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Effects of insulin on sperm cell quality in ram semen cooled at 5°C

Efeitos da insulina sobre a qualidade das células espermáticas no sêmen ovino refrigerado a 5ºC

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Highlights

Insulin improved ram sperm progressive motility after 24 or 48 hours of cooling.
Insulin increased ram sperm linearity and beat caudal frequency during cooling.
Insulin did not affect ram sperm membrane and acrosome integrities during cooling.

Abstract

Insulin is present in the seminal plasma and is involved in sperm activities like motility and capacitation. However, the effects of insulin on the viability of cooled ram sperm are not fully understood. Therefore, the objective of the current study was to evaluate the effect of insulin addition on ram sperm maintained at 5ºC. Sperm samples were collected from six healthy, mature Santa Inês rams. The ejaculates were divided into two aliquots with (insulin group) or without (control group) insulin (3 IU mL^{-1}) in the semen extender, and then cooled at 5°C for 48 hours. Subsequently, the sperm cells were evaluated for motility and kinetics using computer-assisted semen analysis. The samples were evaluated for acrosomal integrity by fluorescein using isothiocyanate combined with peanut agglutinin (FITC-PNA) and membrane functionality by the hypoosmotic swelling test. The semen analysis was performed after 24 or 48 hours of cooling. There was an increased percentage of progressive sperm motility (%), straightness (%), linearity (%) and beat caudal frequency (Hz) in the insulin group after 24 and 48 hours of cooling ($p < 0.05$). However, insulin did not affect total sperm motility, sperm velocities (VSL, VAP and VCL) (μm seg^{-1}), acrosomal integrity and membrane functionality during cooling ($p > 0.05$). In conclusion, the addition of 3 IU mL^{-1} insulin to ram semen extender

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improved the quality of sperm motility after cooling.
Key words: Cooling. Glucose. Semen extender. Sheep. Spermatozoa.

Resumo

A insulina está presente no plasma seminal e participa de atividades espermáticas, como a motilidade e capacitação. Entretanto, os efeitos da insulina sobre a viabilidade do espermatozoide ovino resfriado ainda não estão elucidadas. Desta forma, o objetivo do presente estudo foi avaliar os efeitos da adição de insulina sobre o espermatozoide ovino durante o tempo de armazenamento à 5º C. Amostras espermáticas de seis carneiros da raça Santa Inês foram utilizadas. Os ejaculados foram divididos em duas aliquotas, com (grupo insulina) ou sem (grupo controle) adição de insulina (3 UI mL^{-1}) no diluidor seminal e, posteriormente, resfriados até 5oC e mantidos armazenados por 48 horas. Em seguida, os espermatozoides foram avaliados quanto a motilidade e cinética utilizando um Sistema Computadorizado de Análise de Sêmen (CASA). Adicionalmente, as amostras espermáticas foram analizadas quanto a integridade acrosomal por meio de sondas fluorescentes (FITC-PNA) e, funcionalidade de membrana pelo teste hiposmótico. As análises seminais foram realizadas após 24 ou 48 horas de resfriamento. Foram verificados aumentos de espermatozoides com motilidade progressiva (%), retilinearidade (%), linearidade (%) e frequência de batimento caudal (BCF) (Hz) no grupo insulina após 24 ou 48 horas de resfriamento ($p < 0.05$). Entretanto, não houve efeito da adição de insulina sobre a porcentagem de espermatozoides moveis (%) e das velocidades espermáticas (VSL, VAP e VCL) (μm seg^{-1}), integridade acrossomal e funcionalidade de membrana durante o resfriamento ($p > 0.05$). Conclui-se que adição de insulina (3 UI mL^{-1}) no diluidor seminal melhora a qualidade da motilidade espermática durante o resfriamento.
Palavras-chave: Diluidor seminal. Espermatozoides. Glicose. Ovinos e Resfriamento.

