Effect of adenosine concentration on quality of cooled ram semen

Andréia Santana Bezerra1*, Thialla Emille Costa do Nascimento2, Erick Fonseca de Castilho2, Natália Larissa Cunha Gonçalves2, Sillas Matteus Batista Soares da Silva2, Aline Serrão Cardoso2, Heloíse Karine Rolim de Souza2, Luiz Fernando de Souza Rodrigues2

1 Universidade Federal do Pará, Instituto de Medicina Veterinária, Castanhal, PA, Brasil.  
2 Universidade Federal Rural da Amazônia, Instituto de Saúde e Produção Animal, Belém, PA, Brasil.

*Corresponding author: andreiazootecnistaufra@gmail.com
Received: May 29, 2019  
Accepted: August 25, 2019

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 motility 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 A1 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 spermatic AMP would be deeply involved in spermatic 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 (spermatic mass movement) was evaluated. Subsequently, a coverslip heated at 37 °C was placed on the semen drop to evaluate rectilinear spermatic 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:

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

in which \( Y_{ijk} \) is the quantitative response variable, \( \mu \) is the overall mean, \( B_k \) is the block effect (animal), \( \alpha_i \) is the fixed effect of adenosine level, \( \beta_j \) 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 (x10^9 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≤0.05) of sperm motility compared with other treatments. At the end of 8 h of refrigeration, all treatments containing adenosine were superior (P≤0.05) to the control. At 12 and 16 h of refrigerated storage at 5 °C, higher values (P≤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)               | 0%       | 0.5%     | 0.75%    | 1%       | 1.5%     |
| 4 h                             | 59.6±0.80cA | 69.3±0.85aB | 71.0±0.87aA | 66.8±0.83abA | 63.1±0.96bA |
| 8 h                             | 50.9±1.18bB | 58.8±1.22bB | 61.2±1.34aB | 58.2±1.27aB | 59.3±1.39aA |
| 12 h                            | 40.9±1.24cC | 51.6±1.25aB | 56.6±1.79abB | 48.7±1.19bcB | 43.4±1.59dB |
| 16 h                            | 34.6±1.33bC | 38.3±1.95abD | 43.4±1.03aC | 34.0±1.23bD | 33.5±1.35bC |

Means followed by different lowercase letters in the same row differ from each other (P≤0.05) by Tukey’s test. Means followed by different uppercase letters in the same column differ from each other (P≤0.05) by Tukey’s test.
Rectilinear spermatic motility in function of adenosine concentration showed a quadratic effect (P≤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≤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 spermatozon.

![Figure 1 - Rectilinear progressive motility at each cooled storage time in function of adenosine concentrations.](image)

![Figure 2 - Adenosine monophosphate-activated protein kinase (AMPK) in the head and midpiece of the sperm.](image)

![Figure 3 - Adenosine monophosphate-activated protein kinase (AMPK) in the head and midpiece of the sperm.](image)

**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≤0.05) by the Tukey test.
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 spermatic AMP would be deeply involved in spermatic 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.

**Acknowledgments**

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) – Brasil (Finance Code 001).

**References**

Affonso, F. J.; Carvalho, H. E.; Lançoni, R.; Lemes, K. M.; Leite, T. G.; Oliveira, L. Z.; Celeghini, E. C. C. and Arruda, R. P. 2017. Addition of antioxidants myo-inositol, ferulic acid, and melatonin and their effects on sperm motility, membrane integrity, and reactive oxygen species production in cooled equine semen. Journal of Equine Veterinary Science 59:57-63. https://doi.org/10.1016/j.jevs.2017.09.006

Allai, L.; Druart, X.; Contell, J.; Louanjli, N.; Moula, A. B.; Badi, A.; Essamadi, A.; Nasser, B. and El Amiri, B. 2015. Effect of argan oil on liquid storage of ram semen in Tris or skim milk based extenders. Animal Reproduction Science 160:57-67. https://doi.org/10.1016/j.anireprosci.2015.07.003

Almeida, C. G.; Mendonça, A.; Cunha, R. A. and Ribeiro, J. A. 2003. Adenosine promotes neuronal recovery from reactive oxygen species induced lesion in rat hippocampal slices. Neuroscience Letters 339:127-130. https://doi.org/10.1016/S0304-3940(02)01478-7

Bellezza, I. and Minelli, A. 2017. Adenosine in sperm physiology. Molecular Aspects of Medicine 55:102-109. https://doi.org/10.1016/j.mam.2016.11.009

CBRA - Colégio Brasileiro de Reprodução Animal. 2013. Manual para exame andrológico e avaliação de sêmen animal. 3.ed. CBRA, Belo Horizonte.

