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Cryopreservation of boar semen in 0.5mL straws at low spermatozoa concentration is better than high concentration to maintain sperm viability[1]

Gisele M. Ravagnani[2], Mariana A. Torres[2], Diego F. Leal[2], Simone M.M.K. Martins[2], Frederico O. Papa[3], José A. Dell'Aqua Junior[3], Marco A. Alvarenga[3] and André F.C. Andrade[2]*

ABSTRACT.- Ravagnani G.M., Torres M.A., Leal D.F., Martins S.M.M.K., Papa F.O., Dell'Aqua Junior J.A., Alvarenga M.A. & Andrade A.F.C. 2018. **Cryopreservation of boar semen in 0.5mL straws at low spermatozoa concentration is better than high concentration to maintain sperm viability.** *Pesquisa Veterinária Brasileira 38(9):1726-1730*. Núcleo de Pesquisa em Suínos, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Pirassununga, SP 13635-900, Brazil. E-mail: andrefc@usp.br

To date, no studies have been performed evaluating the effect of boar spermatozoa concentration in 0.5mL freezing straws, leading us to examine this question. Each sperm-rich fraction of the ejaculate (n=25) was diluted at five different sperm concentrations (100, 200, 300, 600 and 800 x 10^6 spermatozoa/mL), packaged in 0.5mL straws, and subsequently frozen. After thawing, the sperm from all of treatment groups were analyzed to determine motility characteristics using a sperm class analyzer (SCA-CASA), and their plasma and acrosomal membrane integrity, mitochondrial membrane potential, sperm membrane lipid peroxidation and fluidity were analyzed by flow cytometry. An increase in spermatozoa concentration above 300x10^6 spermatozoa/mL in a 0.5mL straw impaired ($p<0.05$) the total and progressive motility, curvilinear velocity, straight-line velocity, linearity and beat cross frequency. However, the plasma and acrosomal membrane integrity, mitochondrial membrane potential, membrane lipid peroxidation and fluidity were not influenced ($p>0.05$) by high spermatozoa concentrations at freezing. Therefore, to increase spermatozoa survival and total and progressive motility after thawing, boar spermatozoa should be frozen at concentrations up to 300x10^6 spermatozoa/mL.

INDEX TERMS: Cryopreservation, boar semen, spermatozoa concentration, sperm viability, cryoinjury, cryocapacitation, swine.

RESUMO.- [**A manutenção da viabilidade do sêmen suíno criopreservado em palhetas de 0,5mL em baixas concentrações espermáticas é melhor do que em altas concentrações.**] Até o momento, não foram realizados estudos que avaliassem o efeito da concentração de espermatozoides/mL em palhetas (0,5mL) para a criopreservação, levando-nos a analisar esta questão. Cada fração-rica do ejaculado (n=25) foi diluída em cinco diferentes concentrações de espermatozoides (100, 200, 300, 600 e 800x10^6 espermatozoides/mL), envasadas em palhetas de 0,5mL e posteriormente congeladas. Após a descongelação, os espermatozoides de todos os tratamentos foram avaliados a fim de determinar as características de motilidade usando um sistema de análise computadorizada dos espermatozoides (SCA-CASA). A integridade das membranas plasmática e acrosomal, o potencial de membrana mitocondrial, a peroxidação lipídica e a fluidez da membrana foram analisadas por citometria de fluxo. O aumento na concentração de espermatozoides acima de 300x10^6 espermatozoides/mL diminuiu ($p<0,05$) a motilidade total e progressiva, velocidade curvilínea, velocidade linear, linearidade e frequência de batimento. No entanto, a integridade da membrana plasmática e acrosomal, potencial de membrana mitocondrial, peroxidação lipídica e fluidez de membrana não foram influenciados

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[2] Núcleo de Pesquisa em Suínos, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo (USP), Pirassununga, SP 13635-900, Brazil. *Corresponding author: andrefc@usp.br

[3] Departamento de Reprodução Animal e Radiologia Veterinária, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista (Unesp), Botucatu, SP 18618-970, Brazil.

(p>0,05) por altas concentrações de espermatozoides durante a criopreservação. Portanto, a fim de melhorar a sobrevivência dos espermatozoides suínos e a motilidade total e progressiva após a descongelação, os espermatozoides suínos devem ser congelados a concentrações não superiores a 300x10^6 espermatozoides/mL.

