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Cooling of equine semen at 16°C for 36 hours with addition of different glutathione concentrations

Refrigeração do sêmen equino a 16ºC por 36h com adição de glutationa em diferentes concentrações

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Abstract

Handling equine semen during the refrigeration process reduces sperm viability, and consequently causes membrane lipid peroxidation, among other challenges. The present study aimed to evaluate the *in vitro* effects of glutathione (control, 1. 0, 1. 5, and 2. 5 mM) on equine semen in a cooled protocol of 16ºC for 36 hours. The following variables were evaluated after 0, 12, 24, and 36 hours refrigeration: total sperm motility, vigor, viability, and plasma and acrosomal membrane integrity. Motility was higher with 2. 5mM of glutathione (57. 8 ± 7. 3) after 12 hours of refrigeration compared to the control (53. 2 ± 8. 3) (P < 0. 05). After 36 hours of refrigeration, motility was higher with 1. 5 mM (43. 4 ± 12. 7) and 2. 5mM glutathione (45. 5 ± 6. 2), than it was with 1mM glutathione (38. 2 ± 9) and the control (35. 5 ± 18. 4) (P < 0. 05), respectively. The strength was highest with 1. 5mM glutathione (3. 7 ± 0. 3) after 36 hours compared to the control (3. 2 ± 1. 1), (P < 0. 05). Viability differed between control and 1mM treatments (79. 5 ± 1. 8) only after 24 hours (75. 5 ± 9. 7) (P < 0. 05). Throughout the investigation, no significant differences were noted in plasma and acrosomal membrane integrity (P > 0. 05). The 1. 5 and 2. 5mM glutathione levels were more efficient in protecting sperm cells and yielded higher total motility values after 36 hours of refrigeration.

Key words: Antioxidant, sperm, stallion, cooled semen

Resumo

A manipulação do sêmen equino durante o processo de refrigeração reduz a viabilidade espermática em consequência, entre outros problemas, da peroxidação de lipídios da membrana. Objetivou-se avaliar o efeito *in vitro* da adição de glutationa (controle; 1,0; 1,5 e 2,5mM) em protocolo de refrigeração a 16ºC por 36h. As variáveis avaliadas foram motilidade total, vigor, viabilidade espermática e integridade das membranas plasmática e acrossomal em quatro momentos diferentes de refrigeração (0, 12, 24 e 36h). A motilidade foi superior com 2,5mM de glutationa (57,8±7,3) com 12h de refrigeração quando comparado ao controle (53,2±8,3), P<0,05. Com 36h de refrigeração a motilidade foi superior com 1,5mM (43,4±12,7) e 2,5mM (45,5±6,2) quando comparado com 1mM (38,2±9) e ao controle (35,5±18,4), P<0,05. Com relação ao vigor houve superioridade com 1,5mM de glutationa (3,7±0,3), na avaliação de 36h, em comparação ao controle (3,2±1,1), P<0,05. Para viabilidade só houve diferença em relação ao controle na avaliação de 24h (75,5±9,7) quando comparado ao tratamento 1mM (79,5±1,8), P<0,05. Não houve diferença em relação ao controle para as avaliações de integridade de membrana plasmática e acrosomal, em todos os momentos de avaliação, P>0,05. As concentrações de 1,5 e 2,5MM de glutationa foram mais eficientes para proteção da célula espermática com valores superiores de motilidade total na avaliação de 36h.

Palavras-chave: Antioxidante, espermatozoide, garanhão, sêmen refrigerado

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Introduction

Cooling of equine semen has been the focus of several studies because of the need to optimize fertility for as long as possible, to facilitate greater flexibility in veterinary semen collection, transportation, and insemination at the most opportune times (HECKENBICHLER et al., 2011). The advantages of using cooled semen include reductions in the expenses of animal transportation and accommodation, the stress of transportation, the risk of vehicular accidents, and the incidence of disease from exposure to the pathogens of a new environment (BOWEN, 2011).

The cooling process reduces sperm viability and overall fertility, and induces membrane lipid peroxidation due to high polyunsaturated fatty acid content that makes cells highly susceptible to the action of free radicals and reactive oxygen species (ROS), among other challenges (COCCHIA et al., 2011).

Oxidative effects caused by ROS can be reduced by adding antioxidants to seminal plasma or extenders used for freezing (BAUMBER et al., 2005; OLIVEIRA et al., 2013; ZHANDI; GHADIMI, 2014). Glutathione (ɣ-L-glutamyl-L-cysteinylglycine), which is a tripeptide thiol, widely found throughout the animal body in both somatic cells and gametes, with numerous biological functions, is one of the antioxidants incorporated into the semen of some species. This thiol plays an important role in the antioxidation of endogenous and exogenous compounds, and in maintaining intracellular redox conditions. Glutathione levels represent a natural reservoir of reducing agents, which can be rapidly used by cells as a defense against oxidative stress (LUBERDA, 2005).

