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Original Research

Addition of Glutathione to an Extender for Frozen Equine Semen

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ABSTRACT

The manipulation of equine semen during cryopreservation reduces sperm viability and fertility because of, among other factors, membrane lipid peroxidation that makes cells highly susceptible to free radicals and reactive oxygen species (ROS). The oxidative effect caused by the generation of ROS can be reduced by the addition of antioxidants to the seminal plasma or to the extenders used for freezing. The current study was performed to test the in vitro effect of exogenous glutathione added in five different concentrations (control, 2.5 mM, 5.0 mM, 7.5 mM, and 10 mM [treatments 1-5, respectively]) to the extender for 12 stallions. Analyzed parameters were sperm motility, viability, and acrosome and plasmatic membrane integrity. Total motility was higher in treatments 1 and 2 ($P < .05$); viability, progressive motility, and plasmatic membrane integrity were higher in treatment 2 ($P < .001$). As for acrosome membrane integrity, treatment 3 showed the best results ($P < .05$). The addition of 2.5 mM glutathione to the freezing extender preserves total motility and increases sperm viability, progressive motility, and plasmatic membrane integrity. Concentrations above 2.5 mM were deleterious to spermatozoa.

1. Introduction

Fifty-four years have passed since the first foal was born with the use of frozen semen [1]. Since then, important advances have been observed with the use of this biotechnique; however, pregnancy rates continue to be low and are affected by several factors.

The use of frozen semen created a new dimension for the horse breeding industry by making possible the preservation of this biological material for unlimited time and its worldwide distribution. This optimizes the use of stallions with superior genetics and reduces the costs with transport and diseases. Geographic barriers are abolished, and one can use frozen semen from stallions that are in competition or are recovering from pathologies that would prevent them from mating, and even from dead stallions [2].

The manipulation of equine semen during these processes reduces sperm viability and fertility because of, among others, membrane lipid peroxidation, because of its high polyunsaturated fatty acids content, which makes the cells highly susceptible to free radicals and reactive oxygen species (ROS) [3].

ROS scavengers are present in seminal plasma, and the primary ROS scavengers described in equine semen are glutathione peroxidase, superoxide dismutase, and catalase. Sperm centrifugation used to remove seminal plasma and concentrate spermatozoa before freezing removes antioxidants present in semen, exposing spermatozoa to excessive ROS damage [4].

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A way to improve sperm viability and, consequently, fertilizing capacity would be the addition of antioxidants to the freezing medium. Although most studies that examined the addition of antioxidants to cryopreserved equine semen did not show positive effects on post-thaw parameters and fertility [4], it still depends on the antioxidant type and/or concentration, as well as on the mechanism of action regarding sperm protection [5].

Glutathione is one of the antioxidants added to different semen specimens. It is a thiol tripeptide (γ-glutamyl cysteinyl glycine) with several biological functions found widely in the animal body, not only in somatic cells but also in gametes as well. This thiol has an important role in the antioxidation process of endogenous and exogenous composts, as well as in the maintenance of intracellular redox conditions. Glutathione is a natural reservoir of redox force, which can be quickly used by defend cells against oxidative stress [6]. It is synthesized from glutamate, cysteine, and glycine amino acids. Its reductive power is used to maintain thiol groups in intracellular proteins and other molecules. It acts as a cysteine physiological reservoir and is involved in the regulation of protein synthesis, cellular detoxification, and leukotriene synthesis. The protection by glutathione against oxidative damage is provided by its sulphydryl group (SH), which can be presented in reduced glutathione (GSH) and oxidized glutathione (GSSG) forms. The GSH's attack against ROS is favored by the interaction with enzymes, such as glutathione reductase and glutathione peroxidase (GPx) [6].

The thiol antioxidant system is represented mainly by glutathione, the primary antioxidant in equine semen, abundant in seminal plasma. The amount of GSH in equine seminal plasma is 10 times higher than that in swine [6].

Based on this evidence, the hypothesis of the present study was that the addition of different glutathione concentrations favors equine cryopreserved sperm viability. Lack of information regarding fertility parameters and data discrepancies about the effects of the addition of antioxidants to equine cryopreserved semen indicate the need for more study. Therefore, the objective of the present study was to evaluate the in vitro effect of glutathione addition in five different concentrations to equine spermatozoa subjected to cryopreservation.

