sêmen humano.

A cromatografia gasosa pode ser utilizada para examinar a composição fosfolipídica de espermatozoides antes, durante ou após o trânsito epididimário do

touro, por meio de analises dos espermatozoides obtidos na cauda do epidídimo, ductos eferentes e deferentes (POULOS et al., 1973).

Moraes et al. (2010) utilizaram a cromatografia gasosa para identificação da composição de ácidos graxos presentes na ração de suínos. Esses autores adicionaram fontes de óleo e níveis de suplementação de vitamina E na ração de suínos visando analisar a qualidade do sêmen suíno. Obtendo como resultado um aumento de motilidade progressiva e espermatozoides reativos ao teste hiposmótico nos animais suplementados com óleo de salmão.

Teste de ligação na membrana perivitelina da gema do ovo de galinha

A zona pelúcida envolve o oócito e o embrião quando ainda é jovem, sendo uma matriz extracecular transparente, composta por três glicoproteínas (ZP1, ZP2 e ZP3), na maioria dos mamíferos e, possui um receptor espécie-específico para espermatozoides já capacitados e os induz à reação acrossômica (Sinowatzet al., 2003).

Para a determinação da capacidade de fecundação dos espermatozoides, ensaios *in vitro* são realizados, determinando assim, a habilidade de reação acrossômica, ligação e penetração do espermatozoide na *oolemma*. Estes ensaios têm sido desenvolvidos para humanos, bovinos, suínos, garanhões, entre outras espécies, em que o espermatozoide é incubado com a zona pelúcida por um determinado período de tempo e, depois verifica-se a quantidade de espermatozoides ligados à zona pelúcida, porém este método torna-se de difícil execução devido a obtenção de números insuficientes de zona pelúcida (Amorim, 2008).

Barbatoet al. (1998) realizaram estudos comparando a zona pelúcida com a membrana perivitelina da gema do ovo de galinha e verificaram que há similaridade entre as mesmas, podendo desta forma, substituir os ensaios *in vitro* com zona pelúcida pela membrana perivitelina do ovo, pois os espermatozoides também são capazes de realizar ligação nesta membrana.

Amorim et al. (2007) relataram que os ensaios de ligação com a membrana perivitelina é simples e de fácil obtenção, em que espermatozoides criopreservados se ligam a membrana pertivitelina da gema do ovo de galinha, demonstrando que esta possui receptores para os espermatozóides.

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ACROSOMAL INTEGRITY AND FERTILIZING CAPACITY OF FROZEN GOAT SPERM AFTER ADDITION OF CYCLODEXTRIN-CHOLESTEROL COMPLEX[1]

Contents

This study aimed to evaluate the ability of goat sperm treated of cholesterol to bind to the perivitelline membrane of the hen´s egg yolk and evaluate the integrity of acrossome. Four bucks, from Saanen (2) and Parda Alpine (2)breeds, were used. Three semen collections per animal were performed and diluted according to the following treatments: TG – Tris-glycerol; TGCCC – CCC (1 mg) + Tris-glycerol; TG15CCC – CCC (1 mg) diluted in an isosmotic solution incubated during 15 minutes before the addition of Tris-glycerol; EE – Egg yolk + ethyleneglycol; EECCC – CCC (1 mg) + egg yolk + ethyleneglycol; EE15CCC – CCC (1 mg) diluted in an isosmotic solution incubated during 15 minutes before the addition of egg yolk + ethyleneglycol. Semen was packed into 0.25 mL straws and cooled to 5^{o}C during 1 hour. Cooling was performed in liquid nitrogen vapor for 15 minutes, and sequentially straws were immersed in liquid nitrogen. Semen straws were thawed in water bath at 37^{o}C for 30 seconds. After thawing analyzes of acrosomal integrity, binding assay on the hen´s egg yolk perivitelline membrane (PMEY), sperm motility and vigor were performed. As a result the inclusion of the CCC did not increase sperm binding to the PMEY nor sperm motility and vigor (P>0.05). The EE15CCC treatment was similar to the control treatment (EE) extenders in maintaining acrosomal integrity (P>0.05). There was a negative correlation between the binding assay and acrosomal integrity. However, there was a positive correlation between binding assay and sperm motility.It was concluded that the inclusion of the CCC to diluents did not improve the binding capacity of goat

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sperm; however, the incubation of semen with the CCC before the addition of egg yolk and ethyleneglycol was not able to increase the percentage of spermatozoa with intact acrosome as to differ from the control treatment for both diluents.

Introduction

The freezing process is responsible for a wide variety of lesions on the sperm structure, such as destabilization of its cellular membrane (Holt, 2000). According to Parks and Graham (1992), the primary cause of freezing injury to cells occurs as a consequence of the plasma membrane disruption due to thermal, mechanical, chemical and osmotic stresses imposed upon the membrane.

