





Effect of adenosine concentration on quality of cooled ram semen

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ABSTRACT - This study analyzed the adenosine concentration on quality of ram semen in different refrigerated storage times at 5 °C. The design was in blocks with repeated-measures factorial design. Five levels of adenosine and four refrigeration times were considered as fixed effects and the animal as random effect (block). Ram semen was diluted with Andromed[®] diluent (control) and increasing adenosine levels (0.5, 0.75, 1, and 1.5%). The samples were taken from refrigeration after 4, 8, 12, and 16 h and evaluated for progressive rectilinear sperm motility, sperm vigor, and membrane integrity (supravital test). Data were subjected to analysis of variance with Tukey's test. The regression analysis was conducted to evaluate behavior of adenosine levels. Factor interaction for rectilinear progressive motile variable was verified. There was a quadratic effect of progressive rectilinear motility in function of adenosine levels for each cooled storage time studied, with recommended maximum adenosine concentration of 0.81 and 1.04% at 4 and 8 h, respectively. Adenosine also promoted a protective effect on the membrane integrity. Adenosine added to the diluent increases sperm motility and vigor and protects the sperm membrane integrity.

Keywords: nucleoside, oxidative stress, purinergic, reproduction, spermatozoon

Introduction

Artificial insemination with cooled semen has advantages over its use in reproductive programs, since the refrigeration process allows the spermatozoa to remain viable longer than fresh semen due to metabolic reduction (Allai et al., 2015; Falchi et al., 2018). However, normal metabolism of sperm cells promotes the release of metabolites called reactive oxygen species (ROS), and the excessive production of ROS causes a process known as oxidative stress (Maxwell and Watson, 1996), which is aggravated by the refrigeration process (Zalata et al., 1998).

To avoid the oxidative stress caused by refrigerated semen, some studies have been carried out in different animal species testing substances, some with antioxidant potential (Mata-Campuzano et al., 2014; Affonso et al., 2017), aiming at maintaining spermatozoa quality in a diluent for longer duration.

Adenosine is a purinergic nucleoside formed by a glycosidic bond between a purine adenine base and a D-ribose (Polosa, 2002; Polosa and Holgate, 2006). The literature describes nucleotides and nucleosides as participants in male genital system functions and proposes the presence of purine receptors on

the cell surface of this system. These functions would be related to fertility and reproduction control (Fredholm et al., 1996; Rodrigues et al., 2000; Bellezza and Minelli, 2017).

The presence of A₂ adenosine receptors in spermatozoa was evidenced in previous studies (Fraser, 1990; Fraser and Duncan, 1993; Fénichel et al., 1996). These receptor agonists could stimulate the production of cyclic AMP (Liguori et al., 2005). In addition, cyclic spermatogenic AMP would be deeply involved in spermatogenic function control (Tash and Means, 1988), contributing to ATP formation and increasing sperm motility and vigor.

Adenosine could also have a protective effect on sperm plasma membrane as a potential antioxidant, since this nucleoside is released into different tissues under oxidative stress (Masino et al., 1999). Moreover, when these tissues are treated with adenosine and/or its agonists, significant improvement in lipid peroxidation (Yavuz et al., 1997) and recovery from cellular damage (Almeida et al., 2003; Masino et al., 1999) occur.

Adenosine action as an additive in diluent for animal semen has not been identified; therefore, the objective of this study was to evaluate nucleoside concentrations on quality of refrigerated ram semen at different cooled storage times.

Material and Methods

All procedures performed during the execution of this work were in accordance with the ethical principles of animal experimentation of the National Animal Experimentation Council and were approved by local Comissão de Ética no Uso de Animais (CEUA), protocol no. 037/2017 (CEUA) and 23084.011913/2017-21 (UFRA).

The experiment was carried out in Belém, Pará, Brazil, (1°27'15" South and 48°26'50" West, with an altitude of 14 meters). The study was conducted from December 2017 to March 2018.