2. Materials and Methods

2.1. Semen Collection and Processing

Stallions semen were collected once a day for 7 days to stabilize extragonadal reserve and daily sperm output. Afterward, ejaculates were collected three times per week from 12 fertile light-horse stallions between the ages of 5 and 15 years, using the Colorado model (Equine Artificial Vagina; ARS, Chino-CA, USA) artificial vagina, with a total of 36 ejaculates obtained. Semen samples were collected in a plastic bottle and filtered immediately after collection to create gel-free semen. Only ejaculates with more than 60% motility were used for cryopreservation [7].

The sperm-rich fraction (gel-free) was diluted in a 1:1 ratio using a skim milk-glucose extender with penicillin G procaine [8].

2.2. Addition of Antioxidant and Sample Freezing

Samples were centrifuged at 600 × *g* for 10 minutes, and sperm pellets were resuspended in a freezing extender (Botucrio; Botupharma, Botucatu, Brazil) to a concentration of 200 × 10^6 cells/mL and placed in five (15-mL) sterile centrifuge tubes. These aliquots were used for the treatment groups with the addition of glutathione (G6013; Sigma Chemical Co.) in different treatment concentrations, as follows: treatment 1, control; treatment 2, 2.5 mM; treatment 3, 5 mM; treatment 4, 7.5 mM; and treatment 5, 10 mM. Aliquots were packed into 0.5-mL straws placed in an automated freezing system (TK 3000 SE; TK Tecnologia em Congelação LTDA, Uberaba, Brazil) to stabilize the cooling and freezing rates.

For stabilization, straws were placed on a straw holder inside the cooling tube until it reached 5°C at a rate of −0.25°C/min, remaining 20 minutes in this temperature. After this time, the straw holder was moved to a thermal box containing liquid nitrogen, at a freezing rate of 15°C/min from 5°C until reaching −80°C and from 10°C until reaching −120°C. Once this temperature was reached, the straws were plunged into liquid nitrogen (−196°C) and stored in a liquid nitrogen holding tank.

2.3. Post-Thawing Analysis

Two straws from each treatment, from the same stallion, were thawed in a water bath at 37°C for 30 seconds, 24 hours after storage in the cryogenic container.

The computerized analysis of sperm movement characteristics was performed with an Ivos-Ultimate 12 unit (Hamilton Thorne Biosciences), previously adjusted for equine semen. Three fields were selected for analysis. Measured variables were total motility (% of TM), progressive motility (% of PM), average path velocity (VAP, μm/s), straight-line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), amplitude of lateral head displacement (ALH, μm), beat-cross frequency (BCF, Hz), linearity (% of LIN), and straightness (% of STR).

For the evaluation of viability, the supravital eosin-nigrosin staining technique (LIVE/DEAD) was used, where equal volumes (20 μL) of semen and stain were mixed and transferred to a preheated (37°C) labeled microscope slide and smeared by sliding a cover slip in front of it. The smears were air dried and examined directly. Samples were evaluated by microscopy (magnification ×1,000). Five hundred sperm cells were counted per sample, and unstained cells were classified as those that were viable [9].

To evaluate sperm plasmatic membrane integrity, the hypo-osmotic swelling test (HOST) was used by incubation of 100 μL of semen in 1.0 mL of a sucrose solution of 100 mOsm/L in a water bath at 37°C for 30 minutes. After this time, 20 μL of this solution was analyzed in a humidity chamber, using phase-contrast microscopy at ×1,000 magnification. A total of 200 spermatozoa were counted, and those considered swollen (coiled) were determined to possess membrane integrity after the subtraction of the percentage of tail alterations found in the morphologic evaluation [10].

To evaluate acrosome membrane integrity, trypan blue/Giemsa staining was used. Equal volumes of semen and trypan blue, 0.2% (20 μL; Sigma Chemical Co.) stain were placed on a microscope slide, heated at 37°C, and mixed to prepare a smear, which was air dried. Dried smears were fixed in neutral red solution (Sigma Chemical Co.) for 5 minutes, washed in running water, air dried again, and then immersed in 7.5% Giemsa stain solution (Sigma Chemical Co.) for 4 hours [11].

Evaluation was performed at 1,000× magnification, and 200 spermatozoa were counted and classified as live, that is, acrosomes stained pink or purple and postacrosomal regions were unstained; dead, in which they were stained blue at the postacrosomal region and acrosomes stained purple or pink; true acrosome reaction, that is, acrosomes and postacrosomal regions were unstained; and false acrosome reaction, in which acrosomes were unstained and postacrosomal regions stained blue [12].

2.4. Statistical Analysis

Experimental design was completely randomized. Information obtained in the fieldwork was edited by using an electronic spreadsheet (Excel software; Microsoft Office) [13].

