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Use of galactose to vitrify ram semen in straws

Uso da galactose na vitrificação de sêmen ovino em palhetas

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Abstract
Sperm vitrification is a technique with great potential for cryopreservation of genetic material, with superior effectiveness compared to conventional methods in some species. However, few studies have shown its efficiency with ejaculated sperm of rams and the use of galactose as an extracellular cryoprotectant during vitrification. This study aimed to evaluate the effect of galactose (0.01 M), with or without glycerol addition (3% and 7%) in a commercial extender (Steridyl® - control) for ram sperm cryopreservation in straws, comparing the classic freezing method and vitrification. Ejaculates from six breeding soundness Dorper rams were collected with an artificial vagina, aliquoted, individually diluted (100 × 10^6 sperm/mL) on extenders tested, loaded into 0.25 mL straws, and subjected to a classic freezing method or vitrification. Sperm kinematics, morphology, morphometry, viability, and physical and functional integrity of the sperm membrane were evaluated. The classic freezing method resulted in higher total and progressive motility than vitrification, as no motility was detected in vitrified samples after rewarming ($p<0.05$). The addition of galactose or glycerol to the commercial medium did not benefit both vitrification and the classic freezing method.
Keywords: Semen biotechnology; Ultra-rapid freezing; Cryoprotectants; Reproduction.

Resumo
A vitrificação de espermatozoides é uma técnica que apresenta grande potencial para criopreservação de material genético, e sua eficácia tem sido superior aos métodos convencionais em algumas espécies. No entanto, existem poucos estudos sobre sua eficiência com sêmen ejaculado de carneiros e o uso da galactose como crioprotetor extracelular durante a vitrificação. Objetivou-se com este estudo avaliar o efeito da galactose (0,01 M), associada ou não ao glicerol (3% e 7%), em meio comercial (Steridyl® - controle), na criopreservação de espermatozoides de

carneiros pelo método de palhetas, comparando o método clássico de congelação e a vitrificação. Ejaculados de seis carneiros da raça Dorper em idade reprodutiva foram coletados com vagina artificial, aliquotados, diluídos individualmente (100×10^6 espermatozoides/mL) nos meios testados, envasados em palhetas de 0,25 mL e submetidos à congelação clássica ou vitrificação. Foram analisadas a cinemática, morfologia, morfometria, viabilidade, integridade física e funcional da membrana espermática. A congelação clássica obteve melhores resultados de motilidade total e progressiva do que a vitrificação nos quatro extensores testados, uma vez que as amostras vitrificadas não apresentaram motilidade pós-reaquecimento ($p < 0,05$). A adição de galactose ou glicerol ao meio comercial não trouxe efeito benéfico tanto para a vitrificação quanto congelação clássica.
Palavras-chave: Biotecnologia de sêmen; Congelamento ultrarrápido; Crioprotetores; Reprodução.

Introduction

Sperm cryopreservation is an important tool for animal genetics preservation because it enables biological material storage for an undetermined period[1]. Sperm cryopreservation is associated with reproductive biotechnologies, such as artificial insemination (AI), *in vitro* embryo production (IVP), intracytoplasmic sperm injection (ICSI), increasing genetic gain, and livestock production[2].

To date, the best results with sperm cryopreservation in ovine species have been obtained using the conventional/classic cryopreservation method. However, the aforementioned technique still presents limitations, with intracellular ice crystal formation as one of the main sources of sperm cryoinjuries, causing DNA fragmentation and irreversible rupture of sperm membranes, reducing post-thaw sperm motility[3]. Therefore, new cryopreservation methods have been developed, seeking satisfactory results that can overcome the problems related to classic cryopreservation, with vitrification being considered a promising technique[4].

Sperm vitrification is an ultrarapid and low-cost cryopreservation technique that can be easily executed in the field because it does not require the use of many pieces of equipment. Sperm vitrification is characterized by low cryoprotectant concentration[5] and direct semen immersion in liquid nitrogen, with a temperature reduction rate of up to 10.000 °C/min [4]. These particular features cause a phase-change material from liquid to an amorphous state, when the intracellular fluid acquires a vitreous characteristic, preventing ice crystal formation, and the absence of a defined atomic structure allows the distribution of the vitreous substance among cell organelles[6]. In humans, the sperm vitrification technique is well characterized[7], and good experimental results have been achieved with donkeys[8], horses[9], dogs[10], and cats[6].