Introduction

Cooling ram semen is the main option for cervical artificial insemination (Palacín et al., 2012). However, the cooling process can damage sperm cells, particularly the sperm membrane and acrosome (Gürler et al., 2016). Moreover, these alterations can promote early capacitation and reduce the fertilizing ability (Srivastava et al., 2013). To reduce sperm cryodamage, different additives have been used in semen extender, including the hormone insulin.

Sperm membranes are cytological targets for insulin; indeed, they express the insulin receptor (Carpino, Rago, Guido, Casaburi, & Aquila, 2010). This hormone has similar function among mammals, including glucose uptake and modulation of intracellular and membrane events related to carbohydrate and lipid metabolism (Baumgard, Hausman, & Sanz Fernandez, 2016). In sperm cells, insulin acts on carbohydrate uptake and pathways that modulate sperm motility (Dias, Alves, Silva, & Oliveira, 2014; Laskowski et al., 2016). In this context, men with diabetes mellitus present lower levels of motile sperm cells (Pergialiotis et al., 2016). Recently, lower plasma insulin concentrations were observed in bulls with low fertility (Weerakoon, Sakase, Kohama, & Kawate, 2020), and rats with lower insulin levels had reduced antioxidant capacity and DNA integrity in testicular tissue (Aeeni, Razi, Alizadeh, & Alizadeh, 2021). Moreover, seminal

insulin is associated with sperm quality *in vivo* (Leisegang, Bouic, Menkveld, & Henkel, 2014), and insulin addition to semen samples *in vitro* affects the sperm cell membrane, probably by cholesterol efflux (Carpino et al., 2010).

The freezability of stallion sperm is positively associated with the insulin concentration in the seminal plasma (El-Badry, El Sisy, & Abo El-Maaty, 2016). Moreover, in humans, insulin addition to the semen extender during sperm freezing reduced cryodamage and improved sperm motility (Shokri, Ebrahimi, Ziaeipour, & Nejatbakhsh, 2019). Based on the positive association between insulin and sperm quality, insulin addition in sperm extender could boost the quality of sperm. Of note, in ram sperm (Makarevich, Spalekova, Olexikova, Kubovicova, & Hegedusova, 2014) as well as the sperm of other mammals, the sperm quality was improved when insulin-like growth factor 1 (IGF-1), a protein similar to insulin, was added to the cryopreservation extender (Selvaraju, Krishnan, Archana, & Ravindra, 2016; Silva et al., 2011). However, the studies using insulin have reported divergent results among the species, and there have been few studies in ram. Therefore, the present study aimed to analyze the *in vitro* effects of insulin on ram sperm refrigerated for up to 48 hours.

Material and Methods

Location and animals

The current study was approved by the Graduate Program in Animal Science – UENF and followed the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2012). This study was conducted at Campos dos Goytacazes, RJ, Brazil (21°45'16''S, 41°19'28''W). Six healthy mature rams of the Santa Ines breed, aged 44.3 ± 6.2 months and weighing 79.1 ± 4.8 kg, were used. The study was conducted from June to July. Each week, one ejaculate sample was collected from one of the six rams by the artificial vagina method (IMV®, L´Aigle, France); a total of six ejaculates used in this study.

Chemicals

Tris base, citric acid, glucose, penicillin, streptomycin and fluorescein isothiocyanate-*Pisum sativum* agglutinin (FITC-PSA) were acquired from Sigma-Aldrich Brasil Ltda (São Paulo, SP, Brazil). Mixed bovine-porcine insulin was obtained from Iolin® (Biobrás S.A., Belo Horizonte, MG, Brazil).

Sperm cooling protocol

The semen samples were diluted in Tris-egg yolk extender (3.028 g of Tris, 1.675 g of citric acid, 0.18 g of glucose, 0.031 g of penicillin, 0.05 g of streptomycin, 20 mL of egg yolk and 80 mL of distilled water). Each sample was divided in two aliquots of 2 mL with a concentration of 50×10^6 sptz mL^{-1}. In each aliquot of the insulin group, 3 IU mL^{-1} of mixed bovine-porcine insulin (van Tilburg, Silva, Dias, Quirino, & Fagundes, 2008) was added. The control group did not receive insulin. The sperm samples were cooled, using a cooling machine (Haake, k75 model, Thermo Fisher Scientific®, Newington-NH, USA) at -0.27°C min^{-1} for 2 hours until reaching 5°C. Subsequently, the samples were maintained at 5°C in a refrigerator (Semen Storage Unit, Minitub®, Tiefenbach, Germany). After 24

and 48 hours of cooling, the samples from insulin and control groups were analyzed for motility, kinematics, acrosomal integrity and membrane functionality at 37°C.

