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SEXAGEM DE EMBRIÕES BOVINOS PRODUZIDOS *IN VITRO* COM SÊMEN SELECIONADO POR PERCOLL OU *SWIM-UP*

DISSERTAÇÃO DE MESTRADO

Caroline Antoniazzi Wolf

Santa Maria, RS, Brasil
2007

SEXAGEM DE EMBRIÕES BOVINOS PRODUZIDOS *IN VITRO* COM SÊMEN SELECIONADO POR PERCOLL OU *SWIM-UP*

por

Caroline Antoniazzi Wolf

Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Clínica Médica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do grau de **Mestre em Medicina Veterinária.**

Orientadora: Prof.ª Karin Erica Brass

Santa Maria, RS, Brasil
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A Comissão Examinadora, abaixo assinada,
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elaborada por
Caroline Antoniazzi Wolf

como requisito parcial para obtenção do grau de
Mestre em Medicina Veterinária

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RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria, RS, Brasil.

SEXAGEM DE EMBRIÕES BOVINOS PRODUZIDOS *IN VITRO* COM SÊMEN SELECIONADO POR PERCOLL OU *SWIM-UP*

AUTORA: CAROLINE ANTONIAZZI WOLF
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Santa Maria, 27 de fevereiro de 2007.

O diagnóstico genético pré-implantação (DGP) vem se destacando na área da biotecnologia da reprodução animal por motivos econômicos. Um exemplo de DGP é a predeterminação do sexo da prole. Neste estudo foi verificada a percentagem de embriões bovinos machos e fêmeas produzidos *in vitro* após a fertilização de oócitos com sêmen selecionado por centrifugação em gradiente de densidade de Percoll ou por migração ascendente (*swim-up*). No experimento 1 a seleção espermática foi realizada usando o gradiente descontínuo de Percoll de 90% e 45% (T1) e o *swim-up* (T2). No experimento 2 foi utilizado, além do gradiente descontínuo, um gradiente contínuo de densidade de Percoll de 67,5%, e tempos de centrifugação de 5 e 10 minutos, totalizando 4 tratamentos (TI = contínuo 5 minutos, TII = descontínuo 5 minutos, TIII = contínuo 10 minutos e TIV = descontínuo 10 minutos). A sexagem dos embriões foi realizada através da técnica da reação em cadeia da polimerase (PCR). No T1 (n=185) foram obtidos 48,65% (n=90) de embriões masculinos e 51,35% (n=95) de femininos e no T2 (n=142) 58,45% (n=83) foram machos e 41,55% (n=59) fêmeas. No experimento 2, a percentagem de embriões masculinos e femininos no TI (n=93), TII (n=70), TIII (n=82) e TIV (n=82) foi de 49,46% (n=46) e 50,54% (n=47), 57,14% (n=40) e 42,86% (n=30), 36,59% (n=30) e 63,41% (n=52), e 48,78% (n=40) e 51,22% (n=42), respectivamente. Não houve alteração na percentagem de machos e fêmeas nos tratamentos dos experimentos 1 e 2 quando estes tratamentos foram comparados individualmente com a percentagem teoricamente esperada de 50% de cada sexo. Também não houve alteração na percentagem de machos e fêmeas na comparação entre os dois tratamentos do experimento 1 e entre os quatro tratamentos do experimento 2.

Palavras-chave: seleção espermática; gradiente de densidade; migração ascendente; FIV; cromossomos X e Y; DNA.

ABSTRACT

Masters Dissertation in Veterinary Medicine
Graduate Program in Veterinary Medicine
Federal University of Santa Maria, RS, Brazil

SEXING *IN VITRO* PRODUCED BOVINE EMBRYOS WITH SEMEN SELECTED BY PERCOLL OR SWIM-UP

AUTHOR: CAROLINE ANTONIAZZI WOLF
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Santa Maria, February 27th, 2007.

Preimplantation genetic diagnosis (PGD) is becoming a current issue in animal reproduction biotechnology due to economical reasons. Predetermining the sex of offspring is one example of PGD. This study aimed to determine the percentage of male and female bovine embryos *in vitro* produced after oocyte fertilization with Percoll density gradient centrifugation or with self-migration (swim-up) selected semen. In experiment 1, sperm selection was performed by 90%-45% discontinuous Percoll density gradient centrifugation (T1) and swim-up (T2). In experiment 2, along side the discontinuous gradient, a 67.5% continuous density gradient, and centrifugation time of 5 and 10 minutes were used. A total of 4 treatment groups was defined (TI = continuous, 5 minutes, TII = discontinuous, 5 minutes, TIII = continuous, 10 minutes and TIV = discontinuous, 10 minutes). Polymerase chain reaction (PCR) was used to determine the sex of the embryos. T1 (n=185) resulted in 48.65% (n=90) male embryos and 51.35% (n=95) female embryos and T2 (n=142) in 58.45% (n=83) male and 41.55% (n=59) female embryos. In experiment 2, the percentages of male and female embryos obtained in TI (n=93), TII (n=70), TIII (n=82) and TIV (n=82) were 49.46% (n=46) and 50.54% (n=47), 57.14% (n=40) and 42.86% (n=30), 36.59% (n=30) and 63.41% (n=52) and 48.78% (n=40) and 51.22% (n=42), respectively. There was no difference on the percentage of males and females in all treatment groups from experiments 1 and 2 when these were individually compared to the expected percentage of 50% of each sex. There was also no difference in male and female embryo percentage between treatment groups in experiments 1 and 2.