Six adult rams (Santa Inês breed) presenting a good body condition score (3, in a scale of 1 to 5) were used. Semen collections were performed every three days, yielding a total of six viable ejaculates from each animal, totaling 36 ejaculates.

The samples were taken from males with the aid of estrogen-treated ewes with 1 mg estradiol cypionate (E.C.P - Zoetis®) and appropriately contained. After semen collection, samples were conditioned in a water bath at 37 °C for analysis.

The ejaculates considered for this study had minimum physical patterns of 0.5 mL total volume, sperm vigor of 3, and minimum sperm motility of 80% (CBRA, 2013).

For evaluation of fresh semen, the following parameters were analyzed: volume (mL), appearance (aqueous, opalescent, milky, and creamy), wave motion (scale of 0 to 5), rectilinear sperm motility (%), vigor (scale of 0 to 5), and sperm concentration (spz/mL).

One drop of semen from each ejaculate was placed on a previously heated slide at 37 °C. In a microscope with 10X magnification, the wave motion (spermatogenic mass movement) was evaluated. Subsequently, a coverslip heated at 37 °C was placed on the semen drop to evaluate rectilinear spermatogenic motility and sperm vigor in 400X magnification (CBRA, 2013). All analyses were performed by a trained laboratory technician.

Sperm concentration (spz/mL) was calculated by spectrophotometry (Spectrum Lab 22 PC®), adding 20 µL of fresh semen in 8 mL of saline solution (distilled water and sodium chloride - 3%) in a test tube.

In a 1.5-mL plastic tube containing 1 mL of buffered formal-saline (Hancock, 1956), semen aliquots were conditioned to turbid the solution for spermatozoa morphological analysis by wet preparation. Two hundred cells per slide were assessed using a phase-contrast microscope at 1000X, and sperm defects were determined in percentage (CBRA, 2013).

Sperm viability (membrane damage) were evaluated using eosin-nigrosin stain by supravital test. One hundred cells per slide were assessed and classified as live (% of cells uncolored) and dead (% of cells colored in pink or purple), using a phase-contrast microscope at 1000X (Smith and Murray, 1997). The analysis was conducted according to Mayer et al. (1951) and Swanson and Bearden (1951) methodology.

Following determination of sperm concentration, dilutions of each treatment were performed to maintain a concentration of 50 million spermatozoa per 0.25 mL straw. The semen was diluted with the commercial extender Andromed (Minitube®) (control), and increasing adenosine (Sigma®) levels (0.5, 0.75, 1, and 1.5 %).

Before cooled storage, samples were identified and placed on the semen freezing machine (3000 - TK®) until the refrigeration stage. After 4, 8, 12, and 16 h of cooled storage, samples were conditioned in a 37 °C water bath and evaluated for rectilinear sperm motility, sperm vigor, and supravital test.

The experimental design was in blocks with repeated-measures factorial design. Five levels of adenosine and four refrigeration times were considered as fixed effects and the animal as random effect (block). Data were subjected to the D'Agostino test for analysis of normality. All dependent variables had a normal distribution and were subjected to analysis of variance with comparison of means using Tukey's test. Regression analysis was developed for the different levels of adenosine and cooling times. All analyzes were performed at 5 % level of probability.

The effect of adenosine levels and refrigeration times were evaluated according to the following model:

$$Y_{ijk} = \mu + B_k + \alpha_i + \beta_j + (\alpha \times \beta)_{ij} + e_{ijk}, \quad (1)$$

in which Y_{ijk} is the quantitative response variable, μ is the overall mean, B_k is the block effect (animal), α_i is the fixed effect of adenosine level, β_j is the fixed effect of cooled storage time; $(\alpha \times \beta)_{ij}$ is the interaction effect, and e_{ijk} is the random error.