In sheep, the best results after sperm vitrification were observed using sperm from the epididymal tail, whereas for ejaculated semen, the results are inferior to those of classic cryopreservation[11–13]. Nonetheless, in the aforementioned studies, only sucrose was used as an external cryoprotectant[11–13]. Different carbohydrates, such as galactose, could improve the results after vitrification, as reported for cat oocytes[14] and horse blastocysts[15]. Galactose is a monosaccharide that can inhibit ice recrystallization during the freezing/thawing process, presenting low toxicity at physiological temperatures, with the ability to reduce osmotic stress[16]. It is noteworthy that egg yolk addition to the extender as a cryoprotectant was beneficial to ram sperm[11,12]. Therefore, the goal of the present study was to evaluate the effect of galactose, either with or without glycerol, on ram sperm cryopreservation in straws, by comparing vitrification and classic methods.

Material and methods

Animals

This study was approved by the Ethics Committee for Animal Use of the Federal University of Alagoas (54/2018). Ejaculates from nine Dorper rams, aged between 15 and 24 months and located at Maceió, Alagoas, were collected with the aid of an artificial vagina. The semen samples were evaluated according to the Brazilian College of Animal Reproduction[17]; and ejaculates from six rams were in compliance with the minimal criteria quality for cryopreservation.

Semen evaluation

Immediately after semen collection, the ejaculates were subjected to macroscopic evaluation of volume, color, odor, and aspect. Subsequently, microscopic evaluation of wave motion (0 - 5), progressive motility (0 - 100%), and sperm vigor (0 - 5) were assessed[17]. Sperm concentration was determined spectrophotometrically using a pre-calibrated curve ($r > 0.95$). After diluting a semen aliquot (1:400) in glutaraldehyde buffered solution (sodium citrate solution 2.94%, glutaraldehyde 2%), the absorbance of the diluted sample was determined at 560 nm wavelength (V-1100, J.P. Selecta, Barcelona, Spain).

The physical integrity of the sperm plasma membrane was evaluated using eosin-nigrosin supravital staining (Minitube GmbH, Tiefenbach, Germany)[10]. The functional integrity of the sperm plasma membrane was assessed using a hypoosmotic swelling test. After diluting 10 µL of semen on 90 µL of hypoosmotic solution (7.35 g sodium citrate, 13.5 g fructose, distilled water q.s.p. 1000 mL), the samples were incubated in a water bath at 37 °C for 30 min, followed by the addition of 2 µL of glutaraldehyde buffered solution (5%) to stop the reaction [18]. An aliquot (10 µL) of each sample was loaded onto a slide, covered with a coverslip, and 200 sperm were evaluated under a phase-contrast microscope (400×). The results were expressed as a percentage after subtracting the percentage of coiled tail sperm detected during morphological evaluation, as reported by Oliveira et al.[19].

Sperm morphology was evaluated using a wet chamber, diluting 10 µL of semen in 100 µL of glutaraldehyde buffered solution (5%). Next, 10 µL of each diluted sample was loaded onto a slide, covered with a coverslip, and 200 sperm were evaluated under a phase-contrast microscope (400×), according to CBRA[17].

Cryopreservation Extenders

To evaluate individual cryoresistance, four extenders were used having the Steridyl® (Minitube, Germany) as base extender: 1) Steridyl® (Control); 2) Steridyl® + 0.01 M of galactose; 3) Steridyl® + 0.01 M of galactose + 3% of glycerol; and 4) Steridyl® + 0.01 M of galactose + 7% of glycerol.

Cryopreservation

After determining the sperm concentration of the ejaculate, the semen was aliquoted into four different extenders to a final concentration of 100×10^6 motile sperm/mL. Then, the diluted samples were loaded into 0.25 mL straws and sealed using a MAXI30 sealing machine (Neovet, Uberaba, Brazil). Subsequently, the samples were subjected to either classic cryopreservation or vitrification.

Classic Cryopreservation

The semen straws were cooled for 2 h in an isothermal box (BotuFlex®, Botupharma, Brazil) previously equilibrated at 5 °C. After cooling, the straws were placed horizontally on a rack 7 cm above liquid nitrogen and exposed to nitrogen vapor (15 min) in an isothermal box, and then plunged into liquid nitrogen [20]. The straws were kept in liquid nitrogen tanks (-196 °C) until analyses.

Vitrification

To perform vitrification, the sealed 0.25 mL semen straws were loaded into 0.50 mL open straws and directly immersed into liquid nitrogen[21]. The straws were kept in liquid nitrogen tanks (-196 °C) until analyses.

Post-cryopreservation analyses

The samples subjected to classic cryopreservation were thawed in a water bath at 37 °C/30 s, whereas the vitrified samples were rewarmed in a water bath at 60 °C/5 s[11]. Then, the entire content of each straw was loaded individually into polypropylene tubes at 37 °C in a water bath, and aliquots were used to perform physical and functional analyses of the sperm plasma membrane, as well as sperm morphology. The following sperm analyses were also performed.