Sperm evaluations

Motility and kinematics

The sperm motility and kinematics were assessed with computer-assisted sperm analysis (CASA) using a Ceros 10.8 (Hamilton Thorne Research®, Beverly, MA, USA) set up for ram samples (frames acquired: 30; frame rate: 60 Hz; low average path velocity [VAP] cut-off: 10 μm s^{-1}; medium VAP cut-off: 60 μm s^{-1}; threshold straightness: 80%). Four microliters of each sperm sample was evaluated in a 2X-CEL chambered microscope slide (20 micron 2X-CEL® chamber, Hamilton Thorne Research®), in five different fields with 200× total magnification, with a phase contrast microscope (JANAMED2, Zeiss®, Oberkochen, Germany) at 37°C. The CASA parameters evaluated were: total motility (TM) (%), progressive motility (PM) (%), straightness (STR) (%) and linearity (LIN) (%). The kinematic CASA parameters were: VAP (μm s^{-1}), straight line path velocity (VSL, μm s^{-1}), curvilinear path velocity (VCL, μm s^{-1}), amplitude lateral head displacement (ALH, μm) and beat caudal frequency (BCF, Hz).

Acrosomal integrity

The acrosomal integrity evaluation was performed with a fluorescence technique using a double stain with propidium iodide and FITC-PSA (van Tilburg et al., 2008). In summary, sperm smears were prepared, fixed in methanol for 10 seconds at -18°C and allowed to dry at room temperature. Next, each sperm smear was covered with 80 μL of FITC-PSA (40 μg mL^{-1} in phosphate-buffered saline [PBS]), covered with a cover slip and incubated for 20 minutes protected from light. After removing the coverslip, each sperm smear was covered with 60 μL of propidium iodide (100 μg mL^{-1} in PBS), covered again with the previous cover slip and incubated for 10 minutes protected from light. Next, sperm smears were washed in PBS and allowed to dry at room temperature protected from light.

Sperm cells were examined under a fluorescence microscope (Axio Plan, Zeiss®, Oberkochen, Germany) at 1000× total magnification under oil immersion. Two hundred sperm cells were analysed and the intact acrosome percentage was quantified. Only sperm cells with uniform green fluorescence in the acrosomal region were considered to have an intact acrosome.

Membrane functionality

The membrane functionality was verified using the hypoosmotic test (van Tilburg et al., 2008). The sperm samples were diluted in distilled water and incubated for 5 min at 38°C. Next, 200 spermatozoa per sample were evaluated under phase contrast microscope (JANAMED2, Zeiss®, Oberkochen, Germany) at 400× total magnification. Spermatozoa with tail swelling or curling were considered to have a functional membrane and spermatozoa with a straight tail were considered to have a damaged membrane.

Statistical analysis

All statistical analysis were performed with the Statistical Analysis Software program (SAS®, version 9.0, 1999). The data of six ejaculates are expressed as the mean ± standard deviation. The normality of the data was evaluated with the Shapiro–Wilk test. When necessary, the non-parametric data were transformed by arcsine square root transformation. Data analysis was performed by general linear models (PROC GLM), and the means of the control and insulin groups (24 or 48 hours) were compared using a t test with a significance threshold set as $p<0.05$.

