

# Addition of synthetic amino acids in sheep semen improves its quality after cryopreservation\*

## Adição de aminoácidos sintéticos no sêmen ovino melhora a qualidade espermática após a criopreservação do sêmen

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### Abstract

Some amino acids can protect mammalian sperm cells against oxidation during thermal stress caused by freezing/thawing. Thus, the objective was to evaluate the protective action of the association of the amino acids L-proline (Pro) and L-glutamine (Glu) against the cryoinjury caused to sheep sperm after cryopreservation. Eight ejaculates were collected from four sheep (n=32) and diluted in Tris-Egg Yolk-Glycerol until the final concentration of  $200 \times 10^6$  spz/mL and kept in a water bath at 32 °C. The amino acids were added as follows: control (without adding amino acids), Pro+Glu 1 (100 µM Pro + 500 µM Glu), Pro+Glu 2 (300 µM Pro + 1000 µM Glu), Pro+Glu 3 (500 µM Pro + 1500 µM Glu) and Pro+Glu 4 (700 µM Pro + 2000 µM Glu). Afterwards, the semen was cooled to 5 °C for 2 h, after that period, filled in 0.5 mL straws and then placed under liquid nitrogen vapor (N<sub>2</sub>L), 8 cm from the liquid sheet for 15 min, and then immersed on the N<sub>2</sub>L. The samples were analyzed for sperm motility, plasma membrane and acrosomal membrane integrity, mitochondrial activity and binding test. The variables were subjected to the normality tests (Lilliefors test) and homoscedasticity tests (Cochran and Bartlett test), afterwards the variables of normal distribution were subjected to analysis of variance and the means compared by the Tukey test with a significance level of 5%. The Pro+Glu 3 group exhibited sperm with a greater (P<0.05) motility after thawing. In addition, the highest percentage of plasma and acrosomal membrane integrity were obtained using Pro+Glu 1, Pro+Glu 2 and Pro+Glu 3; and Pro+Glu 2 and Pro+Glu 3, respectively. Amino acids also kept mitochondrial activity high compared to the control, with Pro+Glu 3 resulting in greater activity (P<0.05). Sperm viability was higher (P<0.05) with the use of Pro+Glu 2 and Pro+Glu 3 than in the control. The number of sperm that showed the ability to bind to the egg yolk perivitelline membrane was higher (P<0.05) in semen treated with amino acids. It is concluded that the addition of synthetic amino acids in the semen of sheep before cryopreservation improves sperm quality and fertilization potential and can thus be added in cryopreservation protocols.

**Keywords:** L-proline, L-glutamine, Spermatozoa, Ram, Post-thawed.

### Resumo

Alguns aminoácidos podem proteger as células espermáticas de mamíferos contra a oxidação durante o estresse térmico causado na congelação/descongelação. Dessa forma, objetivou-se avaliar a ação protetora da associação dos aminoácidos L-prolina (Pro) e L-glutamina (Glu) contra as crioinjúrias causadas aos espermatozoides de ovino após a criopreservação. Foram coletados oito ejaculados de quatro carneiros (n=32) e diluídos em Tris-Gema de ovo-Glicerol até a concentração final de  $200 \times 10^6$  spz/mL e, mantidos em banho maria a 32 °C. Os aminoácidos foram adicionados da seguinte forma: controle (sem adição de aminoácidos), Pro+Glu 1 (100 µM Pro + 500 µM Glu), Pro+Glu 2 (300 µM Pro + 1000 µM Glu), Pro+Glu 3 (500 µM Pro + 1500 µM Glu) e Pro+Glu 4 (700 µM Pro + 2000 µM Glu). Depois, o sêmen foi resfriado a 5 °C por 2 h, após esse período, envasado em palhetas de 0,5 mL e então acondicionado sob vapor de nitrogênio líquido (N<sub>2</sub>L), a 8 cm da lâmina líquida por 15 min, e depois imersos no N<sub>2</sub>L. As amostras foram analisadas quanto à motilidade espermática, integridade da membrana plasmática e da membrana acrossomal, atividade mitocondrial e teste de ligação. As variáveis foram submetidas aos testes de normalidade (Teste de Lilliefors) e homocedacidade (Teste de Cochran e Bartlett), posteriormente as variáveis de distribuição normal foram submetidas à análise de variância e as médias comparadas pelo teste de Tukey com nível de significância de 5%. O grupo Pro+Glu 3 exibiu espermatozoides com uma maior (P<0,05) motilidade após o descongelamento. Além disso o maior percentual de integridade da membrana plasmática e acrossomal foram obtidos utilizando Pro+Glu 1, Pro+Glu 2 e Pro+Glu 3; e Pro+Glu 2 e Pro+Glu 3, respectivamente. Os aminoácidos também mantiveram alta a atividade mitocondrial em comparação com o controle, com Pro+Glu 3 resultando numa maior atividade (P<0,05). A viabilidade dos espermatozoides foi maior (P<0,05) com o uso de Pro+Glu 2 e Pro+Glu 3 do que no controle. O número de espermatozoides que apresentaram à capacidade de ligação a membrana perivitelina da gema de ovo foi maior (P<0,05) no sêmen tratado com aminoácidos. Conclui-se que, a adição dos aminoácidos sintéticos no

\*Recebido em 19 de janeiro de 2020 e aceito em 18 de junho de 2020.

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sêmen de ovinos antes da criopreservação melhora a qualidade espermática e o potencial fecundante, podendo assim serem adicionados em protocolos de criopreservação.

**Palavras-chave:** Espermatozoides, L-glutamina, L-prolina Ovinos, Pós-descongelamento.

## Introduction

Conventional cryoprotectants, such as glycerol (Jasko et al., 1992) in the freezing medium, exert toxic chemical and osmotic effects on spermatozoa (Fahy, 1996), reducing motility and viability, thus impairing the fecundity of frozen spermatozoa in artificial insemination programs (Mercado et al., 2009). Thus, there is a need to search for alternative cryoprotectants or methods to minimize the adverse effects of glycerol on spermatozoa. Studies have reported that amino acids exhibit properties that may help protect the sperm cell against damage during the thermal stresses of cryopreservation (Sangeeta et al., 2015). Amino acids appear to benefit sperm metabolic, cryoprotective, oxidative, or osmoregulatory features (Martins-Bessa et al., 2007). However, this protective mechanism is not entirely understood (Sangeeta et al., 2015).