The initial stress suffered by spermatozoa occurs when they are submitted to temperatures lower than 5ºC due to thermal shock by cold. This phase is characterized by the transition of the membrane from its liquid crystalline to a gel state (Parks and Lynch, 1992).

Alterations in the lipid phase and/or an increase in plasma membrane lipid peroxidation lead to a reduction in sperm motility and vigor. As a result of these lipid modifications, mechanical stress occurs in the sperm membranes and it promotes alterations of its components, such as denaturation of membrane proteins, changes in cellular energy metabolism, osmolarity and pH (Watson, 2000). Peroxidation occurs because the sperm membrane has large amounts of polyunsaturated fatty acids and low concentrations of antioxidant enzymes (Poulos et al., 1973). Consequently, peroxidative damage induces the formation of reactive oxygen species, leading to a reduction of sperm viability and fertility (Hsu et al., 1998).

Sperm membranes which contain high cholesterol concentrations showed less susceptibility to thermal shock as human which has a cholesterol: phospholipids

concentrations of 0.99 and rabbit with this ratio of 0.88 (Darin-Bennet and White, 1977), since membrane fluidity is dependent on the cholesterol:phospholipids ratio (Amorim, 2008). Cholesterol is able to increase membrane fluidity when the membrane is cooled to temperatures lower than the transition temperature. According to Graham and Foote, (1987) adding cholesterol to sperm undergoing cryopreservation can minimizeor eliminate the transition phase since it is able to protect the cell during cooling.

Moore et al. (2005) demonstrated that the addition of 1,5 mg cholesterol carried by cyclodextrins during the cryopreservation process of stallion sperm increases cholesterol levels in the sperm membrane and therefore increases cell viability. Adding a cyclodextrin-cholesterol complex (CCC) to bull semen allows propitiating a higher percentage of motile and viable cells after cryopreservation (Amorim et al., 2007).

There are not many studies about the effect that CCC treatment has on goat sperm cryosurvival. Some authors observed that CCC treatment improved pos-thaw sperm motility, although it did not affect plasma membrane and acrossomal membrane integrity (Barrera-Compean et al., 2005). Thomas et al (2008) observed that treatment of buck sperm with 1 mg CCC/120x10^6 sperm after seminal plasma elimination improved both sperm motility and membrane integrity after thawing. Mocé et al. (2010) observed that treatment of buck with 1 mg CCC showed higher percentage of sperm motility that the control treatment whithout addition of CCC. Dias et al (2011) tested different concentrations of CCC in goat sperm cryosurvival without elimination of the seminal plasma, showing superior results in the analysis of sperm motility and vigor when they used 1 mg of CCC.

Moraes et al (2010) used the perivitelline membrane of the hen´s egg yolk for evaluating the ability of bovine sperm treated with cholesterol to bind to the oocyte

membrane. The sperm treated with cholesterol resulted in more sperm binding to the chicken egg perivitelline membrane and cattle zonapellucida. Sperm bound to perivitellime membrane and zona pellucid equally well. Therefore, sperm visualization was better for the perivitelline membrane than zona pellucid of cattle. Reis et al (2003) used the perivitelline membrane of the hen´s egg yolk for predicting the sperm fertility of boar sperm. Purdy (2006) also used this test for evaluated the percentage of post-thaw sperm ram to binding in the perivitelline membrane of the hen´s egg yolk after the sperm held for 0 to 48h at 5ºC prior to cryopreservation.

Barbato et al. (1998) and Amann et al. (1999) demonstrated molecular similarity between the zona pellucid and the pervitelline membrane of the hen´s egg allowing spermatozoa from many species to bind the pervitelline membrane and to predict the fertilizing capacity of sperm.

This study aimed to evaluate the ability of goat sperm treated of cholesterol to bind to the perivitelline membrane of the hen´s egg yolk predicting sperm fertility and to check if the inclusion of cholesterol is able to maintain the integrity of acrossome in frozen goat semen.

Materials and Methods

Local and animals

The samples of semen were collected in the first week of January 2012 in the Caprine Section from Federal University of Viçosa, Minas Gerais, Brazil.Four bucks, Saanen (2) and PardaAlpine (2) breeds, aged 1 and 3 yearswere used, animals were classified as suitable for breeding soundness avaliation, according to the standards recommended by the Brazilian College of Animal Reproduction (CBRA, 1998).

Semen Collection

For obtaining the semen samples a goat with induced estrus (4 mg/kg estrogen intramuscular) 12 hours before the start of samplingwas used. The samples for each buck were interspersed by 48 hours. The ejaculates were obtained by artificial vagina andthe collection tube was coated with dark cloth bag in order to protect the semen from thermal variations and solar ultraviolet radiation.