Computer Assisted Sperm Analysis - CASA

An aliquot (10 µL) of each sample was diluted in 30 µL of Steridyl®, homogenized, and kept in a water bath for 5 min. Then, a 2 µL aliquot was loaded into a sperm counting chamber (HC-B028C, Guangzou Electronics, China) and evaluated under a phase contrast microscope (100×).

Sperm kinematics were assessed using the motility extension of OpenCasa software v1.0[22], with the aid of ImageJ software v1.52. The images were captured at 30 frames

per second (FPS) using a USB camera model OPT12 (Opticam Microscopy Technology) in a phase-contrast microscope FWL 3500TFL (Feldmann Wild Leitz, Manaus, Brazil). Five videos of two seconds were recorded from each sample, and at least 500 sperm were evaluated.

Sperm viability using LIVE/DEAD fluorescent probe

A Live/Dead commercial kit (Invitrogen, EUA) was used for the analysis. Ten microliters of semen were homogenized with 1 µL of SYBR14 (1 mM) and 1 µL of propidium iodide (2.4 mM) into a polypropylene tube. After 10 min of incubation in a water bath (37 °C), 1 µL of glutaraldehyde buffered solution (2 %) was added to fix the sperm. Then, an aliquot (10 µL) was loaded onto a glass slide, covered with a coverslip, and evaluated using fluorescence microscopy at a wavelength range of 450 - 490 nm. The viability extension of OpenCasa software v1.0 (200×) was used to evaluate 200 sperm in each image.

Sperm morphometry

Sperm head morphometry was performed using the same slides prepared for viability analysis (SYBR14 and propidium iodide). The images captured under fluorescence microscopy were evaluated using the morphometry extension of OpenCasa software v1.0, assessing 200 sperm/sample at 400× magnification[22].

Statistics

Classic cryopreservation and vitrification techniques and four different extenders were used to compare sperm traits. The results from each ejaculate within a treatment were grouped and expressed as the mean ± SEM. Data normality was assessed using the Kolmogorov-Smirnov test. The obtained means were compared using one-way ANOVA followed by Tukey's test. All data were analyzed using RStudio software v1.4, and differences were considered significant at $p<0.05$.

Results

For classic cryopreservation, results after the addition of galactose (0.01 M) alone did not differ from the control (Steridyl®) in any sperm trait assessed (Tables 1 and 2). The addition of 3% glycerol reduced sperm viability only ($p<0.05$). However, when glycerol was added at 7%, there were significant reductions in total and progressive motility, viability, and physical and functional plasma membrane integrity. For sperm vitrification, adding galactose (0.01 M) alone or in combination with glycerol at 3% or 7% made no difference ($p>0.05$) to any sperm trait assessed compared with the control extender (Tables 1 and 2). Nevertheless, comparing the cryopreservation methods, sperm vitrification presented lower results compared with classic cryopreservation ($p<0.05$), as no motility was detected after rewarming in any extenders tested, as well

as lower values of viability and physical and functional plasma membrane integrity (Table 1).

Most extenders tested did not influence sperm head morphometry regardless of the cryopreservation technique used, except for extender 4 (Steridyl®+ 0.01 M galactose + 7% glycerol) that resulted in lower values ($p<0.05$) of sperm head area and width after vitrification compared with classic cryopreservation (Table 1).

Within the same cryopreservation technique (vitrification or classic cryopreservation), the addition of galactose (0.01 M) and glycerol (3 or 7%) to the Steridyl® extender did not influence sperm morphology ($p>0.05$). Sperm vitrification resulted in a higher percentage of the coiled tail and a lower percentage of sperm responsive to the hypoosmotic test in all tested extenders, compared with classic cryopreservation ($p<0.05$). Furthermore, the addition of galactose, with or without glycerol, significantly increased the percentage of major sperm defects. Differences between vitrification and classic cryopreservation were also observed for proximal droplets, tail coiled around the head, bent tail, and coiled tail, but without clear distinction among extenders and always in a small percentage of sperm (Table 2).

