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Glutathione for the freezing of cooled equine semen, using different protocols

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Abstract

The aim of this study was to evaluate the effect of *in vitro* addition of glutathione in four different concentrations compared to the control group, using two different freezing systems: controlled-rate (AS) and manual (MS) freezer. The parameters evaluated were motility, strength, plasmatic and acrosomal membrane integrity of spermatozoa from twelve stallions. Ejaculates from stallions were collected three times per week, during four weeks. Gel-free semen was diluted in skim milk extender and cooled (16ºC) for 24 h. After cooling, extended semen was centrifuged at 600 x *g* for 10 min. The supernatant was removed and sperm pellets were re-suspended using the freezing extender with different glutathione concentrations (0, 2.5 mM, 5 mM, 7.5 mM, and 10 mM). Samples were then packed into 0.5 ml straws, which were divided into two parts: one for cryopreservation in a controlled-rate freezer (AS) and the other for a manual system (MS). In this study, the control group showed higher motility and better membrane integrity within treatments for the AS ($P < 0.05$). Furthermore, when an MS group was evaluated, the 2.5 mM glutathione group demonstrated better preservation for total motility and plasmatic membrane integrity. However, concentrations higher than 2.5 mM were deleterious to the spermatozoa in both controlled and manual freezing systems.

Keywords: antioxidant, frozen semen, spermatozoa, stallion.

Introduction

Important advances in the use of frozen semen have occurred in the 57 years since the birth of the first foal using this technology. The cryopreservation of gametes is expensive and requires sophisticated equipment. However, pregnancy rates remain low due to many variables (Miller, 2008). As an arising technology, the implementation relies on the cost and feasibility, and may not be suitable to all breeding farms. Alternative procedures have been used with success to freeze semen immediately post-collection, but this requires the stallion and equipment be at the same place. The collection and shipment of cooled-semen to a specialized facility makes the cryopreservation process easier, without transporting the stallion to reproduction centers (Crockett *et al*., 2001). However, manipulation of equine semen during these processes reduces sperm viability and fertility due to several factors including membrane lipid peroxidation, because of its high polyunsaturated fatty acids content, making spermatozoa highly susceptible to free radicals and reactive oxygen species (ROS; Cocchia *et al*., 2011).

ROS scavengers are present in seminal plasma, with the primary ROS scavengers described in equine semen being 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 (Ball, 2008).

One way to improve sperm viability and, consequently, fertilizing capacity would be the addition of antioxidants to the freezing medium (Oliveira *et al*., 2013). Although most studies that examined the addition of antioxidants to cryopreserved equine semen did not show positive effects on post-thaw parameters and fertility (Ball, 2008), it still depends on the antioxidant type and/or concentration, as well as on the action mechanism regarding sperm protection (Baumber *et al*., 2005).

Glutathione is one of the antioxidants added to different semen specimens. It is a thiol tripeptide (ɣ-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 (Luberda, 2005). 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 present in reduced glutathione (GSH) and oxidized glutathione (GSSG) forms. The

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GSH's attack against ROS is favored by the interaction with enzymes, such as glutathione reductase and glutathione peroxidase (GPx; Luberda, 2005).

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 (Luberda, 2005).

Based on this evidence, the hypothesis of the present study was that the addition of different glutathione concentrations, after 24 h cooling at 16ºC, favors equine cryopreserved sperm viability. We sought to compare two methods of equine frozen semen, controlled-rate freezer and manual system. 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 aim of the present study was to evaluate the *in vitro* effect of glutathione addition in five different concentrations to equine spermatozoa subjected to different methods of cryopreservation after cooling for 24 h at 16ºC.

Materials and Methods

Semen collection and cooling

Stallion 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% total motility were used for cooling (Colégio Brasileiro de Reprodução Animal - CBRA, 1998).

The sperm-rich fraction (gel-free) was diluted in a 2:1 ratio using a skim milk-glucose extender with penicillin G procaine (Kenney *et al*., 1975) and cooled (16ºC) for 24 h in a transport box (Max-Sêmen Express, Agrofarma, São Paulo, Brazil).