Key words: sperm selection; density gradient; self-migration; IVF; X and Y chromosomes; DNA.

LISTA DE ILUSTRAÇÕES

[1] *In vitro* produced bovine embryos after sperm selection by 90%-45% discontinuous Percoll density gradient centrifugation in experiment 1.
[2] DNA extracted from bovine (female and male) leukocytes.

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SUMÁRIO

1 INTRODUÇÃO

A escolha do sexo da prole tem sido uma meta do homem há muito tempo. Na antiguidade acreditava-se que o sexo era determinado pela posição dos testículos e que a amputação do testículo esquerdo resultava no nascimento de indivíduos do sexo masculino, preferido na época. Nos dias de hoje, além dos valores sociais, existe a preocupação em evitar a concepção de uma criança com alguma doença genética recessiva ligada ao cromossomo X (CRAN & JOHNSON, 1996).

Nas espécies animais de produção a escolha do sexo resulta em vantagens econômicas, como ganho genético e otimização do manejo da propriedade. Isso permite aos criadores o planejamento da produção de indivíduos do sexo que gere aumento da produtividade do rebanho. Nos rebanhos bovinos com aptidão leiteira, a produção é dirigida a indivíduos do sexo feminino para reposição do rebanho ou venda. A diminuição no nascimento de machos reduz o prejuízo ocasionado pelo baixo valor dos terneiros, e a produção de leite aumenta devido à seleção genética exercida sobre as reprodutoras. Da mesma forma, em bovinos destinados à produção de carne, as fêmeas são utilizadas na reprodução de animais geneticamente superiores. A escolha de indivíduos machos proporciona um melhor rendimento de carcaça por ocasião do abate.

Técnicas destinadas à seleção do sexo de espermatozóides e embriões pré-implantados vêm sendo desenvolvidas em várias espécies animais. O sucesso da implantação destas técnicas deve-se a eficiência dos programas de inseminação artificial (IA) e de produção *in vitro* (PIV) com subseqüente transferência de embriões (TE). Segundo Johnson (2000), a tecnologia atual de pré-seleção do sexo em animais é baseada na diferença de conteúdo de DNA entre espermatozóides portadores dos cromossomos X e Y. A incorporação desta tecnologia a IA e a fertilização *in vitro* (FIV) aumentará a eficácia desses programas trazendo benefícios para a indústria de produção animal e anulando as perdas originadas pelo descarte de embriões do sexo indesejado na FIV.

Este trabalho teve por objetivo verificar se é possível alterar a percentagem esperada de 50% de cada sexo em embriões bovinos produzidos *in vitro* e, assim,

produzir um maior número de embriões de um determinado sexo de interesse econômico, através de dois métodos de seleção espermática, o de centrifugação em gradiente de densidade de Percoll e o de migração ascendente ou *swim-up.*

2 REVISÃO BIBLIOGRÁFICA

2.1 Métodos de seleção de espermatozóides

Espermatozóides com alta motilidade são selecionados *in vivo* durante a migração no trato reprodutivo feminino. Tecnologias de reprodução assistida (TRA) são aplicadas para simular este processo *in vitro* (CESARI et al., 2006).

Métodos de seleção de espermatozóides são rotineiramente utilizados nos programas de fertilização *in vitro* (FIV) em várias espécies (DODE et al., 2002), como bovinos (CESARI et al., 2006; SAMARDZIJA et al., 2006), eqüinos (ALVARENGA & LEÃO, 2002; NIE et al., 2003), suínos (SUZUKI & NAGAI, 2003), ovinos (PALOMO et al., 1999), caninos (HISHIHUMA & SEKINE, 2004) e humanos (MAKLER et al, 1998; BABBO et al., 1999). Esses métodos são utilizados para separar plasma seminal, crioprotetores (PARRISH et al., 1995), bactérias (KANEKO et al., 1986), *debris* celulares e espermatozóides imóveis dos espermatozóides móveis (HENKEL & SCHILL, 2003). Eles melhoram a qualidade do sêmen (PARRISH et al., 1995) aumentando a percentagem de espermatozóides móveis (SUZUKI et al., 2003) e ao mesmo tempo, iniciam o processo de capacitação espermática (HENKEL & SCHILL, 2003).

Além da FIV, a seleção de espermatozóides também é rotineiramente aplicada com outras TRA como inseminação artificial (IA) e injeção intracitoplasmática de espermatozóides (ICSI) (MAKLER et al., 1998). A fim de explorar ao máximo as vantagens oferecidas pela FIV e as TRA é necessário utilizar os espermatozóides viáveis presentes no sêmen congelado/descongelado (CORREA & ZAVOS, 1996).

Os métodos utilizados para realizar a seleção espermática se baseiam em dois parâmetros, a massa dos espermatozóides e/ou a motilidade dos mesmos (CESARI et al., 2006). A centrifugação em gradiente descontínuo de densidade de Percoll (MAKLER et al., 1998; PALOMO et al., 1999; ALVARENGA & LEÃO, 2002; SUZUKI et al., 2003; OCK et al., 2006) e o método de migração ascendente ou *swim-up* (PARRISH et al., 1986; PARRISH et al., 1995; BABBO et al., 1999; RHEINGANTZ et al., 2002a; MICHAELI et al., 2004) são os métodos mais antigos (CESARI et al., 2006).