TERMOS DE INDEXAÇÃO: Sêmen suíno, criopreservão, crioinjúria, concentração espermática, criocapacitação, suíno.

INTRODUCTION

Frozen-thawed boar semen exhibits fertility rates lower than that of cooled semen because cryopreservation leads to significant loss of spermatozoa viability (Hernández et al. 2007). Thus, an excess of spermatozoa (3 to 5 billion sperm) are used to perform artificial insemination (AI) with frozen-thawed boar semen to guarantee a higher live cell proportion (Ekwall et al. 2007), which requires more thawed straws when performing a single insemination. However, cryopreservation of a high concentration of spermatozoa with good integrity and functionality would allow the use of a smaller number of straws when performing AI facilitating its implementation on swine industry.

On the other hand, the cryopreservation process leads to the formation of extracellular ice crystals and channels of unfrozen medium, as demonstrated by Mazur (1984) and Rodríguez-Martínez & Wallgren (2011). Some extracellular water (approx. 15%) is present in the channels of unfrozen medium (Amann & Pickett 1987) such that during freezing, the spermatozoa are encapsulated in these channels, increasing post-thawed cell survival (Mazur 1984, Ekwall 2009). Furthermore, an increase in spermatozoa concentration up to the limit of channels of unfrozen medium could reduce the possibity of spermatozoa be encapsulated in these channels. Thus, an ideal spermatozoa concentration to cryopreservation is crucial to ensure spermatozoa encapsulation on channels of unfrozen medium increasing post-thawed cell survival.

Thus, we sought to determine the effects of different spermatozoa concentration of frozen-thawed boar semen in 0.5mL straws on the kinetic parameters, plasma and acrosomal membrane integrity, mitochondrial potential, membrane lipid disorder and peroxidation in cryopreserved boar.

MATERIALS AND METHODS

Ethics statement. The experiment was conducted at Swine Research Center, School of Veterinary Medicine and Animal Science, University of São Paulo (USP), in compliance with Ethics Principles in Animal Experimentation, being approved by the the Ethics Committee for the use of Animals in the School of Veterinary Medicine and Animal Science, USP, under protocol 3066/2013.

Reagents and chemicals. Semen extender medium (Botu-Sui®) was purchased from Biotech-Botucatu- Ltd/ME (Botucatu, SP, Brazil). The fluorescent probes Hoechst 33342, C11-BODIPY581/591, Yo-Pro-1 and Merocyanine 540 were purchased from Molecular Probes (Eugene, OR, USA). Unless otherwise stated, all other chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA).

Semen collection, raw semen evaluation, freezing and semen thawing. Five whole sperm-rich fractions were obtained from five cross-bred boars (n=25) using the gloved-hand technique. Only ejaculates with more than 70% of total motility and 80% of normal spermatozoa were used. After the initial raw semen analysis, the semen was diluted (Botu-Sui®) at five different concentrations using a Neubauer Hemocytometer (100x10^6 spermatozoa/mL, 200x10^6 spermatozoa/mL, 300x10^6 spermatozoa/mL, 600x10^6 spermatozoa/mL and 800x 10^6 spermatozoa/mL) and stored in 0.5mL straws (IMV, Laigle, France). The straws were cryopreserved in an automatic machine (TK 3000, TK Tecnologia em Congelação Ltda, Uberaba, Brazil) and cooled at a rate was -0.5°C/min from 25°C to 5°C. The freezing rate was -20°C/min from 5 to -120°C. Subsequently, the straws were immersed in liquid nitrogen at -196°C and stored in goblets within cryogenic tanks (Torres et al. 2016a). Two straws per ejaculate and treatment were thawed in a water bath at 37°C for 30 seconds and subsequently pooled. The thawed semen was diluted to a final concentration of 25x10^6 spermatozoa/mL in a freezing extender.

Computer-assisted sperm analysis (CASA). A sample aliquot (5µL) was placed on a pre-warmed (37°C) cover slide and evaluated by phase contrast microscopy (Nikon, Model Eclipse 80i) at 100x magnification. The SCA (Microptics® - Barcelona/Spain) was pre-adjusted to analyze swine semen, and five fields were analyzed to evaluate the total (MOT) and progressive motility (MOP), curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness (STR), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF).