Table 1. Results (mean ± SEM) of ram sperm parameters after cryopreservation by classic or vitrification method in different extenders

Sperm parameters	Extenders							
	1		2		3		4	
	Classic	Vitrification	Classic	Vitrification	Classic	Vitrification	Classical method	Vitrification
TM (%)	22.7±4.5[a]	0*	19.1±5.4[a]	0*	20.8±7.7[a]	0*	2.2±0.9[b]	0*
PM (%)	13.4±4.3[ab]	0*	14.8±3.9[ab]	0*	18.1±5.5[a]	0*	1.7±1.7[b]	0
Viability (%)	20.6±2.0[a]	0*	17.3±2.4[a]	0*	8.7±1.9[b]	0*	1.9±0.6[b]	0.2±0.2*
PMI (%)	34.5±4.7[a]	0*	27.2±4.0[a]	0.2±0.2*	20.9±3.3[a]	0*	3.7±1.6[b]	0*
Hypoosmotic (%)	38.5±4.4	3.2±1.3*	35.9±3.9	8.8±1.7*	32.6±3.1	7.4±2.6*	41.4±5.4	8.6±2.0*
Area (µm²)	316.0±8.2	298.6±10.6	315.0±4.2	307.2±8.2	307.5±8.1	297.8±7.5	319.1±1.7	293.3±11.3*
Perimeter (µm)	76.3±1.8	79.6±1.6	76.4±1.2	80.3±1.0*	75.8±1.5	78.1±0.9	76.5±1.2	79.4±2.0
Length (µm)	27.2±0.4	27.2±0.4	27.3±0.2	27.5±0.3	27.0±0.3	27.1±0.2	27.4±0.1	27.1±0.4
Width (µm)	15.3±0.2	14.8±0.2	15.3±0.2	15.1±0.2	15.1±0.3	14.8±0.1	15.4±0.1	14.8±0.3*

1: Steridyl®; 2: Steridyl® + 0.01 M galactose; 3: Steridyl® + 0.01 M galactose + 3% Glycerol; 4: Steridyl® + 0.01 M galactose + 7% glycerol.
TM: total motility; PM: progressive motility; Viability (SYBR14 and propidium iodide); PMI: physical integrity of plasma membrane (eosin/nigrosin).
Within a row, asterisks (*) represent significant difference ($p<0.05$) between cryopreservation techniques within extender.
Within a row, different superscript letters indicate differences ($p<0.05$) among extenders.

Table 2. Results (mean ± SEM) of sperm morphology after cryopreservation by classic or vitrification method in different extenders

Sperm parameters	Extenders							
	1		2		3		4	
	Classic	Vitrification	Classic	Vitrification	Classic	Vitrification	Classic	Vitrification
Minor defects (%)	9.0±4.4	2.7±1.8	6.2±2.6	4.8±2.5	4.2±2.1	3.9±2.1	9.7±7.1	5.9±3.8
Major defects (%)	8.8±2.5	18.0±4.4	7.8±1.5	20.8±3.2*	12.0±3.6	21.9±2.6*	9.5±2.1	19.5±3.4*
Total defects (%)	17.8±5.9	20.8±4.4	14.1±3.3	25.7±3.3	16.2±5.1	25.8±2.0	19.2±7.3	25.4±2.8
Proximal droplet (%)	1.5±0.3	0.1±0.1*	1.1±0.2	0	1.8±0.9	0	1.7±0.6	0*
Tail coiled around the head (%)	0	0.1±0.1*	0	0	0.2±0.2	0	0	0
Strong coiled or bent tail (%)	5.9±2.4	17.1±4.4*	5.9±1.4	20.8±3.3*	8.0±3.1	21.7±2.6*	6.9±2.3	19.2±3.6*
Bent tail with distal droplet (%)	0.2±0.2	0	0.2±0.2	0	0.5±0.1	0*	0.2±0.2	0
Coiled or bent tail (%)	3.2±1.7	0.4±0.2	2.5±0.6	0.2±0.2*	0.9±0.4	0.6±0.3	2.2±1.0	0.8±0.4

1: Steridyl®; 2: Steridyl® + 0.01 M galactose; 3: Steridyl® + 0.01 M galactose + 3% Glycerol; 4: Steridyl® + 0.01 M galactose + 7% de glycerol.
Within a row, asterisks (*) represent significant difference ($p<0.05$) between cryopreservation techniques within extender.

Discussion

In the present study, galactose did not improve the results obtained after vitrification of ejaculated ram sperm, unlike in the vitrification of cat oocytes[14] and horse blastocysts[15]. Moreover, the results were lower than those reported for using the same concentration of sucrose with semen from Mouflon rams[11]. The Steridyl® extender was chosen because it is indicated for ruminants and has egg yolk as an additive, and benefits of egg yolk during ram semen vitrification have been demonstrated[23]. After rewarming, no sperm motility in vitrified samples was detected by CASA, associated with low values of viability, physical and functional plasma membrane integrity, crucial sperm traits for the effectiveness of the technique. It is of note that in some rewarmed samples, motility was detected in microcephalic sperm, but because of the head area, the CASA software was unable to identify sperm cells. This information is supported by Isachenko et al.[24], who reported higher resistance to vitrification of sperm from species characterized by smaller sperm head size.