Data analysis was performed using univariate [14] to determine if the experimental error of variables had normal distribution for variance probability and homogeneity. Because the studied variables did not present normal distribution, disobeying the basic premise of analysis of variance, a nonparametric test, the Kruskal-Wallis H test (WinStat module; Microsoft) was used [15].

3. Results

Table 1 shows the results for spermatic kinetics for the different treatments. Total motility was higher in treatments 1 and 2 ($P < .05$). For PM, treatment 2 was higher than the other treatments ($P < .001$). As for straightness (STR and LIN), the highest values were observed in treatments 1 and 5 ($P < .05$).

Data for sperm velocity, from those submitted to cryopreservation in different treatments, is presented in Table 2. VAP results were equivalent for treatments 1, 2, 3, and 5, with differences in treatment 4 ($P < .0001$). Treatment 5 was better ($P < .0001$) regarding VSL, and VCL results were equivalent for treatments 1, 2, 3, and 5, with differences in treatment 4 ($P < .05$). Regarding ALH displacement, treatments 2 and 5 were higher ($P < .05$) than those of the other treatments; BCF was higher for treatment 1 than for the other concentrations ($P < .05$).

Table 1
Results of the post-thaw analysis (CASA) for total motility (TOTMOT), progressive motility (PROGMOT), straightness (STR), and linearity (LIN)

Treatment	TOTMOT	PROGMOT	STR	LIN
1	53.9 ± 5.4^{a}	5.6 ± 3.5^{a}	79.3 ± 6.6^{a}	45.5 ± 7.9^{a}
2	56.0 ± 5.4^{a}	10.5 ± 6.2^{b}	73.9 ± 6.8^{b}	40.6 ± 7.8^{b}
3	46.2 ± 9.5^{b}	4.7 ± 3.9^{ad}	72.2 ± 9.8^{b}	39.0 ± 9.9^{b}
4	19.8 ± 10.9^{c}	1.5 ± 1.1^{c}	74.2 ± 5.8^{b}	34.5 ± 2.8^{c}
5	18.8 ± 10.9^{c}	3.7 ± 2.1^{d}	79.5 ± 8.5^{a}	55.6 ± 11.1^{d}

Different letters in the same column indicate differences ($P < .05$).

Table 3 shows that viability and plasmatic membrane integrity (LIVE/DEAD and HOST) were higher in treatment 2 ($P < .05$). For acrosome membrane integrity (acrosome), treatment 3 had the best results ($P < .05$).

4. Discussion

Data presented in Table 1 show that the addition of more than 2.5 mM glutathione (treatments 3, 4, and 5) reduces total motility, whereas in smaller doses (treatment 2) glutathione was beneficial for increasing progressive motility. This was also reported by Silva et al [16], who added sodium pyruvate and Trolox (soluble form of vitamin E) to the freezing extender used in the cryopreservation of fertile and subfertile stallions' sperm and observed that pyruvate improved total motility with no differences in the other studied parameters (progressive motility, membrane integrity, acrosome, and DNA). However, Baumber et al [5] did not observe an improvement in total and progressive motility after the addition of 10 mM glutathione. Similar results were obtained with the addition of the same antioxidants in equine semen kept under refrigeration [17]. A protective effect of ascorbic acid for membrane integrity was observed, but a deleterious effect on progressive motility was seen [18].

In swine [19], 5 mM glutathione added to the freezing medium did not improve semen parameters or fertilizing capacity. In another experiment conducted in swine [20], the additions of 1 and 5 mM glutathione were evaluated, and an increased motility (total and progressive) was observed after 1 mM addition compared to control, and no differences were observed regarding the other parameters analyzed.

Highly positive correlations between progressive motility and velocity parameters indicate that spermatozoa with good linear progressive velocity and straight path run longer distances in a shorter time. VAP and VSL also have strong positive correlation with fertility and can be used to estimate fertility in semen samples [21]. Better results in treatment 2 were detected for total motility (56.03), progressive motility (10.56), VAP (42.27), and VSL (32.10), indicating that 2.5 mM concentration preserves sperm total motility compared with control.

During hyperactivation, there are dramatic changes in sperm vigor, characterized by a great increase in the amplitude of lateral head displacement, followed by low motility and low flagellar beating frequency but good curvilinear and nonprogressive circular movement. Therefore, high VCL and ALH values and low LIN can be used as criteria to distinguish the movement of hyperactivated spermatozoa [22], which was observed in treatment 5, except for linearity. However, hyperactivation was verified by high ALH (6.2) and VCL (83.5), with low total and progressive motility.