Evaluation of Semen

Immediately after the collection was conducted the analysis of semen: volume (mL), motility (0-100%), sperm vigor (0-5), sperm concentration (sperm / mL), according to the standards recommended by the CBRA (1998).

Sperm motility and vigor were evaluated in light microscopy (100X) using a cover slip pre-heated at 37^{o}C over a drop of semen.

All ejaculates were used in this experiment, they haveshowed progressive motility higher than 70% and vigor higher than 3, as recommended by CBRA, 1998.

In order to calculate thefinalvolumeofdiluenttobeadded and thenumber ofdoses to be made per sperm sample, 10µLoffreshsemenwas dilutedin1.99mL ofHancock solution to determine sperm concentration (haemocytometer method).

Cyclodextrin Preparation

Methyl-β-cyclodextrin was loaded with cholesterol as described by Purdy and Graham (2004). Briefly, 200 mg of cholesterol was diluted in 1 mL of chloroform. In a separate glass test-tube 1 g of methyl-β-cyclodextrin was dissolved in 2 mL of methanol. Then, a 0.45 mL aliquot of the cholesterol solution was added to the cyclodextrin solution and mixed until a clear solution. This mixture was poured into a

glass Petri dish and the solvents were removed by evaporating using a heating dish at 40ºC for 48 hours. The resulting crystals were then allowed to dry for an additional 24 h. After this period, crystals were removed from the dish and stocked in a glass container at 22ºC. A stock solution of cholesterol-loaded cyclodextrin was prepared by adding 50 mg of cyclodextrin loaded cholesterol (CLC) to 1 mL of Tyrode-Albumin-Lactate-Pyruvate (TALP) at 37ºC and mixed briefly using a vortex mixer. This solution was kept frozen at -80ºC until use.

Semen dilution and experimental design

Each ejaculate was split into six treatments: TG – Tris-glycerol (extender composition table 01) ; TGCCC – CCC (1 mg) + Tris-glycerol; TG15CCC – CCC (1 mg) diluted in an isoosmotic solution (Sodium Chloride 0.9%) incubated during 15 minutes before the addition of Tris-glycerol; EE – Egg yolk + ethyleneglycol (extender composition table 02); EECCC – CCC (1 mg) + egg yolk + ethyleneglycol; EE15CCC – CCC (1 mg) diluted in an isoosmotic solution (Sodium Chloride 0.9%) incubated during 15 minutes before the addition of egg yolk + ethyleneglycol.

Semen Freezing

Semen was packed into 0.25 mL straws containing a total of 50 x 10^6 spermatozoa/ straw. Straws were then placed in a 20 mL test-tube coated with refill (plastic bag) and placed into a 240 mL plastic container containing 125 mL of absolute ethyl alcohol (Fürst et al., 2005). The container was placed horizontally inside a refrigerator, with internal temperature of 5°C, a cooling rate of -0.38°C/min, during 45 minutes and 15 additional minutes of equilibration time (Fürst et al., 2005 adapted).

Cooling was performed during 15 minutes in liquid nitrogen vapor. Straws were placed 5 cm above liquid nitrogen (Chirineá et al., 2006). After this period, straws were submerged in liquid nitrogen and stored (-196 ºC) for 10 days.

For thawing, semen aliquots(0.25 mL) was placed in water bath at 37ºC during 30 seconds, were packed into 2.0 mL microtubes so that sperm motility and vigor could be analyzed.

Acrossomal integrity

For evaluating the integrity of the acrosome of thawed semen, 100 µg of the Fluorescein Isothiocyanate conjugated with *Psiumsativum*agglutinin (FITC-PSA) was diluted in Dulbeco Phosphate Buffered Saline (DPBS) and 10% of sodium azide solution at 10% as described by Celeghini et al. (2010). After dilution of the fluorescent probe, 5 µL of FITC-PSA were added to semen. Samples were incubated in water bath at 37°C for eight minutes (Celeghini et al., 2010). Subsequently, a 5 µL aliquot of sample was analyzed with fluorescence microscope[2] containing fluorescence illuminator, density filters ND 6, ND 25 and green filter. One hundred cells were examined per sample at 1000X magnification and classified according to the fluorescence emitted into: intact acrosome (IA; green) or damaged (DA; orange-red).

Binding assay on the hen´s egg yolk

The ability of goat sperm to bind to the perivitelline membrane (PM) was conducted using the chicken egg perivitelline membrane as described by Barbato et al (1998). Perivitelline membrane was prepared by separation the egg yolk from the egg white. The intact yolk was put into a piece of parafilm, the membrane was ruptured and

[2]Olympus BX 53

the yolk gently washed away usingBeltsville Thawing Solution(BTS) keeping the membrane in the parafilm. The membrane was removed from the parafilm and placed into a 10 mL glass vial. The membrane was washed several times with BTS till the solution was clear and no visible yolk was present. The PM was then placed into a Petri dish, gently spread to a single layer and then cut into small squares (1 cm^2), using a spectrophotometer cuvette as a template. Each perivitelline piece was put into a culture tube containing 1 mL of BTS and each PM squares was incubated with 50 x 10^6 thawed sperm from different treatments.