Some amino acids (AA), in particular L-glutamine, have been successfully used to improve sperm quality after cryopreservation in several different species including horses (Trimeche et al., 1999), goats (Ali Al Ahmad et al., 2008; Kundu et al., 2001), and primates (Li et al., 2003). Besides, proline can improve sperm motility, the percentages of live spermatozoa, and acrosome integrity during cryopreservation (Sangeeta et al., 2015). L-glutamine and L-proline also act as antioxidants and reduce lipid peroxidation (Sangeeta et al., 2015). However, the combination of these two amino acids to prevent spermatozoa cryoinjury is not known. In that regard, the addition of amino acids to the diluent medium can help protect the sperm cell against structural damage during thermal stress. Therefore, we aimed to evaluate whether the association of L-glutamine and L-proline amino acids has a protective effect on ovine sperm against cryogenic lesions in cryopreservation.

## Material and methods

### *Ram semen collection and evaluation*

The present study was carried out after the institutional approval of the *Universidade Federal do Vale do São Francisco* (Univasf), under protocol no. 0002/150317, agreeing with the ethical principles of animal experimentation adopted by the Ethics and Deontology Committee on Studies and Research of Univasf.

The experiment was performed during May and June of 2018. The animals were housed in the Sheep farming sector, and the sperm evaluations developed at the *Centro de Pesquisa em Suínos, Espécies Nativas e Silvestre* (CPSENS) at latitude 09°23'55" South and a longitude 40°30'03" West, an altitude of 376 meters, with an annual average rainfall around 300 mm. The region presents average yearly temperature around 27 °C and climate of the Bsh type according to the Köppen-Geiger classification (2007).

We used four adult rams: two Dorper and two Santa Inês breed, that aged between two to four years. We selected the animals by andrological examination. All of them presented sperm characteristics above the minimum standards for the species,

as established by the Brazilian College Journal of Animal Reproduction (CBRA, 2013).

The animals were confined in a facility containing access to natural light, with a size of 2 x 3 m of covered area and a solar area of 2 x 4 m. The average temperature and relative humidity during the experimental period were 28 °C and 47%, respectively. The rams were kept isolated from females, received water *ad libitum*, and food supplied twice a day, with a diet composed of chopped elephant grass (*Pennisetum purpureum*), in addition to concentrated supplementation based on corn bran, soybean bran and mineral mixture. Feed: forage ratio was 60:40 in the form of total mixed diet according to the requirements for animals of this category (NRC 2007).

Eight semen samples were collected from each ram, totaling 32 ejaculates, using an artificial vagina for sheep (Minitub®, Berlin, Germany). Semen was collected from each ram twice a week. After collection, ejaculates were transported in a box to the CPSENS laboratory, in a water bath at 32 °C and appearance was assessed (creamy, milky, opalescent or aqueous), volume (mL), motility (0 to 100%), vigor (1 to 5) and concentration (spermatozoa/mL), according to CBRA recommendations and standards (CBRA, 2013).

Each ejaculate was classified by visual observation according to Mies Filho (1987) in creamy, milky, opalescent, or aqueous, with the volume measured by pipetting. The total and progressive sperm motility were determined using a computerized analysis system (CASA®, Minitub®, Berlin, Germany), equipped with SpermVision® software, using 8 µL subsample of the ejaculate diluted in 40 µL of Tris at 37 °C (Souza et al., 2016).

To evaluate the vigor, we used 10 µL of diluted ejaculate on a heated slide with a coverslip at 37 °C under light microscopy at 100x magnification. The vigor was classified according to the methodology proposed by CBRA (2013), in degree 1 (oscillatory movement), degree 2 (slow movement), degree 3 (intermediate movement), degree 4 (rapid straight progressive movement) and degree 5 (very fast progressive straight movement).

The sperm concentration was determined using the SpermaCue® photometer (Model SMD6, Minitub®, Berlin, Germany) according to the equipment methodology: addition of 4 µL of the ejaculate in 4 mL of distilled water in microcuvettes from the equipment, which was calibrated for the ovine species. Automated reading was performed to obtain the dilution rate for the concentration of 200 x 10<sup>6</sup> spermatozoa/mL.

### *Semen cryopreservation*

After the evaluation, we diluted the ejaculates in Tris-Egg Yolk-Glycerol extender (Souza et al., 2016) to the final concentration of 200 x 10<sup>6</sup> spermatozoa/mL and each ejaculate into five test tubes for Amino acids (AA) treatments: 1) control (no addition of AA); 2) Pro+Glu 1: addition of 100 µM L-Proline (Pro) plus 500 µM L-Glutamine (Glu); 3) Pro+Glu 2: addition of 300 µM Pro plus 1000 µM Glu; 4) Pro+Glu 3: addition of 500 µM Pro plus 1500 µM Glu; and 5) Pro+Glu 4: addition of 700 µM Pro plus 2000 µM Glu.

The tubes (containing the different treatments) were placed into a 100 mL becker with water at 32 °C, and then putting the becker into a cold room at 5 °C for 2 h. After cooling, the samples were stored in 0.5 mL straws, packaged (UltraSeal®, Minitub, Berlin, Germany) and frozen in a styrofoam box at 8 cm above the liquid nitrogen (N<sub>2</sub>L) vapor for 15 min (Souza et al., 2016) before being immersed in N<sub>2</sub>L for storage at -196 °C.

### *Semen thawing and evaluation*

Two straws of each treatment were thawed in a 37 °C automatic thawed machine (Cryofarm®, IMV, Sao Paulo, Sao Paulo, Brazil) for 30 s. We evaluated thawed samples (two straws/treatment) for sperm motility (total and progressive) using CASA, as previously described.

Sperm membrane integrity was performed using two fluorescent probes, Propidium Iodide (PI) and Hoechst 33342 (H33342), as described by Graham et al. (1990). For that, 10 µL of the thawed sample from each treatment were placed into a microtube (1.5 mL), to which 2 µL of each dye (PI and H33342) were added and the samples incubated for 8 min in a 37 °C water bath. After incubation, 10 µL of each sample was placed on a glass slide preheated at 37 °C and the integrity of the plasmatic sperm membrane using a fluorescence microscope (AXIO Image A2®, Carl Zeiss, Berlin, Germany). Sperm were classified as intact (those that present the nucleus stained blue), or injured (those with the pink-colored nucleus), and 200 cells were counted in random fields.