A centrifugação em gradiente descontínuo de densidade de Percoll permite a seleção de espermatozóides com maior motilidade e viabilidade quando comparado ao *swim-up* (SUZUKI et al., 2003). Entretanto, Palomo et al. (1999) observaram maior viabilidade espermática após seleção com *swim-up* quando comparado com o gradiente de Percoll. No sêmen bovino congelado/descongelado não foi observada diferença quantitativa ou qualitativa ao se comparar os dois métodos (CORREA & ZAVOS, 1996). Palomo et al. (1999) observaram que a penetração de oócitos, taxa de clivagem, motilidade, integridade do acrossoma e viabilidade celular com reação de acrossoma também não variaram entre os dois métodos de seleção. Embora a centrifugação possa causar dano celular ao espermatozóide, o Percoll é aparentemente seguro e não apresenta efeitos tóxicos (SUZUKI et al., 2003).

2.1.1 Métodos de separação de espermatozóides portadores dos cromossomos X e Y

Durante a meiose nos testículos, os cromossomos sexuais são segregados individualmente nos espermatócitos, e os espermatozóides haplóides carregam o cromossomo X ou o cromossomo Y. Os espermatozóides portadores dos cromossomos X e Y no sêmen mantém a proporção de 1:1 e a fertilização de um oócito haplóide que carrega o cromossomo X, por um ou outro espermatozóide, determina o sexo do embrião (FLAHERTY & MATTHEWS, 1996).

A seleção pré-concepção do sexo tem gerado grande interesse e controvérsias, especialmente no início da década de 1970 quando Ericsson et al. (1973) (apud FLAHERTY & MATTHEWS, 1996) descreveram o enriquecimento de sêmen humano com espermatozóides portadores do cromossomo Y através de passagem por gradientes descontínuos de albumina.

Desde então, os métodos de seleção espermática *swim-up* (CHECK & KATSOFF, 1993; RHEINGANTZ et al., 2006; YAN et al., 2006) e centrifugação em gradiente de densidade de Percoll (KANEKO et al., 1983; KANEKO et al., 1984; CHECK et al., 2000; HOSSEPIAN de LIMA et al., 2000) têm sofrido modificações na tentativa de enriquecer o sêmen de humanos e bovinos com espermatozóides portadores dos cromossomos X ou Y. Os métodos de sexagem de sêmen pressupõem que existam diferenças fundamentais entre os espermatozóides

portadores dos cromossomos X e Y que podem ser exploradas para enriquecer o sêmen com uma população de espermatozóides ou outra. Estas diferenças incluem tamanho e forma, densidade, motilidade e carga elétrica na superfície da membrana (GLEDHILL, 1988; McEVOY, 1992). Cui & Matthews (1993, apud FLAHERTY & MATHEWS, 1996) observaram que o tamanho, perímetro e área da cabeça, comprimento do colo e da cauda eram maiores em espermatozóides humanos portadores do cromossomo X. No entanto, o único método de separação de espermatozóides (de acordo com o cromossomo sexual que carregam) válido e aceito, até hoje, é a citometria de fluxo (JOHNSON, 2000; PARRILLA et al., 2004).

2.1.1.1 Migração ascendente (*swim-up*)

A separação de espermatozóides portadores dos cromossomos X e Y pelo método *swim-up* se baseia na diferença de motilidade que existe entre eles (CESARI et al., 2006). Os espermatozóides portadores do cromossomo Y se deslocam com maior velocidade (McEVOY, 1992) e penetram soluções viscosas com mais facilidade (FLAHERTY & MATHEWS, 1996) que os espermatozóides portadores do cromossomo X. Desta forma, os espermatozóides mais ágeis alcançariam a superfície do meio mais rapidamente. Diversos tempos de incubação do sêmen foram testados em humanos (YAN et al., 2006) e em bovinos (RHEINGANTZ et al., 2006) sem que se observasse alteração significativa na proporção de espermatozóides portadores dos cromossomos X e Y.

2.1.1.2 Centrifugação em gradiente de densidade de Percoll

O processo de separação de espermatozóides por centrifugação em gradiente de densidade se baseia na diferença de densidade que existe entre os espermatozóides portadores dos cromossomos X ou Y. O cromossomo X possui maior quantidade de DNA que o cromossomo Y (PARRILLA et al., 2004). Em bovinos o cromossomo X contém 3,8% mais DNA que o Y (JOHNSON, 2000). A massa seca da cabeça dos espermatozóides é proporcional ao seu conteúdo de DNA (SUMNER & ROBINSON, 1976), portanto, essa diferença (conteúdo de DNA) produz uma diferença na massa e, conseqüentemente, no peso e na densidade dos espermatozóides. Os primeiros relatos de separação de espermatozóides

portadores dos cromossomos X e Y por centrifugação em gradiente de densidade utilizavam o Percoll (KANEKO et al., 1983; KANEKO et al., 1984). O Percoll é um meio comercial composto por uma solução coloidal de partículas de sílica (15-30nm de diâmetro) encobertas por polivinil pirrolidona (SAMARDZIJA et al., 2006), que elevam a gravidade específica do meio para 1,13g/mL (MAKLER et al., 1998).