The membrane and sperm were co-incubated for one hour, at 37°C in an atmosphere of 5% of CO_2in air. Thirty minutes before the end of the incubation time, 1 µLof Hooechst 33342 solution (1 mg/mL) was added to each tube to get the sperm stained. After incubation, each membrane was put into a tube containing 1 mL of BTS and the membranes were washed 5 times to remove any unbound sperm. For each membrane, all adhered sperm were counted in six random fields; thus, the percentageof adhered spermatozoa was estimated through the correction factor (Barbato et al., 1998):

$$\% \text{ sperm adhered} = 21 \times \text{number of spermatozoa counted} + 31$$

Statistical Analysis

For data analysis, SAEG 9.1 software (SAEG – UFV, 2007) was used. The experimental design was completely randomized, factorial assay 6x4 (six treatments x four animals). The Lilliefors test along with the Cochran and Bartlett test were used to verify data normality and variance homogeneity, respectively. Variables with normal distribution and variance homogeneity were submitted to ANOVA and means were compared by the Duncan's test. When assumptions of ANOVA were not met, data were subjected to the Kruskal-Wallis test. Pearson's linear correlation was made among all

variables. The level of significance adopted was $\alpha = 0.05$. Means and the standard error of mean were assessed by descriptive statistics.

Results

There was no interaction between treatment and animal ($P > 0.05$). Likewise, the addition of cholesterol cyclodextrin complex on goat frozen semen did not increase the sperm ability of binding to perivitelline membrane of the hen's egg yolk ($P > 0.05$, Table 03).

The EE15CCC treatment showed to be able of maintaining the acrosome integrity, but the incubation of semen with the complex before addition of the diluent was no more effective than the control (EE; Table 04). In the control treatment, the Tris-Glycerol diluent provided better acrosome integrity than the other treatments using the same diluent (TGCCC and TG15CCC, Table 04).

The analysis of sperm motility and vigor of fresh and frozen semen showed no difference among treatments ($P > 0.05$, Table 04).

Low correlations were observedbetween binding test and acrosome integrityand sperm motility ($r = -0.25$ and $r = 0.20$, respectively) and low correlation between sperm motility and acrosome integrity ($r = -0.26$).

Discussion

Binding Assay in the hen´s egg yolk perivitelline membrane

In this study there was adhesion of sperm to the perivitelline membrane (PVM) in all treatments. This indicates that the PVM can be used to test the sperm adhesion capacity. This fact was demonstrated by Barbato et al (1998) that by studying different species, verified the adhesion ability of bovine, rat, sheep and humanspermatozoa. The authors pointed out that there are similarities between the glycoproteins of the

zonapellucida of mammals with the composition of the perivitellinemembrane of chicken egg yolk.

In this study, the binding test of the goat sperm to perivitelline membrane of chicken egg yolk did not show difference among treatments. This may be due to excess of sperm ($1x10^6$) incubated with the perivitelline membrane, together with the long incubation time of the PVM with sperm.

It can be seen that binding efficiency varies among species and differences in binding can be detected based on insemination dose (Barbato et al. 1998). Therefore, the insemination dose has great importance in binding test toperivitelline membrane in order to avoid saturation with the sperm due to the limited number of binding sites available on a membrane (Gill et al. 1999).

Thus it is required an adjustment of the insemination dose for each species to maximize the connection/fertilization of sperm to membrane/oocyte, thereby adjusting the test to be used as a way of predicting the fertilizing ability of sperm (Barbato et al. 1998).

Acrossomal Integrity

The maintenance of the acrosome integrity demonstrated by treatment in which the cyclodextrincholesterol complex was previously diluted in isosmotic solution and incubated for 15 minutes before addition of the egg yolk + ethylene glycol (EE15CCC) may have been better than treatment in which the semen was diluted directly with the egg yolk+ ethylene glycol diluent and cholesterol cyclodextrin complex (EECCC) due to the need for complex being incubated with the semen in a medium devoid of lipids. When the complex is added to a diluent containing the egg yolk, cholesterol carried by

cyclodextrinmay be transferred to the lipids in the egg yolk, instead of the sperm membrane (Graham and Purdy, 2004).

However, this prior incubation of semen with the cyclodextrin cholesterol complex before addition of egg yolk +ethylene glycol diluent was probably not able of incorporating cholesterol to the sperm membrane and it did not differ from control.