Addition of antioxidant and sample freezing

After the 24 h of storage, extended semen was centrifuged at 600 x *g* for 10 min, and sperm pellets were resuspended in a freezing extender (Botucrio; Botupharma, Botucatu, Brazil) to a concentration of 200 x 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, USA) 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. The pH and osmolarity were always measured after the addition of different glutathione concentrations. Aliquots were packed into 0.5 ml straws and shared in two parts for the freezing: in a controlled-rate freezer system (AS; TK 3000 SE; TK tecnologia em congelação LTDA, Uberaba, Brazil) and the other in a manual freezing system (MS), to stabilize the cooling and freezing rates.

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

For stabilization in MS, straws were placed in a stainless steel support and kept inside the refrigerator (5ºC) for 20 min. Subsequently, these straws were kept at a height of 6 cm liquid nitrogen for 20 min in an isotherm box and plunged into liquid nitrogen.

Post-thawing analysis

Three straws from each treatment, from the same stallion, were thawed in a water bath at 37ºC for 30 s, 24 h after storage in the cryogenic container. Variables analyzed were total motility, strength, viability and acrosomal and plasmatic membrane integrity

Assessment of sperm motion characteristics

The subjective evaluation of total motility (0-100%) and strength (0-5) was done with a drop of semen (10 µl) placed between a slide and a coverslip previously heated at 37ºC and visualized by a contrast phase optical microscope at 200X magnification. The strenght expresses the speed with which the spermatozoa moves into the field. The result was expressed on a scale 0-5, 0 being no motion and 5 intense speed (CBRA, 1998).

Assessment of viability, plasmatic and acrosomal membrane integrity

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 (37ºC) 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 1,000X). Five hundred sperm cells were counted per sample, and unstained cells were classified as those that were viable (Dott and Foster, 1972).

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 100mOsm/l in a water bath at 37ºC for 30 min. After this time, 20 µl of this solution was analyzed in a humidity chamber, using phase-contrast microscopy at 1,000X 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 (Melo and Henry, 1999).

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 37ºC, and mixed to prepare a smear, which was air dried. Dried smears were fixed in neutral red solution (Sigma Chemical Co.) for 5 min, washed in running water, air dried again, and then immersed in 7.5% Giemsa stain solution (Sigma Chemical Co.) for 4 h (Kútvölgyi *et al*., 2006).

Evaluation was performed at 1,000X 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 that were unstained; and false acrosome reaction, in which acrosomes were unstained and postacrosomal regions stained blue (Didion *et al*., 1989).

Statistical analysis

Data analysis was performed using the SAS UNIVARIATE procedure (Statistical Analysis System, version 9.2) to determine if the experimental error of variables had normal distribution for variance probability and homogeneity. Since the studied variables did not present normal distribution, a non parametric test, Kruskal-Wallis H test (WinStat module; Microsoft) was used.

Results

Table 1 shows the results for total motility and strength for the different treatments and freezing protocols. Total motility was higher ($P < 0.05$) for the control in a controlled rate freezer (T1-AS; 44.6 ± 15.6) when compared to the other treatments and protocols (T2-AS), (19.8 ± 8.6); (T3-AS), (10.4 ± 4.8); (T4-AS), (4.5 ± 2.6); (T5-AS), (5.5 ± 2.6); (T1-MS), (20 ± 10.9); (T2-MS), (28.1 ± 14); (T3-MS), (7.9 ± 2.5); (T4-MS), (9.1 ± 7.4) and (T5-MS), (4.8 ± 3.1). However, in MS the 2.5 mM concentration of glutathione (T2-MS) showed better results in total motility (28.1 ± 14) than the other treatments ($P < 0.05$). The strength results were higher in the control group in both protocols (T1-AS-3.4 ± 0.6)/(T1-MS-3.4 ± 1.1) than the other treatments ($P < 0.05$).