Acrosome integrity was evaluated using fluorochrome (fluorescein isothiocyanate - FITC) conjugated to lecithin (*peanut agglutinin* - PNA) and associated with PI (Souza et al., 2017). This technique allows the differentiation between live or dead sperm with intact or reacted acrosome. The FITC-PNA dye, which stains the cell green, can bind to the outer acrosomal membrane of the sperm being the acrosome reacted or damaged. The PI stains the cell red penetrating the membrane as it is damaged and binding to DNA. A total of 10 µL of a thawed sample of each treatment were added to a microtube (1.5 mL) and 10 µL of each dye (PNA-FITC and PI), then incubated for 20 min in a water bath at 37 °C. After the incubation, 10 µL were removed and placed between the preheated slide at 37 °C to evaluate the acrosomal membrane integrity of the spermatozoa under fluorescence microscopy (AXIO Image A2®, Carl Zeiss, Berlin, Germany).

Spermatozoa were classified into: dead with intact acrosome (when stained red); dead with reacted or damaged acrosome (when stained red and with acrosomal region stained green); alive with intact acrosome (when not observed in fluorescence, only in light field); alive with reacted or damaged acrosome (when stained green). For each sample analyzed, we assessed 200 cells in random fields.

The mitochondrial activity was determined according to Hrudka (1987) by placing 25 µL of the thawed sample of each treatment in a microtube (1.5 mL), adding 25 µL of 3,3'-Diaminobenzidine (DAB) (1 mg/mL Phosphate-Buffered Saline - PBS) and incubating for 1 h in a bath at 37 °C, protected from light. After incubation, a 10 µL drop of semen from each sample was placed to a glass slide and coverslip preheated at 37 °C, and 200 sperm per sample were analyzed were monitored with a Leica optical microscope (DM 750®, Heerbrugg, Switzerland) at 100x

magnification. Spermatozoa were classified according to the four classes scale proposed by Hrudka (1987): Class I (spermatozoa with fully stained middle part, representing high mitochondrial activity/DAB I); Class II: (spermatozoa with colored or active segments and not stained or inactive, with predominance of the active/DAB II); Class III: (spermatozoa with stained or active segments and not stained or inactive, with predominance of inactive/DAB III); Class IV: (spermatozoa with intermediate part completely discolored, without mitochondrial activity/DAB IV).

Samples of 100 µL from each treatment were placed at a microtube (1.5 mL) with 2.5 µL of the SYBR-14/PI (LIVE/DEAD Sperm Viability® - Molecular Probe) dye solution. A total of 8 µL of the solution was placed between a slide and coverslip preheated at 37 °C to evaluate the viability of spermatozoa under fluorescence microscopy (AXIO Image A2®, Carl Zeiss, Berlin, Germany). Spermatozoa were classified as viable (the nucleus of the spermatozoa stained green) and unviable (nuclear region stained red). A minimum 200 cells were counted in random fields from each sample. All cells stain with SYBR-14, permitting cells to be distinguished from egg yolk particles using this protocol, but only non-viable cells stain with PI. SYBR-14 can penetrate the plasmatic membrane of the sperm and color their nuclear region (DNA) by green, and PI penetrates the damaged membrane and stain by red.

The perivitelline membrane binding assay is the ability of ovine sperm to bind the perivitelline membrane of chicken egg yolk (CEPM), as described by Souza et al. (2016). Being, was prepared the perivitelline membranes (PVMs) separating the egg yolk from the albumen. The excess of albumen was removed using a paper towel. The egg yolk, still intact, was placed on a parafilm sheet for the rupture of the membrane and removal of the yolk, then the PMV was washed in Tris diluent, put in a Petri glass dish, soaked at least five times with Tris until the solution cleared, leaving no residuals of egg yolk. The PVM was carefully opened on Tris medium inside the Petri dish and cut into small fragments (1 x 1 cm) using a glass cuvette of the spectrophotometer and a scalpel blade. Each PVM fragment cut was immersed in Tris placed in 1 mL plastic tubes.

The membrane-binding test was performed in test tubes containing the PVMs and inseminated with the addition of 50,000 thawed spermatozoa from each treatment. After insemination, the tubes containing the inseminated PVM were incubated at 37 °C in 5% CO<sub>2</sub> atmosphere for 90 min. Every 30 min, the tubes were slowly stirred so that the membrane remained open. After 70 min of incubation, 10 µL of Hoechst 33342 (1 mg/mL in PBS) was added to each tube to stain the spermatozoa in blue by fluorescence microscopy. At the end of the incubation period, we transferred the PVMs to another tube containing one ml of Tris and washed (addition of 1 mL of Tris followed by shaking and withdrawal) at least five times to remove the spermatozoa that did not bind.

For fluorescence microscopy assessment, we opened the PVMs on a slide to observe it under a fluorescence microscope (AXIO Image A2®, Carl Zeiss, Berlin, Germany). The sperm binding capacity of each treatment was assessed counting the spermatozoa bound to PVM in six random fields of each fragment of PVM, then calculated binding efficiency to PVM as a comparison between the binding capacity of each treatment and the control group.

### Statistical analysis

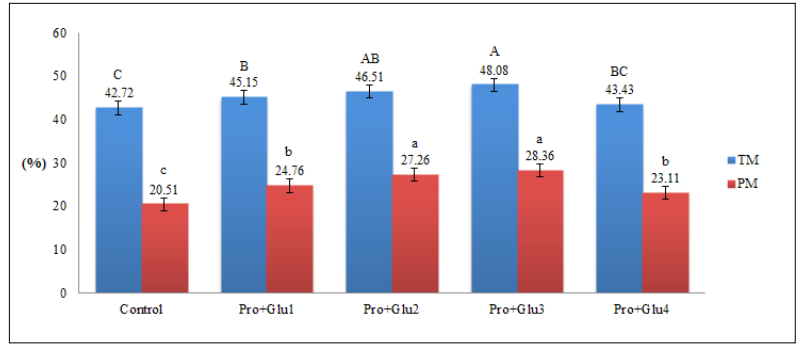
The design was completely randomized block in a 4 x 4 factorial test (4 animals x 4 treatments). The data were analyzed using the program SAS 9.2 by SAS Institute Inc. (Cary, NC, USA). First of all was verified the normality and homoscedasticity by Lilliefors and Cochran and Bartlett test, respectively. Then, the data were analyzed by analysis of variance and the treatment differences separated by the Tukey test ( $P < 0.05$ ). We performed the analysis of variance of the percentage of mobile spermatozoa of each treatment linked to PVM and compared the means by the Tukey test. To find the binding potential of an individual sperm cell, these data were also normalized to take into account the percentage of motile sperm that were added to each PVM, by dividing the number of sperm bound to a PVM by the percentage of motile sperm in that sample.