Glutathione is synthesized from glutamate, cysteine, and glycine amino acids. Its reducing power is used to maintain thiol groups in intracellular proteins and in other molecules. It acts as a physiological reservoir of cysteine and is involved in regulating protein synthesis, cellular detoxification, and leukotriene synthesis (LUBERDA, 2005).

Recent studies on cooling and freezability of equine semen at 5ºC have shown the beneficial effects of glutathione including improved motility, viability, and membrane integrity (OLIVEIRA et al., 2013; ZHANDI; GHADIMI, 2014). Thus, the present study aimed to evaluate the *in vitro* effects of adding glutathione to equine sperm during refrigeration at 16ºC for 36 hours.

Materials and Methods

Twelve stallions (American Quarter Horse and Mangalarga Marchador breeds) aged between four and eight years old were selected and maintained under the same feeding regimen and kept in the metropolitan region of Goiânia, GO, Brazil, from March to May.

After depleting the extragonadal reserves, semen was collected from the stallions every other day, obtaining 36 ejaculates using a Colorado model artificial vagina, with a nylon filter coupled to a collection cup to obtain the gel-free fraction. All stallions exhibited ≥60% total motility and ≥4 strength immediately after collection (CBRA, 2013).

Addition of antioxidant and sample refrigeration

Immediately after collection, the gel-free ejaculate was centrifuged at 600 × *g* for 10 minutes. The resulting sperm pellet was resuspended in a skim milk, glucose, sodium bicarbonate, and amikacin extender (Botu-sêmen®, Biotech Botucatu, Botucatu/SP), containing 0mM (control), 1. 0 mM, 1. 5 mM, and 2. 5 mM of glutathione – G6013 (Sigma Chemical CO, USA), at a final concentration of 50 × 10^6 sperm/mL. The pH of each concentration was measured and did not differ from that of the control. After dilution and homogenization, the semen was cooled in semen transport boxes (Max-sêmen Express®) and kept at 16ºC.

Sperm quality analyses

The following variables were evaluated at four intervals (0, 12, 24, and 36 hours): total motility, strength, sperm viability, and plasma and acrosomal membrane integrity. At each evaluation interval, the samples were removed from the cooling boxes and stabilized in a water bath at 37ºC for five minutes, and the respective *in vitro* analyses were conducted.

Total motility and strength

The percentage of mobile sperm and their velocity were evaluated under a phase contrast optical microscope at 200× magnification (CBRA, 2013).

Sperm viability and plasma membrane integrity

Supravital staining with eosin-nigrosin stain was used to evaluate sperm viability. Immediately after drying the slide, analysis at 1000× magnification was performed by counting 500 sperm and classifying those unstained as having an intact membrane (DOTT; FOSTER, 1972). The hypo-osmotic swelling test was performed with a 100 mOsm/L sucrose solution to evaluate the plasma membrane integrity of sperm (MELO; HENRY, 1999).

Acrosomal membrane integrity

Trypan Blue/Giemsa staining was used to evaluate acrosomal membrane integrity (KÚTVÖLGYI et al., 2006).

Statistical analysis

The investigation was completely randomized with a 4 × 4 factorial scheme. Statistical analysis of the data was performed using the UNIVARIATE procedure (SAS, 2000) to determine whether the experimental errors of the variables exhibited normal probability distribution and homogeneity of variance. As the variables under investigation did not exhibit normal distribution, non-parametric analysis was performed using the Kruskal-Wallis H test, through the H-Test procedure of the WinStat® software package (FITCH, 2006) ($P < 0.05$) and the results were presented as means ± standard deviation.

Results

Reduced motility was noted for all treatments during the evaluation period. However, the 1.5 and 2.5 mM glutathione concentrations were comparatively more effective after 36 hours of cooling, with higher total motility results than other treatment concentrations (Table 1). Motility was higher with 2.5 mM glutathione (57.8 ± 7.3) after 12 hours of cooling in comparison to the control (53.2 ± 8.3) ($P < 0.05$), but did not differ significantly from other treatments. After 36 hours of cooling, motility was higher with the concentrations 1.5mM (43.4 ± 12.7) and 2.5mM (45.5 ± 6.2), in comparison to 1mM glutathione (38.2 ± 9) and the control (35.5 ± 18.4) ($P < 0.05$), respectively. The greatest strength was noted with 1.5mM glutathione (3.7 ± 0.3) after 36 hours of refrigeration in comparison to the control (3.2 ± 1.1) ($P < 0.05$).