Results and Discussion

There was higher progressive sperm motility in the insulin group after 24 hours (control: 31.0% ± 7.6% and insulin: 37.1% ± 8.2%) and 48 hours (control: 25.8% ± 9.1% and insulin: 34.6%± 10.5%) of cooling (Table 1, $p<0.05$). The positive effect from insulin on progressive sperm motility was also observed on the straightness and linearity values after 24 hours (STR: control: 73.1% ± 2.9% and insulin: 78.0% ± 4.4%; LIN: control 42.3% ± 1.7% and insulin: 49.0% ± 6.1%) and 48 hours (STR: control: 68.1% ± 3.8% and insulin: 76.0% ± 8.6%; LIN: control 39.6% ± 2.6% and insulin: 44.1% ± 3.7%) of cooling (Table 1, $p<0.05$). Nevertheless, the total motility was similar between the control and insulin groups after 24 hours (TM: control: 76.3% ± 11.9% and insulin: 77.6% ± 14.3%) or 48 hours (TM: control: 74.6% ± 14.8% and insulin: 78.0% ± 14.7%) of cooling ($p > 0.05$).

Table 1
Motility of ram sperm with or without insulin after 24 and 48 hours of cooling

Variables	24 hours		48 hours	
	Control group	Insulin group	Control group	Insulin group
Total motility (%)	76.3 ± 11.9	77.6 ± 14.3	74.6 ± 14.8	78.0 ± 14.7
Progressive motility (%)	31.0 ± 7.6[a]	37.1 ± 8.2[b]	25.8 ± 9.1[a]	34.6 ± 10.5[b]
Straightness (%)	73.1 ± 2.9[a]	78.0 ± 4.4[b]	68.1 ± 3.8[a]	76.0 ± 8.6[b]
Linearity (%)	42.3 ± 1.7[a]	49.0 ± 6.1[b]	39.6 ± 2.6[a]	44.1 ± 3.7[b]

For each time point and each variable, a different letter between groups represents a significant difference ($p<0.05$).

Table 2 presents the VAP, VSL, VCL, ALH and BCF data. Insulin had no effect on sperm velocities (VAP, VSL or VCL) (μm s^{-1}) and ALH (μm) after 24 or 48 hours of cooling (Table 2, $p>0.05$). However, the BCF was superior for the insulin group after 24 hours (BCF: control: 25.7 ± 3.0 Hz and insulin: 29.3 ± 4.1 Hz) and 48 hours (BCF: control: 22.1 ± 3.2 Hz and insulin: 25.7 ± 3.9 Hz) of cooling (Table 2, $p<0.05$).

Table 2
Kinematics of ram sperm with or without insulin after 24 and 48 hours of cooling

Variables	24 hours		48 hours	
	Control group	Insulin group	Control group	Insulin group
VAP (μm s^{-1})	89.1 ± 14.7	87.2 ± 17.3	95.1 ± 13.1	93.5 ± 12.4
VSL (μm s^{-1})	68.6 ± 11.2	71.0 ± 14.3	66.5 ± 11.7	72.1 ± 11.8
VCL (μm s^{-1})	157.9 ± 27.0	143.5 ± 27.3	169.4 ± 17.1	161.6 ± 18.4
ALH (μm)	7.3 ± 0.9	6.2 ± 0.9	7.2 ± 0.6	6.8 ± 0.4
BCF (Hz)	25.7 ± 3.0[a]	29.3 ± 4.1[b]	22.1 ± 3.2[a]	25.7 ± 3.9[b]

VAP, average path velocity (μm s^{-1}); VSL, straight line velocity (μm s^{-1}); VCL, curvilinear velocity (μm s^{-1}); ALH, amplitude lateral head displacement (μm); BCH, beat caudal frequency (Hz). For each time point and each variable, a different letter between groups represents a significant difference ($p<0.05$).

Regarding acrosomal and membrane integrities (Table 3), insulin did not affect acrosomal integrity by FITC-PSA and hypoosmotic tests analysis after 24 hours (acrosomal integrity: control: 83.8% ± 6.2% and insulin: 88.3% ± 4.2%; hypoosmotic test: control: 42.34% ± 19.6% and 43.9% ± 18.5%) or 48 hours (acrosomal integrity: control: 76.1 ± 4.5% and insulin: 82.7 ± 3.9%; hypoosmotic test: control: 33.5% ± 13.4% and 32.8% ± 16.0%) of cooling ($p>0.05$).