No preparo do gradiente descontínuo, o Percoll é diluído para obter soluções de densidades diferentes e o gradiente é montado em camadas descontínuas de densidade (KANEKO et al., 1983; KANEKO et al., 1984; HOSSEPIAN de LIMA et al., 2000). O sêmen é depositado sobre o gradiente que é centrifugado para promover a separação dos espermatozóides. O melhor resultado obtido após a separação de espermatozóides utilizando o Percoll na centrifugação em gradiente descontínuo de densidade, até hoje, foi o de Iizuka et al. (1987), que obtiveram 94% de espermatozóides portadores do cromossomo X. Embora este resultado não tenha sido alcançado novamente, Kobayashi et al. (2004) demonstraram que a centrifugação em gradiente descontínuo de densidade de Percoll promove um enriquecimento de espermatozóides portadores do cromossomo X no sêmen.

O gradiente contínuo consiste em uma solução com uma densidade única de Percoll. O processo de separação celular por centrifugação em gradiente contínuo de densidade de Percoll permite a separação de neutrófilos de ovelhas sadias e infectadas com *Anaplasma phagocytophilum.* Woldehiwet et al. (2003) obtiveram separação com mais de 95% de pureza utilizando frações contínuas de densidade de Percoll de 70% e 55%, respectivamente.

2.1.1.3 Citometria de fluxo

Este método também é baseado na diferença de conteúdo de DNA entre os cromossomos sexuais e foi validado pelo nascimento de indivíduos vivos, reanálise laboratorial do conteúdo de DNA do sêmen sexado e por biópsia embrionária para determinação do sexo. No entanto, esta técnica não é viável em muitos sistemas de produção que exigem grande número de espermatozóides para fertilização (JOHNSON, 2000). Outros problemas relacionados com o uso de sêmen sexado por citometria de fluxo são baixa fertilidade, baixa sobrevivência após o congelamento, diminuição das taxas de fertilização, clivagem, blastocistos, prenhez e capacitação

parcial dos espermatozóides (WHEELER et al., 2006). A técnica está sendo aprimorada (STAP et al., 1998) através do aumento da velocidade da seleção, o que permite selecionar um maior número de células (JOHNSON, 2000). A sexagem espermática pela citometria de fluxo envolve a coloração dos espermatozóides com o corante Hoechst 33342 associado ao laser ultravioleta, dois agentes de alto potencial mutagênico (YAN et al., 2006). Além disso, o equipamento (citômetro de fluxo) é caro, o que dificulta a disseminação e ampla utilização da técnica (SEIDEL, 2003).

2.2 Sexagem de embriões pela reação em cadeia da polimerase (PCR)

Os avanços observados na reprodução assistida nas últimas décadas tornaram possível participar do processo de fertilização e manipular embriões antes que eles sejam transferidos para o útero (ALMODIN et al., 2005). A tecnologia de embriões está cada vez mais utilizando o diagnóstico genético pré-implantação (DGP), e a sexagem de embriões foi a primeira e é, até hoje, sua aplicação mais utilizada (GARCIA, 2001). Desde o uso comercial da transferência de embriões (TE) em 1970, a sexagem de embriões anterior à transferência tem sido almejada por muitos criadores de gado (THIBIER & NIBART, 1995).

O sexo dos embriões é determinado na concepção quando um espermatozóide portador do cromossomo X ou Y fertiliza um oócito portador do cromossomo X (FLAHERTY & MATTHEWS, 1996), formando um individuo XX ou XY. Sendo assim, o cromossomo Y está presente somente em seres do sexo masculino. A amplificação de DNA cromossômico masculino (cromossomo Y) é uma maneira prática (BREDBACKA et al., 1995) e precisa (SHEA, 1999; CHRENEK et al., 2001; HASLER et al., 2002) de determinar o sexo dos embriões, através da reação em cadeia da polimerase (PCR) (ALVES et al., 2003). A PCR amplifica, exponencialmente, pequenos fragmentos de DNA de regiões específicas do cromossomo Y. A presença ou ausência dessa amplificação indica o sexo do embrião (SHEA, 1999). Esta técnica tem sido muito utilizada (LOPES et al., 2001; HASLER et al., 2002; ALVES et al., 2003) e consiste em uma simulação do processo de replicação de DNA que ocorre nas células *in vivo*. A enzima DNA polimerase promove a síntese de milhões de cópias de seqüências de DNA presentes somente nas células de embriões masculinos (DESCHAMPS et al., 2000), através do uso de

oligonucleotídeos iniciadores (*primers*) específicos desenhados para amplificar regiões do cromossomo Y (SHEA, 1999; PARK et al., 2001; ALVES et al., 2003). A associação dos *primers* masculinos com outros *primers* (que amplificam seqüências autossômicas de DNA bovino) caracteriza a PCR multiplex (RUFINO et al., 2006).

3 CAPÍTULO 1

Sexing *in vitro* produced bovine embryos with semen selected by Percoll or swim-up

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Sexing *in vitro* produced bovine embryos with semen selected by Percoll or swim-up

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Abstract

Preimplantation genetic diagnosis (PGD) is becoming a current issue in animal reproduction biotechnology due to economical reasons. Predetermining the sex of offspring is one example of PGD. This study aimed to determine the percentage of male and female bovine embryos produced *in vitro* after oocyte fertilization with Percoll density gradient centrifugation or with self-migration (swim-up) selected semen. In experiment 1, sperm selection was performed by 90%-45% discontinuous Percoll density gradient centrifugation (T1) and swim-up (T2). In experiment 2, along side the discontinuous gradient, a 67.5% continuous density gradient, and centrifugation time of 5 and 10 minutes were used. A total of 4 treatment groups was defined (TI = continuous, 5 minutes, TII = discontinuous, 5 minutes, TIII = continuous, 10 minutes and TIV = discontinuous, 10 minutes). Polymerase chain reaction (PCR) was used to determine the sex of the embryos. T1 (n=185) resulted in 48.65% (n=90) male embryos and 51.35% (n=95) female embryos and T2 (n=142) in 58.45% (n=83) male and 41.55% (n=59) female embryos. In experiment 2, the percentages of male and female embryos obtained in TI (n=93), TII (n=70), TIII (n=82) and TIV (n=82) were 49.46% (n=46) and 50.54% (n=47), 57.14% (n=40) and 42.86% (n=30), 36.59%