The hen's egg yolk, as well as the soy lecithin, in the Tris-glycerol diluent has an active factor in protecting, the low density protein (LDL), composed of phospholipids, which acts only on the cell surface as a protective film protecting the sperm during the cooling phase (Watson, 1995; Forouzanfar et al. 2010). In the present study, in the Tris-glycerol treatments, only the control treatment (TG) obtained better maintenance of acrosome integrity. The other treatments may have shown inferior results because negative interactions between the components of the diluent and the cyclodextrin.

Combes et al. (2000) observed that stallion spermatozoa incubated in TALP medium jointly with the Methyl-beta-cyclodextrin-cholesterol complex (MβCD) incorporated more cholesterol to their membranes due to the unavailability of additional cholesterol in this medium when compared to the Kenney modified medium. In turn, the effect of cholesterol inclusion in stallion semen was reported to be the same in all diluents used by Zahn et al. (2002), including the one containing egg yolk, without any interaction between the cholesterol treatment and diluents.

Sperm Motility and Vigor

There was no difference on sperm motility and vigor among treatments, since there was no incorporation of cholesterol to the sperm membrane (Silveira et al., unpublished data).

The effect of cholesterol on sperm motility is still controversial (Oliveira et al., 2010). Studies have shown that cholesterol is incorporated into all membranes, and are located in higher concentrations in the acrossomic and mitochondrial membranes

(Moore et al., 2005a). However, corroborating to this study, Zahn et al. (2002) working with frozen equine semen reported no increase in sperm vigor after the inclusion of cholesterol on the extender. Therefore, it is probable that cholesterol was not sufficiently incorporated to the mitochondrial membrane in order to benefit energy production of motile spermatozoa.

Correlation between binding assay in the hen´s egg yolk and integrity acrossome

The binding assay data showed a positive correlation with sperm motility(r= 0.20) and a negative correlation with intact acrosomes (r= -0.25). These correlations may be explained by the cryopreservation process realized on sperm. During cryopreservation, sperm cells undergo intracellular and extracellular stress, resulting in changes in the membrane's conformation, such as reorganization of lipids and proteins. Osmotic changes also occur through the membrane. When spermatozoa are submitted to the cooling and freezing process sperm membranes undergo a transition phase, in which rearrangements between lipids and proteins occur. In this event, membrane lipids are lost causing aggregation between lipids and proteins, leading to loss of membrane selective permeability (Watson, 1995). This results in premature sperm capacitation, due to the disruption of enzymes, including ATPases. Cooled sperm exhibit higher concentrations of intracellular calcium and bicarbonate, which occur in the early stages of *in vivo* sperm capacitation and hyperactivation (Purdy and Graham, 2004). Nevertheless, the correlation between the binding test, sperm motility and acrosome injury may be related to premature capacitation in sperm. As a result of this capacitation the sperm hyperactivationoccurs. These events are necessary for the sperm become able to fertilize the oocyte.

Conclusion

The inclusion of cyclodextrin-cholesterol complex in the cryopreserved goat sperm membrane does not interfere on the fertility assessed by *in vitro*assays.

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Tables

Table 01:Chemical composition of the Tris-Glycerol extender

COMPONENTS	g/L
Tris	2.3
Sodiumcitrate	6.2
Potassiumchloride	0.8
Fructose	1.2
Lactose monohydrate	0.8
Glycine	0.2
Glucose anhydrous	0.5
Taurine	0.005
Gentamicinsulfate	0.24
TylosinTartrate	0.33
LincoSpectin 100	0.383
Glycerol	40.2
Hydratecalciumlactate	0.7
Soylecithin	1.5
Monohydrated citric acid	2.5
Ultrapure water q.s.p.	1000 mL

Adapted of Minitub® - Brazil

Table 02: Chemical composition of the Ethyleneglycol + Egg yolk extender

COMPONENTES	g/L
Glucose	79.9
Sodiumcitrate	12.71
Potassiumchloride	1.59
EDTA	2.65
Sodiumbicarbonate	2.65
Eggyolk	2.5
Ethyleneglycol	2.5
Gentamicinsulfate	0.5
Ultrapure water q.s.p.	1000 mL

Adapted of Minitub® - Brazil

Table 03: Number of frozen / thawed goat sperm adhered to the perivitelline membrane of chicken egg yolk after being incubated for 1 hour at 37 °C with 5% CO_2.

Treatment	Spermatozoa adhered(Mean ± Standard error of mean)
TG	1147.8 ± 257.0^{a}
TGCCC	1282.2 ± 172.9^{a}
TG15CCC	1529.0 ± 227.8^{a}
EE	1028.5 ± 263.9^{a}
EECCC	$1119.5 \pm 2\ 53.2^{a}$
EE15CCC	1047.7 ± 203.0^{a}

P > 0.05 by Kruskal-Wallis test.