Table 1. Results of post-thaw analysis for total motility and strength of equine frozen semen using different concentrations of glutathione and two different freezing systems (controlled rate freezer-AS; Manual-MS) after 24 h of cooling at 16ºC.

Treatment	Motility (%)		Strength (%)	
	AS	MS	AS	MS
1 (Control)	44.6 ± 15.6^{a1}	20.0 ± 10.9^{b1}	3.4 ± 0.6^{a1}	3.4 ± 1.1^{a1}
2 (2.5 mM)	19.8 ± 8.6^{a2}	28.1 ± 14.0^{b2}	1.9 ± 0.9^{a2}	2.6 ± 1.5^{b2}
3 (5 mM)	10.4 ± 4.8^{a3}	7.9 ± 2.5^{a34}	1.1 ± 0.5^{a3}	1.5 ± 0.4^{a3}
4 (7.5 mM)	4.5 ± 2.6^{a4}	9.1 ± 7.4^{b3}	0.3 ± 0.3^{a4}	1.6 ± 0.9^{b3}
5 (10 mM)	5.5 ± 2.6^{a4}	4.8 ± 3.1^{a4}	1.1 ± 0.7^{a3}	1.3 ± 0.5^{a3}

Within a column, values without a common number differed ($P < 0.05$). Within a row, values without a common letter differed ($P < 0.05$).

Table 2 shows that viability was higher ($P < 0.05$) for the control in controlled rate freezer (T1-AS; 57.9 ± 14.7) when compared to the other treatments and protocols (T2-AS), (45 ± 10.2); (T3-AS), (47 ± 20.8); (T4-AS), (26.8 ± 11.9); (T5-AS), (34.1 ± 10.6); (T1-MS), (35.7 ± 8.7); (T2-MS), (45.2 ± 15.5); (T3-MS), (30.8 ± 8.8); (T4-MS), (25.5±11.7) and (T5-MS), (31.3 ± 13.1). However, in MS the 2.5mM concentration of glutathione (T2-MS) showed better results in viability (45.2 ± 15.5) than the other treatments ($P < 0.05$).

Table 3 shows that plasmatic membrane integrity was higher ($P < 0.05$) for the control in controlled rate freezer (T1-AS; 29.3 ± 14.7) when compared to the other treatments and protocols (T2-AS), (22.7 ± 10.4); (T3-AS), (10 ± 3.7); (T4-AS), (4.9 ± 1.9); (T5-AS), (3 ± 1.8); (T1-MS), (5.1 ± 2.8); (T2-MS), (26.3 ± 19.1); (T3-MS), (4.4 ± 1.9); (T4-MS), (7.7 ± 5.4) and (T5-MS), (4.9 ± 2.3). However, in MS the 2.5 mM concentration of glutathione (T2-MS) showed better results in viability (26.3 ± 19.1) than the other treatments ($P < 0.05$). For acrosome membrane integrity the higher results were the control from both protocols. To discuss the results, the relationship of acrosomal integrity will be taken into account only as the percentage of live cells with intact acrosome.

The results for pH and osmolality were 7/374 mmol/kg (control), 6/898 mmol/kg (2.5 mM), 5/888 mmol/kg (5 mM), 5/884 mmol/kg (7.5 mM) and 4/898 mmol/kg (10 mM), respectively.

Table 2. Results of post-thaw analysis for viability (LIVE/DEAD) of equine frozen semen using different concentrations of glutathione and two different freezing systems (controlled rate freezer-AS; Manual-MS) after 24 h of cooling at 16°C.

Treatment	Viability (%)	
	AS	MS
1 (Control)	57.9 ± 14.7^{a1}	35.7 ± 8.7^{b1}
2 (2.5 mM)	45.0 ± 10.2^{a2}	45.2 ± 15.5^{a2}
3 (5 mM)	47.0 ± 20.8^{a2}	30.8 ± 8.8^{b13}
4 (7.5 mM)	26.8 ± 11.9^{a3}	25.5 ± 11.7^{b3}
5 (10 mM)	34.1 ± 10.6^{a4}	31.3 ± 13.1^{b13}

Within a column, values without a common number differed ($P < 0.05$). Within a row, values without a common letter differed ($P < 0.05$).