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(n=30) and 63.41% (n=52) and 48.78% (n=40) and 51.22% (n=42), respectively. There was no difference on the percentage of males and females in all treatment groups from experiments 1 and 2 when these were individually compared to the expected percentage of 50% of each sex. There was also no difference in male and female embryo percentages between treatment groups from experiments 1 and 2.

Key words: sperm selection; density gradient; self-migration; IVF; X- and Y-chromosomes; DNA.

Introduction

Advances in DNA technology enhanced the perspectives of genetic selection in domestic species to increase productivity (Garcia, 2001). Predeterminig the sex of offspring in the dairy and beef cattle industry allows the breeders to plan their production toward a specific sex. The most effective way to achieve this goal is by separating X- from Y- bearing spermatozoa (Stap et al., 1998). According to Cran & Johnson (1996), the only way to effectively separate sperm cell populations is on the basis of their DNA content (Stap et al., 1998). Flow cytometry has been widely used in this purpose (Johnson, 2000; Bodmer et al., 2005; Garner, 2006), however, this technique presents some specific problems, such as broad fluorescence distribution without a distinct X- and Y- peak (Stap et al., 1998).

In attempt to develop a new method for separation of sperm cell populations based on their DNA content, Percoll density gradient centrifugation has been used on human and bovine sperm (Kaneko et al., 1983; Kaneko et al., 1984; Hossepian de Lima et al., 2000). Percoll is a commercial medium composed of colloidal silica particles (15-30nm in diameter) coated with polyvinylpyrrolidone (Samardzija et al., 2006), which increase the specific gravity of the medium to 1.13g/mL (Makler et al.,

1998). It is also used for the isolation of bacteria (Leuschner et al., 1999), neutrophils (Woldehiwet et al., 2003), viruses (Hanabusa et al., 2000) and subcellular particles (Swales & Wright, 2000; Domart-Coulon et al., 2001; Sheoran et al., 2005).

Discontinuous 90%-45% Percoll density gradient centrifugation is widely used to increase sperm motility (Parrish et al., 1995; Alvarenga & Leão, 2002; Suzuki et al., 2003) for *in vitro* fertilization (IVF) and artificial insemination (AI). Swim-up is another commonly used sperm selection method for IVF (Parrish et al., 1986; Parrish et al., 1995; Palomo et al., 1999), because it is the most simple and cheapest method (Rheingantz et al., 2002b; Henkel & Schill, 2003) which selects highly motile spermatozoa that reach the medium surface after incubation.

Taking into account the simplicity and practicity of these sperm selection methods, the aim of the present study was to determine if semen selection by swim-up, 90%-45% discontinuous Percoll density gradient centrifugation and 67.5% continuous Percoll density gradient centrifugation alter the percentage of male and female *in vitro* produced bovine embryos. With the same purpose, 5 and 10 minute centrifugation times were tested on both Percoll gradients.

Materials and Methods

Experiment 1 –

Oocyte recovery

Immature oocytes were recovered from bovine ovaries obtained from a local abbatoir and kept in sterile 0.9% saline solution at 30 to 35^{0}C. Cumulus-oocyte-complexes (COCs) were recovered by aspiration from 2 to 8mm diameter follicles using an 18ga needle attached to a vacuum pump (20mL/minute pression). COCs search, selection (De Loos et al., 1989) and maintenance were performed in folicular

fluid according to Lehmkuhl et al. (2000). Only COCs that had homogeneous cytoplasm and were completely covered by compact and unexpanded cumulus oophorus cells were selected and randomly distributed in the experimental treatment groups.

In vitro maturation (IVM) of oocytes

Immature oocytes were placed on Nunc® plates. Each well contained 400µL of TCM 199 maturation medium supplemented with 26.2mM $NaHCO_3$, 25mM Hepes, 0.2mM sodium pyruvate, 0.01UI/mL FSH, 5µg/mL LH and 10% estrous cow serum (ECS). The medium was covered with mineral oil and previously stabilized at 38^0C for at least 20 minutes. Maturation was performed for 20 to 24 hours in a stove at 38^0C, 5% CO_2 atmosphere and 95% humidity. On each routine, oocytes (average of 30 oocytes/well) were equally distributed between experimental groups.

Sperm preparation and *in vitro* fertilization (IVF)

After maturation, oocytes were transfered to new and sterile Nunc® plates containing 400µL of Talp-Fert fertilizing medium (supplemented with 50µg/mL heparin), covered with mineral oil and previously stabilized at 38^0C for at least 20 minutes. Frozen semen from one *Bos taurus taurus* bull with proven IVF fertility was thawed exposed to 10 seconds at room temperature and 20 seconds immersed in a water bath at 37^0C.

A 90%-45% discontinuous Percoll density gradient (Parrish et al., 1995) used for treatment group 1 (T1) was prepared using 1mL of each Percoll fraction, totalizing 2mL gradient in a conic 15mL tube. For sperm selection, 500µL of thawed semen were layered on the top of the gradient and centrifuged at 120 x g for 10 minutes.