Table 04: Percentage of acrosome integrity (AI), sperm motility of fresh (MOTF) and thawed semen (MOTT), sperm vigor of fresh (VIGF) and thawed semen (VIGT) of goat sperm cryopreserved with or without the addition of the cyclodextrin-cholesterol complex (mean ± standard error of mean).

Treatments	AI	MOTF	VIGF	MOTT	VIGT
TG	71.3 ± 5.9[a]	83.7 ± 6.8[a]	4.2 ± 0.4[a]	25.8 ± 4.0[a]	2.9 ± 0.1[a]
TGCCC	51.0 ± 4.0[b]	83.7 ± 6.8[a]	4.2 ± 0.4[a]	29.5 ± 2.8[a]	2.1 ± 0.2[a]
TG15CCC	42.4 ± 7.1[b]	83.7 ± 6.8[a]	4.2 ± 0.4[a]	35.0 ± 1.5[a]	2.4 ± 0.2[a]
EE	76.5 ± 5.4[a]	83.7 ± 6.8[a]	4.2 ± 0.4[a]	29.2 ± 4.5[a]	2.9 ± 0.3[a]
EECCC	49.0 ± 8.0[b]	83.7 ± 6.8[a]	4.2 ± 0.4[a]	37.1 ± 4.1[a]	2.6 ± 0.4[a]
EE15CCC	77.5 ± 7.4[a]	83.7 ± 6.8[a]	4.2 ± 0.4[a]	26.0 ± 4.0[a]	2.7 ± 0.1[a]

[a, b] Means followed by different letters within the same column differ ($P < 0.05$) by Duncan test

EVALUATION OF CHOLESTEROL INCORPORATION IN GOAT CRYOPRESERVED SPERM MEMBRANE BY THIN LAYER AND GAS CROMATOGRAPHY[3]

Abstract

The objective of this research was to qualify and quantify the concentration of cholesterol in citoplasmatic membranes of goat sperm by the technique of thin layer and gas chromatography, respectively, after the addition of cyclodextrin-cholesterol complex (CCC 1mg). Four bucks, Saanen (2) and Parda Alpine (2) were used. Three semen collections per animal, was diluted in the following treatments: TG – Tris-glycerol; TGCCC – CCC + Tris-glycerol; TG15CCC – CCC diluted in isosmotic solution to semen with 15 minutes of incubation before addition of Tris-glycerol; EE – Egg yolk + ethyleneglycol; EECCC – CCC + egg yolk + ethyleneglycol; EE15CCC – CCC diluted in isosmotic solution to semen with 15 minutes of incubation before addition of egg yolk + ethyleneglycol. Semen was stored in 0.25 ml straws and cooled at 5^{o}C for 1 hour. After thawing, analyzes of thin layer and gas chromatograph were conducted. There were no differences (P> 0.05) among treatments submitted to analysis of gas and thin layer chromatography. It is concluded then that the chromatographic techniques, both gas as thin layer are able to quantify and qualify the cholesterol present in goat sperm cryo-preserved. The concentration of 1 mg cholesterol carried by cyclodextrin and added to the goat semen, did not increase the levels of cholesterol present in semen.

Keywords: spermatozoa; cholesterol; cyclodextrin; goat; chromatography.

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1- Introduction

The phospholipids detected in the mammal´s sperm contain fatty acid in their composition, especially the long chain. This characteristic is essential to promote an excellent fertility, where the decrease in the fatty acids composition reduces sperm concentration and there are negative influences on the sperm motility [1, 2]. The plasma membrane has a mixed composition of phospholipids that may differ from specie to specie, in addition to the transition temperature phase being different for each type of phospholipid [3].

The process of semen cryopreservation induces osmotic stress of the sperm membranes, including the destabilization of this membrane with loss of its functionality and sperm motility [4]. These injuries occur because in the temperature of 37°C the lipids are arranged at random and in a state of fluidity, but when there is a decrease in temperature an elongation of fatty acid chains occurs, resulting in an increase in membrane rigidity, because lipids that have similar structures are grouped, yielding crystalline structures with hexagonal shape. This arrangement favors the movement of proteins to these sites, which fuse and form protein aggregates that result in an increase in membrane permeability and decreased cell metabolism, such effect is called heat shock [5]. This destabilization of the membranes due to cooling results in the acrosome reaction and capacitation disordered, because the changes in membrane fluidity are similar to those that occur during capacitation [6].

Cholesterol is an essential constituent of sperm in mammals. It plays an important role in the fertilization capacity of these cells. The relationship between cholesterol and phospholipid composition of the membrane determines its fluidity, where the membrane regions with high cholesterol have a lower fluidity [7]. Therefore, plasma membranes containing in its composition a higher concentration of cholesterol

(human and rabbit) have less susceptibility to heat shock, since the fluidity of the membrane is dependent on the ratio of cholesterol and phospholipids [8].