Table 2 shows the results of sperm viability following supravital staining. Sperm viability differed ($P < 0.05$) between the control and the 1 mM treatment (79.5 ± 1.8) after 24 hours of cooling (75.5 ± 9.7).

No significant differences were noted in plasma and acrosomal membrane integrity at any of the intervals under evaluation ($P > 0.05$) (Table 3).

Table 1. Effect of different glutathione concentrations on total motility (0-100%) and strength (0-5) of equine semen cooled at 16ºC for different intervals (mean ± standard deviation).

Treatment (mM)	Storage duration (h)			
	0	12	24	36
	% Total Motility			
0	64. 8 ± 6. 0^{a1}	53. 2 ± 8. 3^{a2}	53. 5 ± 7. 2^{ac2}	35. 5 ± 18. 4^{a3}
1	66. 3 ± 5. 4^{a1}	56. 0 ± 5. 6^{ab2}	49. 4 ± 7. 3^{bc3}	38. 2 ± 9. 0^{a4}
1. 5	58. 9 ± 7. 3^{b1}	55. 1 ± 5. 5^{ab1}	46. 2 ± 7. 9^{b2}	43. 4 ± 12. 7^{b2}
2. 5	59. 1 ± 6. 9^{b1}	57. 8 ± 7. 3^{b1}	51. 3 ± 11. 2^{c2}	45. 5 ± 6. 2^{b3}
	Strength (0-5)			
0	4. 2 ± 0. 4^{a1}	3. 8 ± 0. 5^{ab2}	3. 6 ± 0. 5^{a2}	3. 2 ± 1. 1^{a3}
1	3. 8 ± 0. 5^{b1}	3. 7 ± 0. 5^{ab1}	3. 4 ± 0. 5^{ab2}	3. 4 ± 0. 7^{ab2}
1. 5	3. 7 ± 0. 4^{b1}	3. 6 ± 0. 5^{a1}	3. 2 ± 0. 4^{b2}	3. 7 ± 0. 3^{b1}
2. 5	3. 8 ± 0. 3^{b1}	3. 8 ± 0. 4^{b1}	3. 2 ± 0. 5^{b2}	3. 4 ± 0. 5^{ab2}

Different letters in the same column and different numbers in the same row indicate significant difference (P < 0. 05), Kruskal-Wallis H test.

Table 2. Effect of different glutathione concentrations on sperm viability of equine semen cooled at 16ºC for different intervals (mean ± standard deviation).

Treatment (mM)	Storage duration (h)			
	0	12	24	36
	Viability (%)			
0	84. 2 ± 3. 3^{ac1}	84. 3 ± 2. 7^{a1}	75. 5 ± 9. 7^{a2}	68. 1 ± 13. 4^{a3}
1	84. 6 ± 3. 9^{a1}	76. 4 ± 5. 5^{b2}	79. 5 ± 1. 8^{b2}	68. 8 ± 9. 2^{a3}
1. 5	79. 9 ± 4. 0^{b1}	76. 7 ± 7. 0^{b1}	76. 7 ± 9. 4^{ab1}	66. 5 ± 8. 3^{a2}
2. 5	82. 6 ± 3. 9^{c1}	81. 4 ± 6. 6^{c1}	76. 4 ± 3. 5^{ab2}	67. 6 ± 7. 5^{a3}

Different letters in the same column and different numbers in the same row indicate significant difference (P < 0. 05), Kruskal-Wallis H test.

Table 3. Effect of different glutathione concentrations on plasma and acrosomal membrane integrity of equine semen cooled at 16ºC for different intervals (mean ± standard deviation).

Treatment (mM)	Storage duration (h)			
	0	12	24	36
	Plasma membrane integrity (%)			
0	65. 2 ± 4. 8^{a1}	62. 8 ± 6. 1^{a1}	56. 2 ± 14. 5^{a2}	54. 1 ± 11. 8^{a2}
1	64. 7 ± 2. 5^{a1}	59. 2 ± 5. 9^{ab2}	52. 0 ± 11. 6^{a3}	56. 0 ± 3. 2^{a23}
1. 5	65. 4 ± 6. 9^{a1}	57. 9 ± 9. 2^{b2}	43. 0 ± 12. 05^{b3}	45. 4 ± 7. 4^{b3}
2. 5	64. 2 ± 2. 8^{a1}	53. 1 ± 10. 2^{c2}	52. 9 ± 14. 8^{a2}	51. 8 ± 13. 5^{a2}
	Acrosomal membrane integrity (%)			
0	75. 2 ± 8. 9^{a1}	73. 3 ± 7. 6^{a12}	72. 1 ± 9. 6^{a12}	70. 5 ± 10. 4^{a2}
1	75. 8 ± 2. 2^{a1}	73. 9 ± 7. 4^{a1}	68. 7 ± 7. 5^{b2}	67. 5 ± 5. 8^{a2}
1. 5	74. 2 ± 3. 3^{a1}	70. 8 ± 7. 7^{b2}	70. 1 ± 11. 9^{a2}	67. 9 ± 7. 8^{a2}
2. 5	74. 1 ± 4. 5^{a1}	72. 6 ± 9. 6^{a12}	70. 8 ± 8. 8^{a2}	68. 7 ± 4. 4^{a2}