The supernatant was discarded, the pellet recoverd, ressuspended in 4mL of Talp-Sperm and centrifuged at 50 x g for 10 minutes to remove residual Percoll. Two-hundred microliters from the washed pellet were recovered.

In group 2 (T2), the self-migration (swim-up) technique described by Parrish et al. (1986) was used. Three semen fractions of 100µL each were placed on the botton of three conic tubes containing 1mL of Talp-Sperm medium. Tubes were kept at 38^0C immersed in a water bath for 1 hour. Eight-hundred microliters from the upper Talp-Sperm medium layer were transfered to 4mL Talp-Sperm and centrifuged for 10 minutes at 50 x g. Two-hundred microliters from the pellet were recovered.

Fertilization was performed by the addition of 1 to $2x10^6$ spermatozoa/mL to the fertilizing medium containing oocytes. The gametes were incubated for 18 to 22 hours under the same conditions of maturation.

Embryo culture

Presumptive zygotes were desnuded by mechanical agitation (vortex) for 1.5 minutes and washed with TCM-Hepes. Then, they were transfered to SOFaaci culture medium (supplemented with 5% ECS and 0.022mg/mL sodium pyruvate) covered with mineral oil, previously stabilized. On the next day, day 2 (D2) of culture (day zero (D0) = IFV day), fertilization ratio was evaluated by identifying cellular division (cleavage). Embryos were cultured for four days in Foil Bag System according to Vajta et al. (1997) under an atmosphere of 5% CO_2, 5% O_2 and 90% N_2. On the fifth (D5) day of culture, embryo morphology was evaluated and they were classified according to their developmental stage and prepared for sexing.

Embryo digestion

Morulae were washed with BSA free-PBS, with polyvinilalcohol (PVA). Embryos were individually transfered to 0.2mL PCR tubes previously filled with 10µL of ultrapure water, immersed in liquid nitrogen for 30 seconds and stored at -20^{0}C, pending DNA extraction. For DNA extraction, embryos were treated with proteinase K (16mg/mL) at 37^{0}C for 1 hour and 98^{0}C for 10 minutes. Embryo DNA was divided into two samples for two PCR rounds (reaction 1 and 2). Reaction 2 confirmed results from reaction 1.

DNA extraction from bovine leukocytes

To obtain DNA control samples, 5mL of blood were collected from bovine (male and female) through jugular vein puncture. Blood was kept in 15mL glass tubes with 100µL of 5% EDTA and stored at -20^{0}C. For extraction, 10µL of 20% SDS and 15µL of proteinase K (1mg/mL) were added to 100µL of blood and the mixture was incubated at 55^{0}C for 2 hours. Phenol was added (v:v) to the mixture and the tube was vortexed and then centrifuged for 5 minutes at 8000 x g. This procedure was repeated with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1). The supernatant was mixed with 0.5 volume of 7.5M ammonium acetate and 2 volumes of 95% cold ethanol and centrifuged for 10 minutes at 8000 x g. The pellet was washed twice with 250µL of 70% cold ethanol and ressuspended in 500µL of ultrapure water.

Sexing by PCR technique

Three specific primer pairs designed to amplify only bovine DNA were used. One primer pair, called BOV, amplifies a 280bp region of an autossomic gene from

bovine DNA, which sequences are 5' – AGG TCG CGA GAT TGG TCG CTA GGT CAT GCA – 3' and 5' – AAG ACC TCG AGA GAC CCT CTT CAA CAC GT – 3'. Y1 and Y2 primer pairs amplify a 210bp and a 250bp region, respectively, only from male bovine DNA. Y1 sequences are 5' – CCT CCC CTT GTT CAA ACG CCC GGA ATC ATT 3' and 5' – TGC TTG ACT GCA GGG ACC GAG AGG TTT GGG – 3 and Y2 sequences are 5' – ATC AGT GCA GGG ACC GAG ATG – 3' and 5' – AAG CAG CCG ATA AAC ACT CCT T – 3'. BOV and Y1 primers were used in the same PCR round (reaction 1), while Y2 primers were used in reaction 2. Reaction conditions were: 75mM Tris-HCl pH 9, 50mM KCl, 20mM $(NH_4)_2SO_4$, 1.5mM $MgCl_2$, 160µM dNTPs, 0.17µM of each primer and 1U Taq DNA polymerase. Reactions had a final volume of 30µL. Positive (male and female bovine DNA extracted from leukocytes) and negative (water) controls were used in all PCR reactions. DNA from all samples was denatured at 94^0C for 5 minutes in a PTC-100^{TM} thermal cycler. Reaction 1 ran 40 cycles at 94^0C for 60 seconds, 58^0C for 30 seconds and 72^0C for 60 seconds, followed by an additional time of 7 minutes at 72^0C. Reaction 2 ran 38 cycles at 94^0C for 60 seconds, 58^0C for 60 seconds and 72^0C for 60 seconds, followed by an additional 7 minutes at 72^0C.

Sex identification

Amplification products were loaded on 2% agarose gels stained with ethidium bromide and visualized under UV light. Electrophoresis conditions were 90V, 100W, 150mA for 1.5 hours. Female embryos were identified when only a 280bp band was detected on the first PCR round (reaction 1) and no band on the corresponding reaction 2. The presence of two bands (280bp and 210bp) on reaction 1 indicated a

male embryo, and this result only was confirmed when a 250bp band was seen on the corresponding reaction 2 (figure 1).