Flow cytometry analysis. The samples for staining and flow cytometry analysis were diluted (5x10^6 spermatozoa/mL) in TALP medium and stained with Hoechst 33342 (H342, 33µg/mL in 150µL, PMT–photomultiplier tubes with a 450±10nm band pass filter) for 10 min at 37°C, this probe was used to exclude particles with similar scatter properties as spermatozoa from analysis (De Andrade et al. 2012). Samples were analyzed in a BD FACSAria flow cytometer (Becton Dickinson, San Jose, CA, USA) controlled by BD FACSDiva 6.0 software (Becton Dickinson).

Frozen-thawed boar semen was analyzed to simultaneous integrity of plasma, acrosomal membranes and mitochondrial potential by flow cytometry (Torres et al. 2016b). For this propose, following probes association was used: Hoechst 33342 (H342), propidium iodide (PI -10µg/mL), *Pisum sativum* agglutinin conjugated to FITC (PSA-13.4µg/mL) and JC-1 (4.08µM). The plasma (IP) and acrosomal membrane (IA) integrity and mitochondrial membrane potential (HM) were also assessed individually.

Peroxidation of plasma membrane lipids was performed with the same protocol described by De Andrade et al. (2012) by the association of H342, C11- BODIPY$^{581/591}$ probe (3.3µg/mL) and PI (10µg/mL).

Plasma membrane fluidity was performed by the association of H342, Yo-Pro-1 (25nM) and Merocyanine 540 (2,7µM) and evaluated by flow crytometry cytometry (Rathi et al. 2001).

Statistical analysis. The data were analyzed using the MIXED procedure in SAS software (Statistical Analisys System 2002) according to a randomized block that contained the treatments as the main factor. Each boar (n=5) was considered one block, and the experimental unit was 1/5 of the ejaculate. The effects of the treatments were evaluated using the PDDIF test. The effects were considered significant when p<0.05. The results are expressed as the means ± SD.

RESULTS

Concentrations greater than 300x10^6 spermatozoa/mL were detrimental (p<0.05) to total and progressive motility. Concentrations of up to 600x10^6 spermatozoa/mL were

deleterious (p< 0.05) to VSL, VAP and BCF. The LIN and STR remained stable at concentrations of up to 200x10^6 spermatozoa/mL (p>0.05) and decreased (p<0.05) at 300 x10^6 spermatozoa/mL and higher (Table 1).

None of the concentrations of boar semen used for cryopreservation were harmful (p>0.05) to IPIAH (spermatozoa with simultaneous plasma, acrosomal membranes integrity and high mitochondrial potential), IP, IA or HM spermatozoa population (Table 2).

Lower (p<0.05) levels of lipid peroxidation were observed in the cryopreserved samples at 100x10^6 spermatozoa/mL, and the levels remained unchanged (p>0.05) at concentrations up to 200x10^6 spermatozoa/mL. The spermatozoa frozen at 600x10^6 spermatozoa/mL led to higher (p<0.05) levels of lipid peroxidation (Table 3).

The plasma membrane remained stable (p>0.05) regardless of the spermatozoa concentration and was unaffected at concentrations up to 800x10^6 spermatozoa/mL (Table 3).

DISCUSSION

The cryopreservation of spermatozoa still remains a challenge, as all of the spermatozoa compartments are subjected to harmful freezing effects and only approximately half of spermatozoa survive after thawing (Vadnais & Althouse 2011, De Andrade et al. 2012). Thus, the cryopreservation of high concentrations of spermatozoa might be a useful method to increase the absolute number of viable spermatozoa when inseminating frozen-thawed semen samples (Alvarez et al. 2012).