No benefit of galactose addition was detected on sperm quality, either post-thaw or rewarming. Unlike oocyte and embryo vitrification[4], sperm vitrification uses low cryoprotectant concentration or even no cryoprotectant[7], seeking lower osmotic stress and sperm damage[24]. Therefore, low galactose concentration was added to extenders, since ram sperm are potentially sensitive to high carbohydrate concentrations in vitrification extenders and better results were reported for rewarming performed with low sucrose concentration[11,25].

To evaluate the influence of the penetrant cryoprotectant, 0.01 M glycerol was added at 3% and 7% to the extender with 0.01 M of galactose. No positive effect was detected since the samples had low viability and no motility after rewarming with both glycerol concentrations tested. These findings are similar to those reported by Jiménez-Rabadán et al.[25] and Pradiee et al.[11], both reporting negative effects of glycerol on sperm vitrification. It is worth mentioning that for classic cryopreservation, the addition of 3% glycerol was not deleterious, with results similar to both Steridyl® and Steridyl® with 0.01 M of galactose. However, 7% glycerol reduced the total and progressive motility, viability, and physical and functional plasma membrane integrity parameters. Although glycerol toxicity was an expected outcome because the commercial extender has glycerol as a constituent, concentrations higher than 6% can be toxic to ram sperm[26]. It was not possible to predict the effects of the association between galactose and higher glycerol concentration during vitrification, and the same extender was used for classic semen cryopreservation to avoid compromising the experimental design.

No difference between extenders within the same cryopreservation technique was detected on functional membrane integrity evaluated by hypoosmotic test; this could be related to the addition of 0.01 M of galactose since the osmoprotectant effect is one of the characteristics of extracellular cryoprotectants[14]. It is important to note that simple hypoosmotic test is unable to detect sperm subpopulations with high osmotic resistance or sperm that quickly return to the initial volume after active transport of water and ions through the membrane (being classified as non-reactive to the test), or

cells with very low osmotic resistance that disrupt during the test (consequently not identified), or even non-viable cells from the beginning of the test not responsive to hypoosmotic conditions[27].

Within the same cryopreservation technique, no differences in sperm morphology were detected for different extenders. Comparisons between cryopreservation techniques demonstrated that vitrification resulted in a higher percentage of sperm with a strong coiled tail compared with classic cryopreservation for all extenders tested. This result is similar to that reported by Arando et al.[28] who showed that vitrification of sperm from Merino rams increased the percentage of tail defects compared with classic cryopreservation; this might be explained by tail damages observed using transmission electron microscopy, such as vesicles and dilated areas beneath the sperm membrane, as well as shape and size modifications on sperm mitochondria. Most likely, these damages occur during rewarming; according to Curry and Watson[29], ram's sperm is more sensitive than human's when transferred from hyperosmotic to isosmotic medium which can reflect on sperm quality differences observed after vitrification between species.

Osmotic resistance is an important parameter for sperm quality after vitrification, as better results for ram sperm vitrification were observed with sperm originating from the epididymis tail[12], with no difference between classic cryopreservation and vitrification on total sperm motility, morphology, and physical integrity of the plasma membrane. The higher cryoresistance of epididymal sperm compared with ejaculated sperm was confirmed by Martínez-Fresneda et al.[13], showing that after contact with the seminal plasma, the sperm initiate capacitation-like changes, increasing tyrosine phosphorylation, reducing osmotic resistance, and consequent cryoresistance.

Sperm morphometry is a parameter that can be used to evaluate the physical effects of extenders and cryopreservation methods on sperm dimensions. In the present study, there was no difference among the extenders within the cryopreservation technique. Comparing the cryopreservation techniques, vitrified sperm in the extender with 7% glycerol presented reduced area and width compared to those cryopreserved by the classic method. This finding can be a consequence of the higher percentage of sperm with loss of functional and physical integrity of the plasma membrane, associated with the high osmolarity of the medium, reducing the rehydration in the smaller percentage of sperm with functional membrane after rewarming, resulting in smaller sperm head size, as reported by Arando et al.[28].

Conclusion

It was concluded that the addition of galactose to the Steridyl® extender, with or without glycerol, is ineffective in vitrifying ejaculated ram sperm in straws, and does not improve post-thaw sperm parameters obtained when the classic cryopreservation method is used.

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Conflict of interest

The authors declare no conflict of interest.

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