Statistical analysis

Experimental design was of random blocks, with each routine being considered one block. All treatment groups were performed simultaneously and data processed by χ^2 (chi-square) test with 5% significance. Male and female percentages obtained on each group were compared with the theoretical percentage of 50% of each sex. Male and female percentages were also compared between treatment groups. Data were processed using the SAS statistical program (1996).

Experiment 2 –

Oocyte recovery, IVM and sperm preparation were performed as described in experiment 1. IVM and IVF, however were performed in 200µL drops of maturation and fertilizing medium (average of 24 oocytes/drop), respectively, with each drop representing one treatment group.

Sperm selection was performed by discontinuous Percoll density gradient centrifugation (same as experiment 1) and continuous Percoll density gradient. A total of four treatment groups was set: TI, TII, TIII and TIV. Continuous gradient was obtained homogenizing the tube contents in order to mix 90%-45% Percoll fractions, resulting in 2mL of a 67.5% gradient. Gradients were centrifuged at 200 x g. Treatment groups distribution is presented in table 1.

The other steps were performed according to experiment 1. The only change made was that the embryos stayed for 6 days in Foil Bag System and morulae and

blastocysts were recovered from SOFaaci medium at the end of 7 days (D7) of culture.

Results

In experiment 1, 327 (65.4%) out of 500 embryos had their sex determined by PCR. From these, 185 (56.57%) were produced by IVF with Percoll (T1) and 142 (43.42%) with swim-up (T2). The percentages of male and female embryos obtained in the two treatment groups and the comparison between them are presented in table 2.

In experiment 2, 345 embryos were produced *in vitro*, from which 329 (95.36%) were submited to PCR. From these embryos, 327 (99.4%) had their sex determined by PCR. Results of the four treatment groups and the comparison among them are presented in table 3.

Discussion

In experiment 1, even though swim-up selected sperm resulted in more male embryos (58.45%) neither selection methods showed a deviation on the 50% expected percentage for male and female embryos. In comparison with discontinuous Percoll density gradient, swim-up showed a tendency to produce more ($p<0.1$) male embryos (table 2). As described by Johnson (2000), the Y-chromosome is lighter and smaller than the X-chromosome. The DNA content difference between them is quantified in 3.8%. In addition, McEvoy (1992) believes that Y- bearing sperm is faster than X- bearing sperm. Rheingantz et al. (2002a), performing swim-up with 45 minute incubation time, observed a signifficant male and female ratio deviation ($p<0.05$) toward male embryos. These data suggest that swim-up favours Y- bearing

sperm selection, which migrate faster to reach medium surface. However, in 2006, Rheingantz et al. could not repeat the results after performing swim-up with 15, 45 and 90 minutes of incubation period.

Discontinuous 90%-45% Percoll density gradient centrifugation did not show signifficant difference ($p>0.05$) on the percentage of male and female embryos (table 2). This may be explained by the small gradient volume used (2mL). The mechanism of enrichment of X- bearing spermatozoa by discontinuous Percoll gradients is not fully understood (Kobayashi et al., 2004). Even though there is controversy, it is believed that separation occurs as a result of X- and Y- bearing spermatozoa buoyant density difference. When Percoll density gradients are prepared in discontinuous fractions, sperm layered on the top of the gradient (less Percoll concentration) naturally penetrate it. The amount of penetration depends on sperm mass and motility. However, when a gradient is centrifuged the effect of sperm motility is minimized and their mass difference effect is maximized (McEvoy, 1992). It makes the heavier spermatozoa reach the bottom faster; however, the separation threshold between X- and Y- bearing bovine sperm is small because their difference in DNA content is only 3.8% (Johnson, 2000). Using flow cytometry, Johnson (2000) observed that in species where the difference in DNA content is greater, such as the *Chinchilla langier* (7.5%), a 100% pure selection is possible, but in species where this diference is smaller, like human beings (2.8%), purity decreases. In cattle the difference (3.8%) is close to the minimum (3.5%) necessary for separation to occur (Johnson, 2000). The use of larger volume gradients in order to make spermatozoon penetration more difficult could be an alternative. Smaller volumes (1 to 4mL), may not be enough to promote separation. Greater volumes (over 7mL) and higher Percoll concentrations (close to 90%) would turn the swimming down of lighter sperm

more difficult. Although they are faster (McEvoy, 1992), their mass is smaller and this effect is maximized during centrifugation. Supporting the hypothesis of larger volumes Hossepian de Lima et al. (2000), using 8 and 12mL gradients obtained about 75% females after IVP of bovine embryos with Percoll selected sperm.

Another factor is the speed of sperm penetrating the Percoll density gradient. Heavier spermatozoa should settle down faster than lighter spermatozoa, therefore centrifugation time could positively influence X- bearing sperm moving down the gradient. The shorter the centrifugation time, less time the Y- bearing sperm (lighter ones) would have to reach the bottom towards highest Percoll concentration (90%). Centrifugation time used in the experiment 1 was 10 minutes and it probably exceeded the time needed to separate sperm of different mass with the tested gradients.