### Results

Figure 1 displays the effects of the association of amino acids on sperm motility after thawing of ovine spermatozoa. The total and progressive motility of the spermatozoa that received the different concentrations of the synthetic amino acids was higher when compared to the control (Figure 1;  $P < 0.05$ ). Spermatozoa added with 500  $\mu\text{M}$  of L-Proline, and 1500  $\mu\text{M}$  of L-Glutamine (Pro+Glu 3) in the semen presented total and progressive motility higher ( $P < 0.05$ ) than the other associations of the amino acids tested and the control (Figure 1).

The addition of Pro+Glu 1, Pro+Glu 2, and Pro+Glu 3 concentrations showed higher plasmatic membrane integrity (PMI) of spermatozoa compared to control (Table 1;  $P < 0.05$ ). However, the addition of the amino acids Pro+Glu 4 resulted in lower PM than the control (Table 1;  $P < 0.05$ ). The integrity of the acrosomal membrane of the spermatozoa that received the Pro+Glu 2 and Pro+Glu 3 concentrations were higher than in the other treatments (Table 1;  $P < 0.05$ ). The control and Pro+Glu 4 did not differ among themselves (Table 1;  $P < 0.05$ ).

Table 2 displays the results concerning mitochondrial spermatozoa activity after thawing. Spermatozoa receiving different concentrations of synthetic amino acids showed higher mitochondrial activity (DAB I) compared to control (Table 2;  $P < 0.05$ ). The Pro+Glu 3 group presented the highest percentage of DAB I ( $P < 0.05$ ). There was no difference between the tested treatments in the rate of active sperm cells (DAB II, Table 2,  $P < 0.05$ ). Spermatozoa treated with the Pro+Glu 3 solution displayed a lower rate of inactivity after thawing (DAB III) compared to the other treatments and the control ( $P < 0.05$ ; Table 2). However, although DAB II was similar, the addition of Pro+Glu 3 promoted a lower percentage ( $P < 0.05$ ) of inactive cells (DAB III) and without mitochondrial activity (DAB IV) when compared to other treatments (Table 2).



**Figure 1:** Total (TM) and progressive (PM) (mean  $\pm$  P.D.; %) motile spermatozoa of sheep after addition of different concentrations of synthetic amino acids in semen. Different letters indicate differences,  $P < 0.05$ , using the Tukey test.

**Table 1:** Plasmatic membrane (PMI) and acrosomal membrane integrity (AMI) (mean  $\pm$  S.D.; %) of thawed sheep spermatozoa after addition of different concentrations of synthetic amino acids in semen

Treatments	PMI	AMI
Control <sup>1</sup>	56.4 $\pm$ 2.1 <sup>C</sup>	70.7 $\pm$ 3.3 <sup>C</sup>
Pro+Glu1 <sup>2</sup>	60.0 $\pm$ 2.2 <sup>B</sup>	76.6 $\pm$ 3.3 <sup>B</sup>
Pro+Glu2 <sup>3</sup>	67.1 $\pm$ 2.1 <sup>AB</sup>	81.1 $\pm$ 3.4 <sup>A</sup>
Pro+Glu3 <sup>4</sup>	72.4 $\pm$ 2.2 <sup>A</sup>	83.4 $\pm$ 3.3 <sup>A</sup>
Pro+Glu4 <sup>5</sup>	51.2 $\pm$ 2.3 <sup>D</sup>	70.1 $\pm$ 3.1 <sup>C</sup>

<sup>A,B,C,D</sup> Numbers followed by different letters in the same column differ from one another by the Tukey test ( $P < 0.05$ ).

<sup>1</sup> without adding amino acids; <sup>2</sup> 100  $\mu\text{M}$  Pro + 500  $\mu\text{M}$  Glu; <sup>3</sup> 300  $\mu\text{M}$  Pro + 1000  $\mu\text{M}$  Glu; <sup>4</sup> 500  $\mu\text{M}$  Pro + 1500  $\mu\text{M}$  Glu; <sup>5</sup> 700  $\mu\text{M}$  Pro + 2000  $\mu\text{M}$  Glu.

**Table 2:** Mitochondrial activity (%) of thawed ovine spermatozoa after addition of different concentrations of synthetic amino acids in semen

Treatments	DAB I (%)	DAB II (%)	DAB III (%)	DAB IV (%)
Control <sup>1</sup>	124 $\pm$ 3 <sup>D</sup>	18 $\pm$ 3 <sup>A</sup>	18 $\pm$ 3 <sup>A</sup>	40 $\pm$ 3 <sup>A</sup>
Pro+Glu1 <sup>2</sup>	134 $\pm$ 3 <sup>C</sup>	18 $\pm$ 3 <sup>A</sup>	19 $\pm$ 3 <sup>A</sup>	29 $\pm$ 3 <sup>B</sup>
Pro+Glu2 <sup>3</sup>	147 $\pm$ 3 <sup>B</sup>	18 $\pm$ 3 <sup>A</sup>	17 $\pm$ 3 <sup>A</sup>	18 $\pm$ 3 <sup>C</sup>
Pro+Glu3 <sup>4</sup>	161 $\pm$ 3 <sup>A</sup>	20 $\pm$ 3 <sup>A</sup>	10 $\pm$ 3 <sup>B</sup>	9 $\pm$ 3 <sup>D</sup>
Pro+Glu4 <sup>5</sup>	130 $\pm$ 3 <sup>C</sup>	20 $\pm$ 3 <sup>A</sup>	20 $\pm$ 3 <sup>A</sup>	30 $\pm$ 3 <sup>B</sup>