Results

For fresh semen, all parameters were in agreement with the values recommended by CBRA (2013) for ram ejaculates collected through the artificial vagina. The mean values of volume (mL), appearance, wave motion (0-5), motility (%), sperm vigor (0-5), supravital test (%), sperm concentration ($\times 10^9$ spz/mL), and normal sperm (%) were 1.01 ± 0.17 , 3.44 ± 0.44 , 3.97 ± 0.40 , 82.78 ± 3.07 , 4.08 ± 0.40 , 85.06 ± 6.27 , 3.85 ± 0.58 , and 74.24 ± 18.65 , respectively.

The effects of adenosine levels and refrigeration times showed interaction for variable progressive rectilinear motility. After cooled storage for 4 h, adenosine concentrations of 0.5, 0.75, and 1% resulted in higher values ($P \leq 0.05$) of sperm motility compared with other treatments. At the end of 8 h of refrigeration, all treatments containing adenosine were superior ($P \leq 0.05$) to the control. At 12 and 16 h of refrigerated storage at 5 °C, higher values ($P \leq 0.05$) of sperm motility were observed in treatments of 0.5 and 0.75% adenosine addition (Table 1).

Table 1 - Mean values and standard deviations of rectilinear sperm motility of cooled ram semen according to adenosine concentration and cooled storage time at 5 °C

Cooling time (°C)	Adenosine concentration				
	0%	0.5%	0.75%	1%	1.5%
4 h	59.6±9.08cA	69.3±8.54abA	71.0±7.87aA	66.8±8.03abA	63.1±8.96bcA
8 h	50.9±8.18bB	58.8±12.60aB	61.2±13.48aB	58.2±12.70aB	59.3±11.39aA
12 h	40.9±12.40dC	51.6±12.56abC	56.6±9.76aB	48.7±11.96bcC	43.4±15.99cdB
16 h	34.6±13.83bC	38.3±9.85abD	43.4±10.03aC	34.0±12.30bD	33.5±13.55bC

Means followed by different lowercase letters in the same row differ from each other ($P \leq 0.05$) by Tukey's test.

Means followed by different uppercase letters in the same column differ from each other ($P \leq 0.05$) by Tukey's test.

Rectilinear spermatic motility in function of adenosine concentration showed a quadratic effect ($P \leq 0.05$) during the four studied refrigeration times, with maximum point referring to 0.81, 1.04, 0.77, and 0.67% adenosine addition at 4, 8, 12, and 16 h of cooled storage, respectively (Figure 1).

No significant interaction between adenosine levels and refrigeration time effect for sperm vigor and supravital test was observed. In view of this, the effect of each factor was studied separately.

Higher values ($P \leq 0.05$) were recorded for sperm vigor and supravital test, in the treatments containing 0.5 and 0.75% adenosine compared with the others, and equal between these two treatments ($P > 0.05$) (Table 2). The supravital test and sperm vigor displayed a quadratic effect, showing an optimal adenosine level of 0.82 and 0.67%, respectively (Figures 2 and 3).

Discussion

Fresh semen values were similar to those observed in experiments that used Santa Inês breed in the same location (Maia et al., 2011; Frazão Sobrinho et al., 2014).

Adenosine monophosphate-activated protein kinase (AMPK) is located in the head and the midpiece of the sperm (Zhu et al., 2018), and its presence promotes adenosine rephosphorylation to form adenosine monophosphate (AMP) (Rodrigues et al., 2000). Therefore, adenosine added to the dilution at 0.5 to 1% concentrations may have favored AMP formation, with consequent energy production, observed by the increase in motility and sperm vigor in the different refrigeration times studied. The increase in the values of these variables should provide an increase in fertilization rate, since fertilizing capacity and sperm hyperactivation closely depend on the production of adenosine triphosphate (ATP) in midpiece of the spermatozoon.