When there is a decrease in ALH and an increase in flagellar beating, spermatozoa gain energy during the path. High extender viscosity diminishes flagellar wave amplitude [23]. Generally, elevated ALH is not desired because it affects cellular progression; therefore, higher ALH values denote lower seminal quality. According to Table 2, ALH was lower in treatments 3 and 4 and higher in treatment 5.

Table 2
Results of the post-thaw analysis for average path velocity (VAP), progressive linear velocity, curvilinear velocity (VCL), amplitude of lateral head displacement (ALH) and beat-cross frequency (BCF)

Treatment	VAP (μm/s)	VSL	VCL (μm/s)	ALH (μm)	BCF (Hz)
1	42.3 ± 5.5[a]	32.7 ± 2.4[a]	79.6 ± 12.2[a]	5.2 ± 1.3[a]	39.8 ± 5.3[a]
2	42.2 ± 8.0[a]	32.1 ± 6.2[ab]	78.8 ± 12.6[a]	5.4 ± 1.7[ab]	36.5 ± 4.2[b]
3	41.4 ± 6.0[a]	27.7 ± 7.9[bc]	81.2 ± 9.2[a]	2.8 ± 1.7[c]	35.2 ± 4.9[b]
4	29.2 ± 10.6[b]	25.6 ± 6.2[c]	68.5 ± 15.2[b]	2.6 ± 1.4[c]	31.2 ± 1.7[c]
5	41.8 ± 18.3[a]	48.5 ± 19.5[d]	83.4 ± 11.3[a]	6.2 ± 3.0[b]	36.8 ± 6.9[b]

VSL, straight-line velocity.
Different letters in the same column indicate differences ($P < .05$).

A possible explanation for the latter result is a more viscous and acid medium. Under microscopic evaluation, a viscous layer was observed in the samples with the highest glutathione concentrations, which affected cellular movement, and spermatozoa had to put more effort to move.

In treatment 1 (control), flagellar beating frequency was higher than that in the other glutathione concentrations; however, the group that presented the lowest ALH and the highest flagellar beating frequency was treatment 4. This demonstrates, for this characteristic alone, a better energetic yield during the path.

However, spermatic movement alone is inconsistently correlated with fertility, as sperm motility represents only one of the several basic requests for the spermatozoon to conclude its biological function, represented by oocyte fertilization [24].

Regarding sperm membrane (plasmatic and acrosome) integrity, as presented in Table 3, there was a significant drop in values, which is expected after freezing. Ice crystal formation that occurs during freezing, described by Mazur [25], leads to an increase in salt concentration in the nonfrozen fraction. High salt concentrations can dehydrate sperm cells, deforming them and causing damage to membrane structure and denaturation of proteins. Consequently, after freezing, spermatozoa suffer irreversible damage characterized by abnormal movement (circular or backwards), a rapid drop in motility, damages in the acrosome and plasmatic membrane, reduction in metabolism, and loss of intracellular components [26].

However, the addition of 2.5 mM glutathione was highly efficient for preservation of viability and membrane plasmatic integrity compared with the other treatments.

Nevertheless, additional studies are needed to evaluate glutathione dose-response and determine an ideal concentration, as well as more experiments are necessary to prove the beneficial effect of this antioxidant in the improvement of fertility rates of mares inseminated with frozen semen.

Table 3
Results of the post-thaw analysis for viability (Live/Dead), plasmatic membrane integrity (Host), and acrosome membrane integrity (Acrosome)

Treatment	Live/Dead	Host	Acrosome
1	59.5 ± 7.5[ab]	30.4 ± 10.8[a]	66.2 ± 10.6[a]
2	61.9 ± 12.9[a]	44.5 ± 26.9[b]	60.2 ± 10.3[b]
3	54.6 ± 6.6[b]	24.2 ± 14.3[a]	72.3 ± 8.7[c]
4	39.8 ± 13.7[c]	13.6 ± 5.6[c]	65.7 ± 10.9[a]
5	31.9 ± 12.9[d]	16.5 ± 11.3[c]	56.0 ± 7.9[b]

Different letters in the same column indicate differences ($P < .05$).

5. Conclusions

The addition of 2.5 mM glutathione to the freezing extender preserved total motility and improved progressive motility, viability, and plasmatic membrane integrity. Glutathione concentrations above 2.5 mM were deleterious to spermatozoa.

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