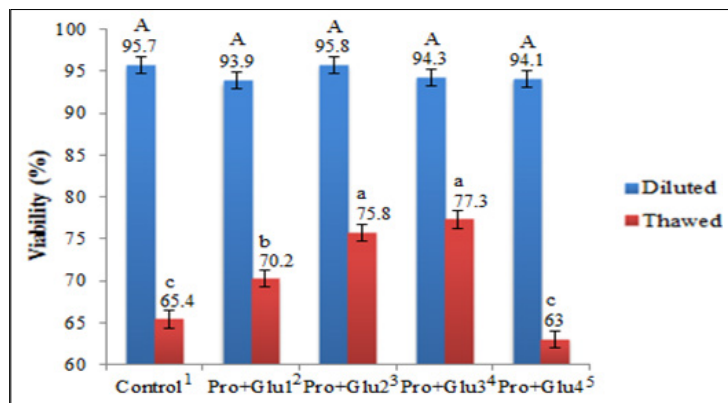
<sup>A,B,C,D</sup> Numbers followed by different letters in the same column differ from one another by the Tukey test ( $P < 0.05$ ).

<sup>1</sup> without adding amino acids; <sup>2</sup> 100  $\mu\text{M}$  Pro + 500  $\mu\text{M}$  Glu; <sup>3</sup> 300  $\mu\text{M}$  Pro + 1000  $\mu\text{M}$  Glu; <sup>4</sup> 500  $\mu\text{M}$  Pro + 1500  $\mu\text{M}$  Glu; <sup>5</sup> 700  $\mu\text{M}$  Pro + 2000  $\mu\text{M}$  Glu.



The sperm viability of the spermatozoa treated with the synthetic amino acids in the Pro+Glu 2 and Pro+Glu 3 concentrations displayed higher than the other treatments tested (Figure 2;  $P < 0.05$ ). It should be highlighted that we assessed the sperm viability after the semen dilution and before semen freezing when we observed no differences in viability between treatments (Figure 2;  $P > 0.05$ ).

Table 3 highlights the addition of synthetic amino acids increased the number of spermatozoa bound to the perivitelline membrane of the egg yolk as compared to the control ( $P < 0.05$ ). Among the different concentrations, Pro+Glu 2 and Pro+Glu 3 provided the highest number of binding cells ( $P < 0.05$ ).



**Figure 2:** Viability (mean  $\pm$  P.D; %) ovine spermatozoa after addition of different concentrations of synthetic amino acids in semen. Different letters indicate differences,  $P < 0.05$ , using the Tukey test.

<sup>1</sup> without adding amino acids; <sup>2</sup> 100  $\mu$ M Pro + 500  $\mu$ M Glu; <sup>3</sup> 300  $\mu$ M Pro + 1000  $\mu$ M Glu; <sup>4</sup> 500  $\mu$ M Pro + 1500  $\mu$ M Glu; <sup>5</sup> 700  $\mu$ M Pro + 2000  $\mu$ M Glu.

**Table 3:** Number (mean  $\pm$  S.D.) of thawed spermatozoa of sheep bound to perivitelline egg yolk membrane (NSB) and their binding efficiency (BEF) after addition of different concentrations of synthetic amino acids in semen

Treatments	NSB	BEF	P value
Control <sup>1</sup>	<b>139,6 <math>\pm</math> 3,4<sup>D</sup></b>	<b>1,0</b>	<b>P&lt;0,05</b>
Pro+Glu1 <sup>2</sup>	<b>176,5 <math>\pm</math> 3,3<sup>B</sup></b>	<b>1,3</b>	
Pro+Glu2 <sup>3</sup>	<b>212,1 <math>\pm</math> 3,4<sup>A</sup></b>	<b>1,5</b>	
Pro+Glu3 <sup>4</sup>	<b>202,3 <math>\pm</math> 3,4<sup>A</sup></b>	<b>1,4</b>	
Pro+Glu4 <sup>5</sup>	<b>155,1 <math>\pm</math> 3,3<sup>C</sup></b>	<b>1,1</b>	

<sup>A,B,C,D</sup> Numbers followed by different letters in the same column differ from one another by the Tukey test ( $P < 0,05$ ).

<sup>1</sup> without adding amino acids; <sup>2</sup> 100  $\mu$ M Pro + 500  $\mu$ M Glu; <sup>3</sup> 300  $\mu$ M Pro + 1000  $\mu$ M Glu; <sup>4</sup> 500  $\mu$ M Pro + 1500  $\mu$ M Glu; <sup>5</sup> 700  $\mu$ M Pro + 2000  $\mu$ M Glu.

## Discussion

Several studies described the benefits of using amino acids on preserving spermatozoa against the damages caused by cryopreservation (Koskinen et al., 1989; Renard et al., 1996; Sanchez-Partidata et al., 1998; Bilodeau et al., 2001, Sangeeta et al., 2015). Our study illustrates that amino acid at different concentrations to sheep semen before the cryopreservation process improves sperm quality.

The addition of 500  $\mu$ M proline and 1500  $\mu$ M glutamine to the semen maintained higher motility after thawing. Sperm cells, contain a large number of densely packed mitochondria around the dense fibers that surround the axoneme, which is responsible for the production of adenosine triphosphate (ATP) and the mitochondrial ATP synthesis depends on the high mitochondrial membrane potential, whose increase in this potential results to improve the mitochondrial function (Perumal et al., 2013). Spermatozoa treated with amino acids (Pro+Glu 3) may have raised intracellular ATP levels, and amino acids are capable of protecting of mitochondria membrane against freeze and thaw damage.