The addition of the cholesterol molecule to the sperm cryopreserved may minimize or eliminate the phase transition from liquid crystalline to the plasma membrane that occurs during the cooling and freezing, as the cholesterol molecule is capable of increasing membrane fluidity when subjected to the cooling process [9].

Cyclodextrins are cyclic oligosaccharides of glucose that have a hydrophobic core able to incorporate lipids [10]. Moore et al. [11] demonstrated that the addition of cyclodextrins loading cholesterol during the cryopreservation process, allows the cells to maintain a high cholesterol content, thus preventing the pre maturation for cell capacity and increasing its feasibility. These observations have also been made previously by Combes et al [12] in stallion semen and Purdy & Graham [13] in bull semen, when employed cyclodextrin loading cholesterol prior to cryopreservation, with increase in the percentage of motile and viable cells after freezing and thawing.

Thus, the cyclodextrinsmay be used to alter the content of cholesterol from plasma membranes [14, 15], because when cyclodextrins loaded with cholesterolinserted into the membrane, the survival rate of cells after cryopreservation is increased [16]. Therefore, the aim of the present research was to check the incorporation of cholesterol carried by cyclodextrin into the plasma membrane of goat sperm after cryopreservation using the technique of gas chromatography and thin layer.

2- Material and Methods

2.1 Local

The experiment was conducted from January to July 2012in the Caprine Section from Federal University of Viçosa, Minas Gerais, Brazil. The city is located at

an altitude of 649 m, parallel 20º 45'14 "south latitude and the meridian of 42º 52'54" west longitude. The average annual temperature is 18.5 º C and the climate of the region is classified, according to the KöppenCwa - highland tropical climate with dry winter and rainy summer.

2.2 Animals

Four males of Saanen (2) and Alpine Brown (2), aged 1 and 3 years, clinically normal, classified as useful for breeding soundness examination, according to the standards recommended by the Brazilian College of Animal Reproduction (CBRA) were used [17].

2.3 Treatments

The experimental design was completely randomized with six treatments and tested with three replicates per treatment: TG: negative control for the extenderTris-glycerol; TGCCC: CCC (1 mg) + Tris-glycerol; TG15CCC: CCC (1 mg) diluted in isosmotic solution of semen (saline) with 15 minute incubation before addition of Tris-glycerol; EE: negative control for the extender egg yolk + ethylene glycol; EECCC: CCC (1 mg) + egg yolk + ethylene glycol; EE15CCC: CCC (1 mg) was diluted in isosmotic solution of semen (saline) with 15 minute incubation before addition of the egg yolk + ethylene glycol[18].The treatments 2,3,5 and 6 show different ways to incorporate the cholesterol in the semen.

2.6 Cyclodextrin Preparation

Briefly, 200 mg of cholesterol was diluted in 1 mL of chloroform. In a separate glass test-tube 1 g of methyl-β-cyclodextrin was dissolved in 2 mL of methanol. Then, a

0.45 mL aliquot of the cholesterol solution was added to the cyclodextrin solution and mixed until a clear solution. This mixture was poured into a glass Petri dish and the solvents were removed by evaporating using a heating dish at 40 ºC for 48 hours. The resulting crystals were then allowed to dry for an additional 24 h. After this period, crystals were removed from the dish and stocked in a glass container at 22ºC. A stock solution of cholesterol-loaded cyclodextrin was prepared by adding 50 mg of cyclodextrin loaded cholesterol (CLC) to 1 mL of Tyrode-Albumin-Lactate-Pyruvate (TALP) at 37ºC and mixed briefly using a vortex mixer. This solution was kept frozen at -80 ºC until use [13].

2.8 Thin Layer Chromatography

A pallet of each treatment was thawed in a water bath at 37°C for 30 seconds and conditioned in 1.5 mLmicrotubes (eppendorf®) and centrifuged at 700g for 20 minutes. Subsequently, the supernatant was removed and added 1 mL of saline and the pellet was re-suspended. The centrifugation procedure was repeated three times. After the third centrifugation and removal of supernatant 100 mL of chloroform and 200 of methanol was added and the pellet was re-suspended and centrifuged for another 20 minutes in rotation similar to the previous [19]. At the end of centrifugation the supernatant was placed in a 1.5 mL plastic tube (eppendorf®) for conducting the thin layer chromatography.

The samples were injected in a plate of silica gel G with 5 cm of length, using a capillary, with a distance of about 1 cm between each sample. At first, the samples were placed on the plate and the cholesterol pattern diluted in hexane at a ratio of 200 mg: 20 mL [19]. After the cholesterol pattern, about 5 µL of each samples in ascending order were injected on the plate.