Different letters in the same column and different numbers in the same row indicate significant difference (P < 0. 05), Kruskal-Wallis H test.

Discussion

We selected specific intervals within a 36-hour duration of cooling to test sperm integrity with free radical production, to assess the effects of antioxidant addition, and to mimic the time that would be necessary to send cooled semen from large urban centers to distant locations. The passive cooling system at 16ºC was selected, as it is low-cost and easily accessible to owners and field veterinarians, and is the most commonly used system for semen transport in Brazil (OLIVEIRA et al., 2015).

A decline in motility was noted throughout the evaluation period, accentuated from 24 hours of cooling, a finding that was consistent with the observations of Zhandi and Ghadimi (2014). This decrease could be attributed to thermal shock when sperm are subjected to temperatures lower than 20ºC. Characteristic changes include an abnormal movement pattern and rapid decline in sperm motility, membrane damage, reduced metabolism, loss of enzymes and other intracellular components (AURICH, 2005), and lipid peroxidation in the presence of oxygen ions. Sperm membranes contain large quantities of unsaturated fatty acids, which are particularly susceptible to peroxidation, leading to a subsequent loss of membrane integrity and cell function, and reduced motility (AURICH et al., 1997).

The 1. 5 and 2. 5 mM glutathione concentrations demonstrated better results in maintaining motility (43. 4% and 45. 5%, respectively) after 36 hours of cooling, in comparison to the control (35. 5%) ($P < 0. 05$). This difference can be attributed to the mechanism by which glutathione slows the oxidation rate by inhibiting the production, or the deleterious effects of free radicals and ROS (BALL et al., 2001). Zhandi and Ghadimi (2014) compared the effects of 5 and 10 mM concentrations of glutathione, incorporated into the INRA82 extender for semen refrigeration at 5ºC for 48 hours, on Caspian stallion semen and obtained better motility results for 5mM glutathione (41. 18%) compared to the control (38. 73%). In addition, they reported motility of 33. 53% for 10 mM glutathione ($P < 0. 05$) (ZHANDI; GHADIMI, 2014).

As expected, reduced sperm viability was noted in all treatments within 36 hours of cooling. Sperm viability was higher at the 1mM glutathione concentration (79. 5%) after 24 hours of cooling in comparison to the control (75. 5%). However, no significant differences were noted among other concentrations. Zhandi and Ghadimi (2014) found no significant effects of glutathione on stallion semen viability cooled for up to 48 hours at 5ºC with the INRA82 extender.

No significant differences were noted in plasma and acrosomal membrane integrity for any glutathione concentration. On the other hand, Zhandi and Ghadimi (2014) obtained better results for plasma membrane integrity with 5mM glutathione at both 24 hours and 48 hours of cooling, as evaluated by the hypo-osmotic test. Baumber et al. (2005) also reported no effects on viability or acrosomal integrity, mitochondrial membrane potential or lipoperoxidation levels, after freezing equine sperm with 10 mM glutathione, suggesting that ROS production did not significantly affect these variables and that sperm motility would be a better indicator of oxidative stress in this species.

There is no consensus in the scientific literature regarding the effects of glutathione when added to a cooling or freezing extender, since some studies report positive effects in swine (GADEA et al., 2005) and equines (OLIVEIRA et al., 2013), and no significant effects in other species (BAUMBER et al., 2005). The disparity in the results of the present study and those of the cited experiments could be attributed to variations in age, breed, species, and composition of the extender in which glutathione was incorporated. The addition of glutathione to the extender used to cooling equine sperm can retard the loss of motility and sperm viability, particularly when there is a need for its cooling at 16ºC for up to 36 hours.

Conclusion

The 1. 5 and 2. 5 mM glutathione concentrations yielded higher total motility values at 36 hours of cooling and were more efficient at protecting equine

sperm, when cooled for longer than 24 hours in a passive commercial cooling system at 16ºC. In light of the fact that total sperm motility is one of the variables that is highly correlated with fertility after artificial insemination, these glutathione concentrations could represent an important antioxidant addition to be incorporated into this extender for cooling and transport.

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