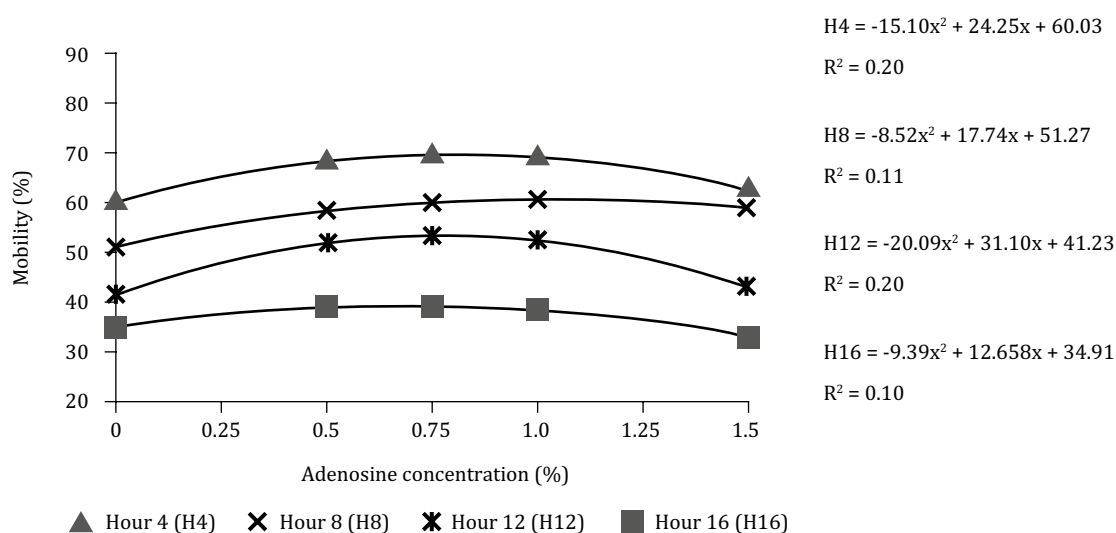


Figure 1 - Rectilinear progressive motility at each cooled storage time in function of adenosine concentrations.

Table 2 - Mean values and standard deviations of spermatic vigor and supravital test of cooled ram semen according to cooled storage time at 5 °C

Parameter	Adenosine concentration				
	0%	0.5%	0.75%	1%	1.5%
Spermatic vigor	2.42±2.42b	2.72±2.72a	2.81±2.81a	2.49±2.49b	2.36±2.36b
Supravital test	47.47±14.13c	55.17±15.12a	58.15±14.17a	50.96±14.69b	51.17±15.18b

Means followed by different letters in the same row differ from each other ($P \leq 0.05$) by the Tukey test.

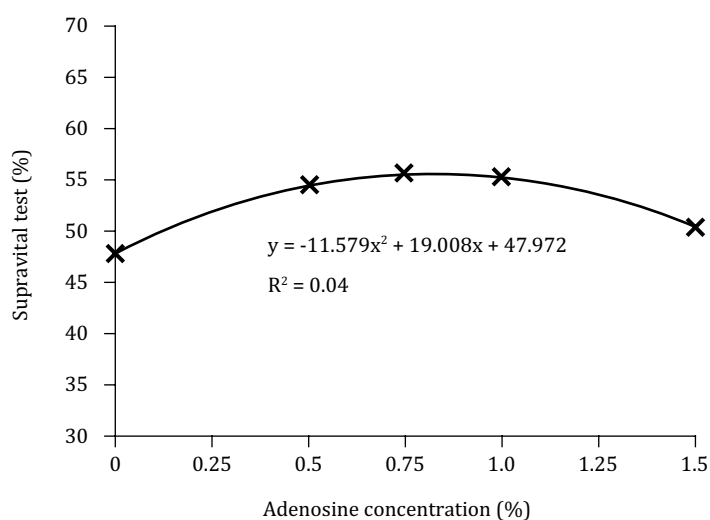


Figure 2 - Supravital test in function of adenosine concentrations.