Cryopreservation of high concentrations of boar semen was harmful to sperm kinematic parameters, and both the total and progressive motility were decreased at concentrations higher than 300x10^6 spermatozoa/mL, whereas the VSL, VAP and BCF were only impaired at 800x10^6 spermatozoa/mL. In rams, the total spermatozoa motility appears to be less affected by high concentrations and did not exhibit additional harmful effects at 800x10^6 spermatozoa/mL, unlike the progressive motility, in which a deleterious effect was observed at

Table 1. Mean ± SD of kinematics sperm of frozen-thawed boar semen cryopreserved at five spermatozoa concentrations in 0.5mL straws

Sperm characteristics	Treatments (x 10^6 spermatozoa/mL)				
	100	200	300	600	800
MOT (%)	20.31 ± 10.82 ^{a}	19.87 ± 9.53 ^{a}	20.70 ± 9.25 ^{a}	14.60 ± 7.80 ^{b}	9.90 ± 5.82 ^{c}
MOP (%)	13.45 ± 9.02 ^{a}	12.69 ± 8.52 ^{a}	12.40 ± 7.21 ^{a}	7.86 ± 5.11 ^{b}	4.42 ± 3.63 ^{c}
VCL (µm/s)	56.82 ± 15.18	60.50 ± 18.21	59.90 ± 12.85	55.52 ± 15.43	44.39 ± 15.60
VSL (µm/s)	31.28 ± 11.35 ^{a}	33.80 ± 12.92 ^{a}	31.13 ± 9.55 ^{a}	26.23 ± 13.07 ^{a}	20.76 ± 12.10 ^{b}
VAP (µm/s)	39.02 ± 13.18 ^{a}	41.57 ± 14.85 ^{a}	40.10 ± 10.85 ^{a}	34.11 ± 15.67 ^{a}	25.47 ± 13.11 ^{b}
LIN (%)	54.00 ± 8.78 ^{a}	54.35 ± 10.61 ^{a}	49.36 ± 10.50 $^{a\,b}$	49.10 ± 11.24 $^{a\,b}$	44.92 ± 14.34 ^{b}
STR (%)	81.26 ± 5.46 ^{a}	77.89 ± 8.95 ^{a}	77.61 ± 6.97 $^{a\,b}$	74.72 ± 9.45 $^{a\,b}$	72.64 ± 11.54 ^{b}
ALH (µm)	2.60 ± 0.46	2.57 ± 0.57	2.81 ± 0.38	2.70 ± 0.49	2.50 ± 0.62
BCF (HZ)	9.35 ± 1.21 ^{a}	8.80 ± 2.02 ^{a}	8.85 ± 2.09 ^{a}	7.78 ± 2.68 $^{a\,b}$	6.46 ± 3.34 ^{b}

MOT = Total motility, MOP = progressive motility, VCL = curvilinear velocity, VSL = straight-line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, ALH = lateral amplitude of head displacement, BFC = beat cross frequency; ^{a,b} Values within a row with different superscripts differ significantly at P<0.05.

Table 2. Mean ± SD of spermatozoa membranes integrity of frozen-thawed boar semen cryopreserved at five spermatozoa concentrations in 0.5mL straws

	Treatments (x 10^6 spermatozoa/mL)				
	100	200	300	600	800
IPIAH (%)	6.00 ± 4.10	7.18 ± 5.01	8.18 ± 5.56	7.13 ± 5.34	5.58 ± 3.93
MI (%)	23.63 ± 20.50	19.58 ± 11.61	22.38 ± 16.07	25.16 ± 19.55	11.13 ± 6.73
AI (%)	21.04 ± 18.94	14.56 ± 11.46	23.28 ± 25.46	15.20 ± 9.77	11.04 ± 7.90
HM (%)	10.76 ± 11.32	9.82 ± 6.26	14.36 ± 12.95	9.24 ± 5.95	9.00 ± 8.10

IPIAH = plasma and acrosomal membrane integrity and high mitochondrial membrane potential, MI = plasma membrane integrity, AI = acrosomal membrane integrity, HM = high mitochondrial membrane potential.