The plate containing the samples was placed in a glass container with hexane: formic acid at a ratio of 3:2 and sealed for approximately 2 minutes so that the passage of the mobile phase through the stationary phase by capillary action could occur. Then, the plate was removed from the container and steeped in a solution of vanillin sulfuric and dry hot steam. After drying, a test was held to observe the flow of the samples made by capillarity, comparing them with the cholesterol pattern flow, which presented itself in the form of bands of pink [19].

2.8 Gas Chromatography

For gas chromatography analysis samples were subjected to the same procedures performed on thin layer chromatography. However, after the fourth centrifugation and removal of supernatant was added 400 µL of chloroform and 600 µL of methanol pellet was re-suspended and centrifuged for over 20 minutes in rotation similar to the previous [20]. At the end of centrifugation, the supernatant was stored in tube vial for gas chromatography.

Analyzes were carried on a gas chromatograph Model GC - 17A, Shimadzu®, equipped with FID detector. For registration and analysis of chromatograms, the device was attached to a notebook, using the program GC Solution. Compounds were separated and identified on a DB1 capillary column of size 30 m x 0.25 mm.

For chromatographic separation 1µL sample was injected with aid of syringe of 10 µL (Hamilton®) system Split = 1. Nitrogen gas was used as a carrier with linear speed set to 43.2 cm / s and the gases hydrogen and synthetic air formed the flame in detector [20].

The temperatures of the injector and detector were controlled at 350ºC. The initial temperature of column was 100 °C (maintained for 2 min), increasing in 20°C per

minute until reaching 350°C (maintained for 25 minutes), resulting 39 minute of analysis. The gas flow arrestin the column was 1 mL /minute [20].

2.9 Statistical Analysis

The statistical program SAS 9.0 [21] was used for data analysis. Data referring to gas chromatography was submitted to square root transformation ($x' = \sqrt{x}$) to normalize the data, then submitted to analysis of variance (ANOVA) and Duncan Test at 5% probability. Mean and standard error of the mean were determined as descriptive statistics, also at SAS 9.0 [21].

3- Results

There were no differences (P> 0.05) among treatments analyzed for gas (Table 01) and thin-layer chromatography. Even though this last test has subjective evaluation, the results of this may be related to those obtained by gas chromatography.

Table 01:Cholesterol present in cryopreserved goat sperm after addition of cholesterol cyclodextrin complex (CCC) in the extender.

Treatments	**Gas Chromatography (ppm)**
TG	26.3±11.5
TGCCC	27.4±15.8
TG15CCC	16.6±4.5
EE	8.6±1.9
EECCC	22.2±13.9
EE15CCC	8.16±0.61

* P>0.05 ANOVA; Mean ± Standard error by Duncan test.

4- Discussion

The present research demonstrated that the cholesterol contained in goat sperm can also be identified by thin layer and gas chromatography as well as quantified by gas chromatography, with similar results for identification of compound between the two techniques.

The thin layer chromatography showed that there was no incorporation of cholesterol in the sperm submitted to incubation treatment with the cholesterol-cyclodextrin complex (CCC) compared to the control sample.This may be explained by the small amount of the CCC (1 mg), without incorporation of cholesterol to provide adequate difference between the treatments. According to Purdy and Graham [13], the concentration of cholesterol associated with sperm increases in a linear form according to the increase in the concentration of cholesterol-cyclodextrin complex incubated with the sperm. The concentrations of cholesterol obtained by the method of gas chromatography did not differ among treatments.Purdy and Graham [13] performed the technique of high performance liquid chromatography and showed that only bovine sperm submitted to treatment with 5 mg of cholesterol-cyclodextrin complex have had higher concentration of cholesterol compared to the control group.

In the present research, it was used 1 mg of CCC based on previous experiment conducted by Dias et al. [22] in goats, where different concentrations of cyclodextrin-cholesterol complex were tested (0.5 at 3 mg), showing advanced results in the analysis of physical sperm when used 1 mg of CCC.

Purdy and Graham [13] reported that the complex should be incubated with the sperm, diluted in medium devoid of lipids to prevent most of the cholesterol carried by cyclodextrin to be transferred to lipid droplets contained in the base diluents chicken egg yolk instead of sperm membrane. However, in this research, the treatment that received prior dilution of the complex with the semen showed no difference from other

treatments, either in the chicken egg yolk extender as well asTris-glycerol extender. To perform the chromatographic analysis, it was necessary to complete the washing of sperm aiming to removal of the dilution used for cryopreservation of semen. This procedure was necessary because the dilutive interference will not occur in the analysis because while withdrawing the same, it can be observed if there was really incorporation of the sperm membrane cholesterol.

5- Conclusions

The gas and thin layer chromatographytechnique are able to quantify and qualify the cholesterol present in goat sperm cryopreserved.

The concentration of 1 mg cholesterol is not sufficient to increase the concentration of cholesterol in the goat sperm cryopreserved.

6- References

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