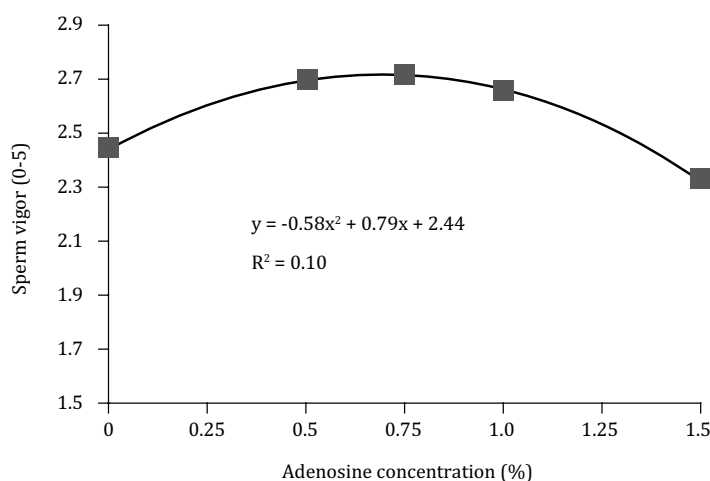


Figure 3 - Sperm vigor in function of adenosine concentrations.

An increase in ATP production and sperm motility as well as an improvement in membrane integrity and in the acrosome reaction was observed in goat sperm when researchers added acadesin (AMP analog) in goat semen dilution (Zhu et al., 2018). Other authors observed that AMP produced by adenosine kinase has effects on sperm motility of mice (Vadnais et al., 2013).

The beneficial adenosine effect on maintaining the quality of cooled ram semen may also be related to the presence of A_2 adenosine receptors in spermatozoa, as evidenced in previous studies (Fraser, 1990; Fraser and Duncan, 1993; Fénichel et al., 1996).

A_2 receptor agonists of this nucleoside could stimulate the cyclic AMP production in human (Liguori et al., 2005) and mouse (Fraser and Duncan, 1993) spermatozoa. Cyclic spermatoc AMP would be deeply involved in spermatoc function control (Tash and Means, 1988), as well as in motility control and fertilization capacity (Harrison, 2003). Shen et al. (1993) observed sperm motility increase in humans following the administration of an A_2 -specific receptor agonist. Thus, purinergic nucleoside addition may work as an A_2 agonist, which contributes to ATP formation, increasing sperm motility and vigor up to 16 h on refrigeration compared with the control treatment.

Likewise, adenosine presence, mainly at 0.5 and 0.75% concentrations, prolonged the spermatozoa quality subjected to refrigeration up to 16 h. On the other hand, when adenosine was added in a higher

proportion (1.5%), at 4, 12, and 16 h of refrigeration, there may have been a refractory effect on the spermatic cell metabolism, causing a negative effect.

An adenosine protective effect on integrity of sperm plasma membrane was observed, evidenced by the supravital test. This finding corroborates Masino et al. (1999) and Almeida et al. (2003). According to Almeida et al. (2003), adenosine also promotes induced neural lesion recovery by ROS. Thus, purinergic nucleoside addition may have functioned as a potential antioxidant, improving sperm quality.

Conclusions

Adenosine added to the diluent, mainly at 0.5 to 0.75%, increases sperm motility and vigor and protects the sperm membrane integrity. We recommend a short-medium term for refrigerated storage to maintain semen quality.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: E.F. Castilho. Data curation: T.E.C. Nascimento, N.L.C. Gonçalves and S.M.B.S. Silva. Formal analysis: A.S. Bezerra. Funding acquisition: L.F.S. Rodrigues. Investigation: E.F. Castilho and A.S. Cardoso. Methodology: T.E.C. Nascimento, E.F. Castilho, N.L.C. Gonçalves, S.M.B.S. Silva and A.S. Cardoso. Project administration: E.F. Castilho and L.F.S. Rodrigues. Resources: A.S. Bezerra and A.S. Cardoso. Software: A.S. Bezerra. Supervision: L.F.S. Rodrigues. Writing-original draft: A.S. Bezerra. Writing-review & editing: A.S. Bezerra, H.K.R. Souza and L.F.S. Rodrigues.

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