Proline oxidizes to glutamate, which occurs in two enzymatic steps coupled to a non-enzymatic reaction in the mitochondria. The oxidation of proline to  $\Delta^1$ -pyrroline-5-carboxylate (P5C) occurs first by the FAD-dependent enzyme, proline dehydrogenase (PRODH). P5C is a molecule which spontaneously interconverts in semi-aldehyde glutamate (SAG) in the mitochondrial environment by a non-enzymatic reaction. In the second step, the NAD<sup>+</sup>-dependent enzyme  $\Delta^1$ -pyrroline-5-carboxylate dehydrogenase (P5CDH), completes the conversion of proline to glutamate by the catalysis of SAG oxidation. Glutamate by itself is deaminated, entering the Krebs Cycle as  $\alpha$ -ketoglutarate (Tanner, 2008). Thereby, the catalytic mechanism of the PRODH enzyme involves the transfer of electrons from the proline substrate to the FAD cofactor, which in turn is reduced by the electron transport chain II complex. This fact allows the PRODH to transfer electrons to the respiratory chain (Hansford and Sacktor, 1970; Adams and Frank, 1980; Elthon and Stewart, 1982). Thus, the oxidation of proline increases the activity of respiratory complexes, improving the respiratory efficiency of the mitochondria and increasing the synthesis of ATP, consequently, increasing sperm motility. The action of the amino acids on the high mitochondrial activity observed in this study is directly related to the effect of proline.

The damage to plasma membrane integrity causes an increase in the membrane permeability and a reduction in the spermatozoa's ability to control the intracellular passage of ions and small molecules, which destabilize the membrane, therefore directly compromising sperm motility (Baumber et al., 2000). The high concentration of proline and glutamine into the cell and the consequent increase of its osmotic pressure and hypertonicity can explain the effect of different levels of proline and glutamine on membrane integrity (Badr et al., 2015). The increase of the osmotic pressure increases the permeability of the plasmatic membrane. Consequently, the spermatozoa are more prone to the effects of cryoinjury (Shoae and Zamiri, 2008; Ashrafi et al., 2011). Our results also highlighted that Pro+Glu 2 and Pro+Glu 3 concentrations increased cell integrity, as spermatozoa treated with these solutions maintained their plasma and acrosomal membranes intact after the freezing and thawing processes.

This study showed there was an increase in the integrity of the plasmatic and acrosomal membranes when treated with Pro+Glu 1 Pro+Glu 2 and that treatment Pro+Glu 3 increased sperm viability. We observed no deleterious effect on the plasmatic and acrosomal membrane of sheep spermatozoa during the

cryopreservation process by increasing the production of reactive oxygen species (ROS). The amino acids used in this research neutralized the effects of lipid peroxidation, associated with inhibition of membrane rupture and consequent reduction of lipid peroxidation. This effect of the amino acids explains why the use of the concentration of the amino acids Pro+Glu 3 provided higher resistance to oxidative damage and injuries during temperature changes, stabilizing the permeability of the plasmatic membrane and preserving its integrity during cryopreservation.

Several studies associate the protective effect of the amino acids to their ability to form a protective layer on the surface of spermatozoa. These positively charged molecules can electrostatically interact with the phosphate group of phospholipids in the spermatic membrane (Anchordoguy et al., 1988), acting as a barrier against the formation of ice crystals, and preventing thermal shock (Kundu et al., 2001). Amino acids also favor osmolarity in the sperm cell (Billard and Menezo, 1984), promoting the higher maintenance of sperm viability observed in our experiment. Lahnsteiner et al. (1992) noted that amino acids have a stabilizing effect on the cell membrane, preventing membrane injury, and inhibiting plasmolysis (Rudolph and Crowe, 1985). The stabilization provided by amino acids also prevents sperm membrane lipid loss during cryopreservation (Alvarez and Storey, 1983), thus explaining the positive results found.

Proline interacts with the bilayer of phospholipids, stabilizing the structure and function of the plasmatic membrane during freezing (Rudolph et al., 1986), also acts as a principal osmoprotectant, participating in response to osmotic stress (Tonelli et al., 2004). Glutamine plays a regulatory role in several cell-specific processes, including metabolism, cellular integrity, protein synthesis, redox potential, and gene expression (Curi et al., 2005). In this way, glutamine modulated the activation of heat shock proteins (HSP), which are related to the cellular antiapoptotic response (Lester, 2008). Activation of these proteins corresponds to one of the leading signaling ways that contribute to the increase in the cell's ability to survive alterations in its homeostasis due to exposure to stressors, such as ROSs (Lester, 2008). Increased glutamine availability in cells can increase HSP expression, enhancing the cell's ability to resist thermal shock lesions (Fassano, 2011). Accordingly, we can understand the main beneficial effects of the addition of these two amino acids, especially Pro+Glu 3, on the sperm cell that will undergo the cryopreservation process.

Other beneficial effects of amino acids on the cryopreserved sperm parameters of sheep can be attributed to the antioxidant activity that neutralizes ROS and protects them from toxic oxygen metabolites and consequent lipid peroxidation of the plasmatic membrane during cryopreservation (Alvarez and Storey, 1983; Bilodeau et al., 2001). Therefore, possibly, the amino acids proline and glutamine increase the total antioxidative capacity with elevated activity of the antioxidant enzymes.

## Acknowledgments

To FUNCAPE, for the scholarship. To the Center for Research on Swine, Native, and Wild Species (CPSENS) of CPGCA/UNIVASF for all infrastructure (APQ-1072-5.04/12 and APQ-0227-5.04 / 10) and support on conducting the research. The Research and Postgraduation Dean's Office of UNIVASF for the financial support for equipment acquisition (PRPPGi-Univasf N° 17/2013). This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) Finance Code 001.

Amino acids increased sperm cell resistance against oxidative stress since proline activates the action of antioxidant enzymes such as superoxide dismutase and catalase, involved in ROS metabolism, which minimizes peroxidation (Duan et al., 2008; Chutipajit et al., 2009). Glutamine influences the antioxidant system in the intracellular environment, undergoing hydrolysis and increasing the availability of glutamate, which is essential for the synthesis of glutathione (Giacomelli et al., 2009). Thus, the sperm cell is less damaged and has improved sperm quality by the evaluations performed in this study. The amino acids used here and their concentrations tested promoted a protective function against peroxidation of the spermatic membrane during cryopreservation, thus preserving the integrity of the spermatozoa and, consequently, their fertility rate.

Sheep spermatozoa are particularly vulnerable to oxidative stress due to this plasma membrane composition, which has a high ratio of polyunsaturated fatty acids: saturated fatty acids and a lower rate of cholesterol to phospholipids (Maxwell and Watson, 1996). Exposure to drastic changes in temperature, when used in the cryopreservation of semen (Mata-Campuzano et al. 2012), results in apoptosis and DNA damage and compromises their fertilizing capacity (Martinez-Pastor et al. 2009). Although *in vitro* tests cannot reliably assess the spermatozoon's fertility potential (Moce and Graham 2008), they may help to evaluate sperm fertility with low cost, and rapid response (Moraes et al., 2015).