Table 3. Mean ± SD of median of fluorescence intensity of C11- BODIPY$^{581/591}$ and Merocyanine 540 in frozen-thawed boar semen cryopreserved in 0.5mL straws

	Treatments (x 10^6 spermatozoa/mL)				
	100	200	300	600	800
C11- BODIPY$^{581/591}$	148.96 ± 23.79^c	162.17 ± 28.06^{b,c}	172.12 ± 31.63^{a,b}	185.72 ± 34.93^a	168.72 ± 30.02^{a,b,c}
Merocyanine 540	1352.75 ± 104.18	1343.83 ± 131.33	1369.08 ± 138.87	1382.64 ± 121.63	1350.61 ± 156.64

a.u. = arbitrary units; ^{a,b} Values within a row with different superscripts differ significantly at P<0.05.

concentration as low as $400x10^6$ spermatozoa/mL (Alvarez et al. 2012). As previously reported, in jacks, high spermatozoa concentrations at freezing negatively impact sperm kinetics; furthermore, the total and progressive motility appear to be more sensitive to an increase in spermatozoa concentration at freezing compared with other sperm kinetics parameters (Contri et al. 2012). The cryopreservation of boar semen with spermatozoa concentration up to the limit of unfrozen channels ($300x10^6$ spermatozoa/mL, as the evidence of present results) perhaps reduces encapsulated spermatozoa leading to damage in the spermatozoal tails. Besides that, spermatozoa frozen up to $300x10^6$ spermatozoa/mL had a higher ratio of cryoprotectant agents per cell than samples at a higher sperm concentration. The higher the amount of cryoprotectant per sperm cell, possibly the higher the percentage of unfrozen water channels (Nascimento et al. 2008).

The integrity of post-thawed spermatozoa compartments ensures the maintenance of sperm fertility (Rodríguez-Martínez 2007). Damage can be caused by physical (crystal ice) and biochemical (osmotic) changes (Watson 1995). We hypothesized that the increase in spermatozoa concentration in the 0.5 straws at freezing would reduce the chances that the spermatozoa would be encapsulated in the channels not frozen. However, our results did not demonstrate that the increase in spermatozoa concentrations at freezing (up to $800x10^6$ spermatozoa/mL) had a harmful effect on sperm membrane integrity or mitochondrial membrane potential, and it is possible that spermatozoa encapsulation in the channels not frozen was not prevented in this study. Although the plasma and acrosomal membranes are the most sensitive sperm compartments to cold shock (Salamon & Maxwell 1995), the acrosome seems to be more resistant to the additional damage caused by an increase in spermatozoa concentration at freezing, similar to the results described in jacks (Contri et al. 2012), stallions (Nascimento et al. 2008), rams (Alvarez et al. 2012) and dogs (Okano et al. 2004).

Furthermore, regarding mitochondrial membrane potential, equine spermatozoa are less sensitive (impaired at concentrations up to $400x10^6$ spermatozoa/mL) to the increase in spermatozoa concentration at freezing than the plasma membrane (Nascimento et al. 2008). However, our results were not able to demonstrate similar results, and it is possible that boar spermatozoa are not as greatly influenced by the concentration at freezing as equine spermatozoa.

The increase in spermatozoa death after cryopreservation increases free radical generation (Aitken et al. 1989), leading us to believe that lipid peroxidation would increase upon freezing at high spermatozoa concentrations. Indeed, the increase in spermatozoa concentration at freezing increased lipid peroxidation of the sperm membranes, and concentration of $600x10^6$ spermatozoa/mL at freezing were the most detrimental. To the best of our knowledge, the effect of increasing spermatozoa concentrations at freezing on the peroxidation of plasma membrane lipids has rarely been studied. Nevertheless, human spermatozoa are more resistant to lipid peroxidation when increased spermatozoa concentrations ($5\text{-}40x10^6$ spermatozoa/mL) are used at freezing (Wang et al. 1997).

Capacitation-like changes are commonly observed in frozen-thawed semen samples (Neild et al. 2003). Ram spermatozoa have been proven to be very sensitive to an increase in concentration at freezing with regard to the early membrane changes (measured by PI/YO-PRO-1) when cryopreserved at $800x10^6$ spermatozoa/mL (Alvarez et al. 2012). This result differs from boar spermatozoa, in which membrane fluidity was not influenced by the increase in spermatozoa concentration at freezing, as verified in our study.

CONCLUSIONS

To achieve high viability after thawing, boar spermatozoa should be frozen at concentrations up to $300x10^6$ spermatozoa/mL, which would allow the majority of the spermatozoa to be encapsulated in the channels not frozen.

The concentration of boar spermatozoa at freezing must be carefully assessed.

More studies are required to define the ideal value because there is a large range of concentrations from 300 to 600 million spermatozoa/mL.

Conflict of interest statement.- The authors have no competing interests.

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