Table 3
Acrosomal integrity and hypoosmotic test of ram sperm with or without insulin after 24 and 48 hours of cooling

Variables	24 hours		48 hours	
	Control group	Insulin group	Control group	Insulin group
Acrosomal integrity (%)	83.8 ± 6.2	88.3 ± 4.2	76.1 ± 4.5	82.7 ± 3.9
Hypoosmotic test (%)	42.3 ± 19.6	43.9 ± 18.5	33.5 ± 13.4	32.8 ± 16.0

Although insulin did not affect total motility, the progressive motility was positively affected ($p<0.05$). As progressive sperm motility is associated with high fertility in sheep (Yániz, Palacín, Vicente-Fiel, Sanchez-Nadal, & Santolaria, 2015), future studies evaluating insulin in ram semen extender should verify fertility by artificial insemination. Sperm motility is glycolysis dependent, and the majority of adenosine triphosphate (ATP) in mature sperm is produced by this metabolic pathway. However, cooling can alter the fluidity of cell membranes, leading to phospholipid grouping, loss of membrane proteins and other negative impacts on membrane functions (Morris, Acton, Murray, & Fonseca, 2012). The insulin in semen extender could promote higher glucose uptake by sperm cells and, consequently, greater intra-sperm glucose for tail metabolism. The

straightness, linearity and BCF were higher in the insulin group than the control group ($p<0.05$). In humans, sperm cells exposed to insulin showed phosphorylation of pathways related to insulin receptors and sperm motility (Aitken et al., 2021). It is important to point out that glycolysis in the caudal portion of sperm cells is critical for sperm motility, and glycolytic enzymes are concentrated in this region, producing ATP mainly for flagellar activity (Zhu et al., 2020). Interestingly, sperm cells are capable of liberating insulin, which could participate in sperm capacitation (Aitken et al., 2021). Hyperactivated ram sperm cells show a strong circular movement that is common to capacitated sperm during fertilization (Curtis, Kirkman-Brown, Connoly, & Gaffney, 2012); however, our results did not show hyperactivated sperm movement in the insulin group.

In our experiment, insulin did not affect acrosomal integrity and membrane functionality. In swine, the addition of insulin to cryopreservation media did not affect the sperm viability (Cunha et al., 2012). Studies evaluating specific insulin effects on the sperm cell membrane are scarce in domestic animals. However, in men, Cappello et al. (2012) verified the effects of insulin on sperm cell membranes associated with cholesterol efflux and the acrosome reaction. In addition, Shokri et al. (2019) observed that insulin addition to semen extender reduced reactive oxygen species and DNA damage in human cryopreserved sperm cells. In our current study, the insulin concentration tested was perhaps not high enough to promote significant changes in the acrosome and membrane of the ram sperm cells during the storage at 5°C. Future studies of insulin effects on ram sperm cells should consider specific aspects of sperm membranes and sperm cells like cholesterol efflux, reactive oxygen species and tyrosine phosphorylation under different insulin concentrations.

Curiously, bovine genomic studies identified a relation between IGF-1 receptor genes and sperm motility (Hering, Olenski, & Kaminski, 2014). Moreover, in humans, sperm proteome analysis highlighted the insulin pathway in male gametes (Wang et al., 2013), which could be related to sperm motility and the acrosome reaction. In rodents, alterations in sperm insulin metabolism reduced sperm viability and motility (Meneses et al., 2019). Recently, lower plasma insulin concentrations were associated with inferior bull fertility (Weerakoon et al., 2020). We observed some positive insulin effects on sperm motility during cooling. However, the sperm cell pathways associated with these insulin effects on sperm quality and male fertility still need to be elucidated in rams. Molecular studies associated with sperm function analysis can improve the comprehension of the roles of insulin on male gamete physiology.

Conclusion

The addition of 3 IU mL^{-1} insulin in sperm cooling extender improves the sperm motility quality in rams.

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