Our results show that the addition of amino acids in the semen increased the sperm binding capacity, especially when using the concentration of 500  $\mu$ M Proline plus 1500  $\mu$ M Glutamine. Thus, we show that proline and glutamine promote improved sperm quality due to the increase in the number of sperm cells that can bind to the perivitelline membrane.

Based on the results obtained, amino acids protect spermatozoa, particularly during cryopreservation. The association of the amino acids tested is responsible for the improvement of antioxidant activity, reduction of lipid peroxidation, preservation of the functional integrity of the plasmatic membrane, and mitochondrial function of spermatozoa.

## Conclusion

The addition of the synthetic amino acids in the concentration in the sheep semen before the cryopreservation improves the sperm quality and the fertilizing potential by the protection caused to the spermatozoa of cryoinjuries.

## Conflict of interest

The authors declare no conflict of interest that could be perceived as prejudicing the impartiality of the present research.

## References

- Adams, E., Frak, L. 1980. Metabolism of proline and the hydroxylprolines. *Annu. Rev. Biochem.* 49, 1005-1061.
- Ali Al Ahmad, M., Chatagnon, G., Amirat-Briand, L., Moussa, M., Tainturier, D., Anton, M., Fieni, F. 2008. Use of glutamine and low density lipoproteins isolated from egg yolk to improve buck semen freezing. *Reprod. Domest. Anim.* 43, 429-436.
- Alvarez, J. G., Storey, B. T. 1983. Taurine, hypotaurine, epinephrine and albumin inhibit lipid peroxidation in rabbit spermatozoa and protect against loss of motility. *Biol. Reprod.* 29, 548-555.
- Anchordoguy, T., Carpenter, J.F., Loomis, S.H., Crowe, J.H. 1988. Mechanisms of interaction of amino acids with phospholipid bilayers during freezing. *Biochim. Biophys. Acta* 946, 299-306.
- Ashrafi, I., Kohram, H., Najjian, H., Bahreini, M., Mirzakhani, H. 2011. Effect of controlled and uncontrolled cooling rate on motility parameters of cryopreserved ram spermatozoa. *BMC Res. Notes* 4, 547.
- Badr, M.R., Rawash, Z.M., Ghada, H., Abd El Rahman, Assi, M.M., Hasan, H.M. 2015. Effect of amino acids on ram sperm freezability, biochemical ultrastructure changes and fertilizing potentials. *Assiut Vet. Med. J.* 61:146.
- Baumber, J., Bll, B.A., Gravance, C.G. 2000. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential and membrane lipid peroxidation. *J. Androl.* 21:6, 895-902.
- Billard, R., Menezo, Y. 1984. The amino acids alanine on Rain-bow trout seminal fluid and blood plasma: a comparison with carp. *Aquaculture, Cryobiology* 46: 17-25.
- Bilodeau, J.F., Blanchette, S., Gagnon, C., Sirard, M.A. 2001. Thiols prevent H<sub>2</sub>O<sub>2</sub>-mediated loss of sperm motility in cryopreserved bull semen. *Theriogenology* 56:275-286.
- Colégio Brasileiro de Reprodução Animal (CBRA). 2013. Manual para exame andrológico e avaliação de sêmen animal. 3.ed. Belo Horizonte, 104p.
- Chutipaijit, S., Suriyan, C., Kanokporn, S. 2009. Differential accumulations of proline and flavonoids in *indicarice* varieties against salinity. *Pak. J. Bot.* 41, 2497-2506.
- Curi, R., Lagranha, C.J., Doi, S.Q., Sellitti, D.F., Procopio, J., Pithon-Curi, T.C., Corless, M., Newsholm, P. 2005. Molecular mechanisms of glutamine action. *J. Cell. Physiol.* 204, 392-401.
- Duan, J.J., Li, J., Guo, S.R., Kang, Y.Y. 2008. Exogenous spermidine affects polyamine metabolism in salinity-stressed *Cucumis sativus* roots and enhances short-term salinity tolerance. *J. Plant Physiol.* 165:1620-1635.
- Elthon, T. E., Stewart, C. R. 1982. Proline Oxidation in Corn Mitochondria: Involvement of NAD, Relationship to Ornithine Metabolism, and Sidedness on the Inner Membrane. *Plant Physiol.* 70: 2, 567-572.
- Fahy, GM. 1996. The relevance of cryoprotectant toxicity to cryobiology. *Cryobiology* 23:1, 1-13.
- Fassano, A. 2011. Zonulin and Its Regulation of Intestinal Barrier Function: The Biological Door to Inflammation, Autoimmunity, and Cancer. *Physiol Rev.* 91, 151-175.
- Giacomelli, F.C., Silveira, N.P., Stepánek, P. 2009. Dynamics of PMMA-PHSA Hard Spheres under External Electric Field at Low Temperatures: a Singular Dynamic Light Scattering Experiment. *Macromolecules.* 42, 3818-3822.
- Graham, J.K., Kunze, E., Hammerstedt, R.H. 1990. Analysis of sperm cell viability, acrosomal integrity, and mitochondrial function using flow cytometry. *Biol. Reprod.* 43(1):55-64.
- Hansford, R. G., Sacktor, B. 1970. The control of the oxidation of proline by isolated flight muscle mitochondria. *J. Biol. Chem.* 245:5, 991-994.
- Hrudka, F. 1987. Cytochemical and ultracytochemical demonstration of cytochrome-c oxidase in spermatozoa and dynamics of changes accompanying ageing or induced by stress. *Int. J. Androl.* 10:6, 809-828.
- Jasko, D.J., Little, T.V., Lein, D.H. 1992. Comparison of spermatozoal movement and semen characteristics, with fertility in stallion: 64 cases (1987-1988). *J Am Vet Med Assoc* 200:979.
- Köppen-Geiger Peel, M.C., Finlayson, B.L., McMahon, T.A. 2007. Updated world map of the Köppen-Geiger climate classification. *Hydrol. Earth Syst. Sci.* 11, 1633-1644.
- Koskinen, E., Junnila, M., Katila, T., Soini, H., 1989. A preliminary study on the use of betaine as a cryoprotective agent in deep freezing of stallion semen. *J. Vet. Med.* 39, 110-114.
- Kundu, C.N., Das, K., Majumder, G.C., 2001. Effect of amino acids on goat cauda epididymal sperm cryopreservation using a chemically defined model system. *Cryobiology* 42, 21-27.
- Lahnsteiner, F., Weismann, T., Patzner, R.A. 1992. Fine structural changes in spermatozoa of the grayling, *Thymallus thymallus* (Pisces: Teleostei), during routine cryopreservation. *Aquaculture* 103,73-84.
- Lester, D.R. 2008. Gluten measurement and its relationship to food toxicity for celiac disease patients. *Plant Methods*, 28, 4-26.
- Li, Y., Si, W., Zhang, X., Dinnyes, A., Ji, W., 2003. Effect of amino acids on cryopreservation of cynomolgus monkey (*macaca fascicularis*) sperm. *Am. J. Primatol.* 59, 159-165.
- Martinez-Pastor, F., Aisen, E., Fernández-Santos, M.R., Estes, M.C., Maroto Morales A., García-Álviz, O., Garde, J.J. 2009. Reactive oxygen species generators affect quality parameters and apoptosis markers differently in red deer spermatozoa. *Reproduction* 137:2, 225-235.
- Martins-Bessa, A., Rocha, A., Mayenco-Aguirre, A. 2007. Incorporation of taurine and hypotaurine did not improve the efficiency of the Uppsala Equex II extender for dog semen freezing. *Theriogenology* 68, 1088-1096.
- Mata-Campuzano, M., Alvarez-Rodríguez, M., Del Olmo, E., Fernández-Santos, M.R., Garde, J.J., Martínez-Pastor, F. 2012. Quality, oxidative markers and DNA damage (DNA) fragmentation of red deer thawed spermatozoa after incubation at 37°C in presence of several antioxidants. *Theriogenology* 78:5, 1005-1019.
- Maxwell, W.M.C., Watson, P.F., 1996. Recent progress in preservation of ram semen. *Anim. Reprod. Sci.* 42, 55-65.
- Mercado, E.D.E., Hernandez, M., Sanz, E., Rodriguez, A., Gomez, E., Vazquez, J.M., Martinez, E.A., Roca, J. 2009. Evaluation of L-glutamine for cryopreservation of boar spermatozoa. *Anim. Reprod. Sci.* 115, 149-157.
- Mies Filho, A. 1987. Inseminação artificial. Porto Alegre: Sulina, v.2, 701p.
- Moce, E., Graham J.K. 2008. In vitro evaluation of sperm quality. *Anim. Reprod. Sci.* 105(1/2):104-118.
- Moraes, E.A., Matos, W.C.G., Graham, J.K., Ferrari Junior, W.D. 2015. Cholesterol-loaded-cyclodextrin improve the quality of stallion spermatozoa after cryopreservation. *Anim. Reprod. Sci.* 158, 19-24.