In experiment 2, there was no difference on male and female embryo percentages in none of the four treatments (table 3) when compared to the 50% expected percentage for both sexes. The same happened when treatments were compared to each other. The only treatment group that showed a tendency ($p<0.1$) to produce more female embryos (63.4%) was TIII where semen was selected by continuous Percoll density gradient centrifugation for 10 minutes (table 3). Continuous Percoll density gradients have already been used to separate or isolate other biological structures (Leuschner et al., 1999; Sheoran et al., 2005; Hanabusa et al., 2000), but have not yet been used to separate X- from Y- bearing sperm. Nevertheless, Woldehiwet et al. (2003) could isolate leukocytes from normal and infected sheep (with *Anaplasma phagocytophilum*) attaining high purity (>95%) using 70% and 55% continuous Percoll density gradients, respectively. These data suggest

that continuous Percoll densities can be further explored in an attempt to develop other methods to separate different weight sperm cell populations.

Conclusions

It can be concluded that neither the sperm selection methods (Percoll discontinuous (90%-45%) and continuous (67.5%) gradient centrifugation and swim-up) nor the centrifugation times (5 and 10 minutes) used in this study were capable of altering the percentage of male and female embryos after *in vitro* production of bovine embryos.

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Table 1 – Distribution of treatment groups based on Percoll gradient and centrifugation time in experiment 2.

Treatments	Percoll density gradient	Centrifugation time (minutes)
TI	Continuous 67.5%	5
TII	Discontinuous 90%-45%	5
TIII	Continuous 67.5%	10
TIV	Discontinuous 90%-45%	10

Table 2 – Percentage of male and female embryos obtained in experiment 1 after discontinuous Percoll density gradient centrifugation (90%-45%; T1) and swim-up (T2) compared with the expected percentage of 50% of each sex and between treatments.

Treatments	Males n (%)	Females n (%)	Embryos n	χ^2	p>0,05
T1	90 (48.65)	95 (51.35)	185	0.0676	0.7949
T2	83 (58.45)	59 (41.55)	142	2.0442	0.1529
Total	173 (52.90)	154 (47.09)	327	3.0980	0.0784

Table 3 – Percentage of male and female embryos obtained in experiment 2 after continuous (67.5% for 5 (TI) and 10 (TIII) minutes) and discontinuous (90-45%; for 5 (TII) and 10 (TIV) minutes) Percoll density gradient compared with the expected percentage of 50% of each sex and among treatments.

Treatments	Males n (%)	Females n (%)	Embryos N	χ^2	p>0,05
TI	46 (49.46)	47 (50.54)	93	0.0054	0.9415
TII	40 (57.14)	30 (42.86)	70	0.7179	0.3968
TIII	30 (36.59)	52 (63.41)	82	3.0053	0.0830
TIV	40 (48.78)	42 (51.22)	82	0.0244	0.8759
Total	156 (47.71)	171 (52.29)	327	6.7166	0.0815

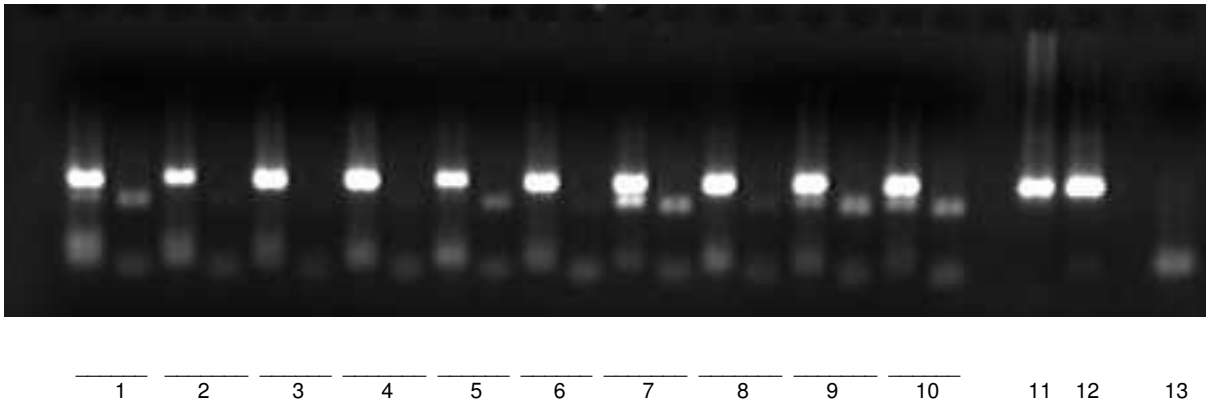

Figure 1 – 2% agarose gel electrophoresis of bovine male and female embryos[3]. Lanes 1, 5, 7, 9 and 10: male embryos (280bp and 210bp bands in reaction 1 and 250bp band in reaction 2) and lanes 2, 3, 4, 6 and 8: female embryos (280bp band in reaction 1 and no band in reaction 2). Reaction 1 controls – lanes 11 and 12: bovine female and male DNA[4], respectively and lane 13: water.

[3] *In vitro* produced bovine embryos after sperm selection by 90%-45% discontinuous Percoll density gradient centrifugation in experiment 1.
[4] DNA extracted from bovine (female and male) leukocytes.

4 CONCLUSÕES

Com base nos dados obtidos neste estudo, conclui-se que não é possível alterar a percentagem de machos e fêmeas na produção *in vitro* de embriões bovinos após fertilização com sêmen selecionado pelos métodos de centrifugação em gradiente descontínuo de densidade de Percoll nas concentrações de 90% e 45% durante 5 e 10 minutos e gradiente contínuo de densidade de Percoll de 67,5% de concentração por 5 e 10 minutos e de migração ascendente ou *swim-up*.

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