Falchi, L.; Galleri, G.; Zedda, M. T.; Pau, S.; Bogliolo, L.; Ariu, F. and Ledda, S. 2018. Liquid storage of ram semen for 96 h: Effects on kinematic parameters, membranes and DNA integrity, and ROS production. Livestock Science 207:1-6. https://doi.org/10.1016/j.livsci.2017.11.001

Fénichel, P.; Gharib, A.; Emiliozzi, C.; Donzeau, M. and Ménézo, Y. 1996. Stimulation of human sperm during capacitation in vitro by an adenosine agonist with specificity for A2 receptors. Biology of Reproduction 54:1405-1411. https://doi.org/10.1095/biolreprod54.6.1405
Fraser, L. R. and Duncan, A. E. 1993. Adenosine analogues with specificity for A₁ receptors bind to mouse spermatozoa and stimulate adenylyl cyclase activity in uncapacitated suspensions. Journal of Reproduction and Fertility 98:187-194. https://doi.org/10.1530/jrf.0.0980187

Fraser, L. R. 1990. Adenosine and its analogues, possibly acting at A₁ receptors, stimulate mouse sperm fertilizing ability during early stages of capacitation. Journal of Reproduction and Fertility 89:467-476. https://doi.org/10.1530/jrf.00890467

Frazão Sobrinho, J. M.; Castelo Branco, M. A.; Sousa Júnior, A.; Nascimento, I. M. R.; Mota, L. H. C. M.; Carvalho, Y. N. T.; Ferreira, S. B.; Costa, D. N. M.; Moraes Júnior, F. J. and Souza, J. A. T. 2014. Características dos sêmen de carneiros Dorper, Santa Inês e sem padrão racial definido, pré e pós-congelação, nos períodos chuvoso e seco. Arquivo Brasileiro de Medicina Veterinária e Zootecnia 66:969-976. https://doi.org/10.1590/1678-6465

Fredholm, B. B.; Burnstock, G.; Harden, T. K. and Spedding, M. 1996. Receptor nomenclature. Drug Development Research 39:461-466. https://doi.org/10.2174/0169411993903040115

Harrison, R. A. P. 2003. Cyclic AMP signalling during mammalian sperm capacitation - Still largely Terra Incognita. Reproduction in Domestic Animals 38:102-110. https://doi.org/10.1046/j1439-0531.2003.00400.x

Ligouri, L.; de Lamiranra, E.; Minelli, A. and Gagnon, C. 2005. Various protein kinases regulate human sperm acrosome reaction and the associated phosphorylation of Tyr residues and of the Thr-Glu-Tyr motif. Molecular Human Reproduction 11:211-221. https://doi.org/10.1093/molehr/gah154

Maia, M. S.; Medeiros, I. M. and Lima, C. A. C. 2011. Características reprodutivas de carneiros no Nordeste do Brasil: parâmetros seminais. Revista Brasileira de Reprodução Animal 35:175-179.

Masino, S. A.; Mesches, M. H.; Bickford, P. C. and Dunwiddie, T. V. 1999. Acute peroxide treatment of rat hippocampal slices induces adenosine-mediated inhibition of excitatory transmission in area CA1. Neuroscience Letters 274:91-94. https://doi.org/10.1016/S0304-3940(99)00693-X

Mayer, D. T.; Squiers, C. D.; Bogart, R. and Oloufa, M. M. 1951. The technique for characterizing mammalian spermatozoa as dead or living by differential staining. Journal of Animal Science 10:226-235. https://doi.org/10.2527/jas1951.104981x

Polosa, R. 2002. Adenosine-receptor subtypes: Their relevance to adenosine-mediated responses in asthma and chronic obstructive pulmonary disease. European Respiratory Journal 20:488-496. https://doi.org/10.1183/09031936.02.01132002

Polosa, R. and Holgate, S. T. 2006. Adenosine receptors as promising therapeutic targets for drug development in chronic obstructive pulmonary disease. European Respiratory Journal 20:488-496. https://doi.org/10.1183/09031936.02.01132002

Shen, M. R.; Linden, J.; Chen, S. S.; and Wu, S. N. 1993. Identification of adenosine receptors in human spermatozoa. Clinical and Experimental Pharmacology and Physiology 20:527-534. https://doi.org/10.1111/j.1440-1681.1993.tb0736x

Smith, J. F. and Murray, G. R. 1997. Evaluation of different staining techniques for determination of membrane status in spermatozoa. In: Proceedings of the New Zealand Society of Animal Production 57:246-250.

Swanson, E. W. and Bearden, H. J. 1951. An eosin-nigrosin stain for differentiating live and dead bovine spermatozoa. Journal of Animal Science 10:981-987. https://doi.org/10.2527/jas1951.104981x

Tash, J. S. and Means, A. R. 1988. cAMP-dependent regulatory processes in the acquisition and control of sperm flagellar movement. Progress in Clinical and Biological Research 267:335-355.

Vadnais, M. L.; Aghajanian, H. K.; Lin, A. and Gerton, G. L. 2013. Signaling in sperm: Toward a molecular understanding of the acquisition of sperm motility in the mouse epididymis. Biology of Reproduction 89:1-10. https://doi.org/10.1095/biolreprod.111.110163

Yavuz, O.; Türkściçek, N.; Bilgihan, A.; Dogulu, F. and Aykol, S. 1997. The effect of 2-chloroadenosine on lipid peroxide level during experimental cerebral ischemia-reperfusion in Gerbils. Free Radical Biology and Medicine 22:337-341. https://doi.org/10.1016/S0891-5849(97)82138-7

Zalata, A. A.; Christophe, A. B.; Depuydt, C. E.; Schoonjans, F. and Comhaire, F. H. 1998. White blood cells cause oxidative damage to the fatty acid composition of phospholipids of human spermatozoa. International Journal of Andrology 21:154-162. https://doi.org/10.1111/j.1365-2605.1998.00112.x

Zhu, Z.; Li, R.; Ma, G.; Bai, W.; Fan, X.; Lv, Y.; Luo, J. and Zeng, W. 2018. 5'-AMP-Activated protein kinase regulates goat sperm functions via energy metabolism In vitro. Cellular Physiology and Biochemistry 47:2420-2431. https://doi.org/10.1159/000491616