- National Requirement Council - NRC. 2007. Nutrient Requirements of Small Ruminants: Sheep, Goats, Cervids, and New World Camelids. National Academy of Science, Washington, D.C. 347p.
- Perumal, P., Vupru, K., Rajkhowa, C. 2013. Effect of addition of taurine on the liquid storage (5°C) of mithun (*Bos frontalis*) semen. *Vet. Med. Int.* (4):1-7.
- Renard, P., Grizard, G., Griveau, J.F., Sion, B., Boucher, D., Le Lannou, D., 1996. Improvement of motility and fertilization potential of postthaw human sperm using glutamine. *Cryobiology* 33, 311–319.
- Rudolph, A.S., Crowe, J.H. 1985. Membrane stabilization during freezing: the role of two natural cryoprotectants, trehalose and proline. *Cryobiology* 22: 367-377.
- Rudolph, A.S., Crowe, J.H., Crowe, L.M., 1986. Effects of three stabilizing agents proline, betaine, and trehalose on membrane phospholipids. *Arch. Biochem. Biophys.* 245(1), 134–143.
- Sangeeta, S., Arangasamy, A., Kulkarni, S., Selvaraju, S. 2015. Role of amino acids as additives on sperm motility, plasma membrane integrity and lipid peroxidation levels at pre-freeze and post-thawed ram semen. *Anim. Reprod. Sci.* 161, 82-88.
- SAS System for Windows (Statistical Analysis System), versão 9.2. Cary, USA: SAS Institute Inc; 2002-2008.
- Scanchez-Partidata, L.G., Maxwell, W.M.C., Setchell, B. P., 1998. Effect of compatible solutes and diluents composition on the post-thaw motility of ram sperm. *Reprod. Fertil. Develop.* 10, 347-357.
- Shoae, A., Zamiri, M.J. 2008. Effect of butylated hydroxytoluene on bull spermatozoa frozen in egg yolk-citrate extender. *Anim. Reprod. Sci.* 104(2/4):414-418.
- Souza, W.L., Moraes, E.A., Costa, J.M.S., Sousa, P.H.F., Lopes Junior, E.S., Oliveira, R.P., Toniolli R. 2016. Efeito de diferentes concentrações de melatonina em espermatozoides de carneiros sobre estresse oxidativo após criopreservação. *Pesq. Vet. Bras.* 36(7):657-664.
- Souza, W.L., Moraes, E.A., Toniolli, R. 2017. Adição de antioxidantes ao sêmen de carneiros e seus efeitos após a descongelamento. *Pesqui. Vet. Bras.* 37, 471-478.
- Tanner, J.J. 2008. Structural biology of proline catabolism. *Amino Acids* 35:4, 719-30.
- Tonelli, R. R., Silber, A.M., Almeida-de-Faria, M., Hirata, I.Y., Colli, W., Alves, M.J. 2004. L-proline is essential for the intracellular differentiation of *Trypanosoma cruzi*. *Cell Microbiol.* 6: 8, 733-41.
- Trimeche, A., Yvon, J.M., Vidament, M., Palmer, E., Magistrini, M. 1999. Effects of glutamine, proline, histidine and betaine on post-thaw motility of stallion spermatozoa. *Theriogenology* 52, 181-191.