Table 3. Results of post-thaw analysis for plasmatic membrane integrity and acrosomal membrane integrity of equine frozen semen using different concentrations of glutathione and two different freezing systems (controlled rate freezer-AS; Manual-MS) after 24 h of cooling at 16°C.

Treatment	Plasmatic membrane integrity (%)		Acrossomal membrane integrity (%)	
	AS	MS	AS	MS
1 (Control)	29.3 ± 14.7^{a1}	5.1 ± 2.8^{b1}	71.1 ± 2.8^{a1}	68.0 ± 4.3^{a1}
2 (2.5 mM)	22.7 ± 10.4^{a2}	26.3 ± 19.1^{a2}	62.6 ± 5.9^{a2}	60.0 ± 8.5^{a2}
3 (5 mM)	10.0 ± 3.7^{a3}	4.4 ± 1.9^{b1}	56.4 ± 9.5^{a3}	69.7 ± 12.9^{b1}
4 (7.5 mM)	4.9 ± 1.9^{a4}	7.7 ± 5.4^{a1}	50.0 ± 19.1^{a4}	53.6 ± 5.5^{a3}
5 (10 mM)	3.0 ± 1.8^{a4}	4.9 ± 2.3^{a1}	52.4 ± 13.5^{a34}	59.4 ± 0.3^{b2}

Within a column, values without a common number differed ($P < 0.05$). Within a row, values without a common letter differed ($P < 0.05$)

Discussion

The cryopreservation of equine semen is limited by factors such as the high cost of equipment, the need for specialized technicians, and on farm locations to manipulate semen (Crockett *et al*., 2001). This facilitates the necessity of shipping cooled-semen to specialized laboratories.

Cooled equine semen is used to allow its shipment for variable periods of time. During transportation the semen should keep the fertility and that depends on factors like the storage temperature, composition of the extender and spermatic concentration per insemination dose (Heckenbichler *et al*., 2011).

The cooled shipped system that we chose maintains the semen at 16ºC for 24 h. It is a low cost procedure, widely used during the breeding season and allows the shipment of semen for long distances (Melo *et al*., 2007). It was chosen to reproduce the real situation faced by the farms.

Data presented in Table 1, 2 and 3 show that the addition of more than 2.5 mM glutathione (treatments 3, 4 and 5) reduced total motility, strength, viability, and integrity of the plasmatic and acrosomal membrane, whereas in smaller doses (treatment 2) glutathione was beneficial for increasing total motility, viability and plasmatic membrane integrity in manual system freezing. This was also reported by Silva *et al*. (2009), 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 parameters studied. However, Baumber *et al*. (2005) 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 (Ball *et al*., 2001). A protective effect of ascorbic acid for membrane integrity was observed, but a deleterious effect on progressive motility was seen (Aurich *et al*., 1997).

One possible explanation for the reduction in all evaluated variables (total motility, strength, viability and plasmatic membrane integrity) in treatments 3, 4 and 5 was probably due to a more viscous and acid medium after addition of these glutathione concentrations. 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, such as we observed in other experiments (Oliveira *et al*., 2013).

The pH reduction in the vehicle reduces the longevity and spermatic fertility, which occurs in the

absence of reduction mechanisms to the hydrogen ions at the extra cellular environment. The low pH induces a reversible decrease in sperm motility (Acott and Carr, 1984) that could have masked the glutathione action, in the same way described by Aurich *et al.* (1997), where a reduction in sperm motility of stallions with low pH resulting from the addition of antioxidative to the vehicle was verified.

The addition of glutathione at four different concentrations evaluated in this study did not result in an improvement of the quality of the cryopreserved spermatozoa after cooling for 24 h and cryopreserving in an controlled rate freezer form (AS; Tables 1, 2 and 3). Of the samples cryopreserved in MS, it was verified that treatment 2 (2.5 mM) improved total motility, viability and plasmatic membrane integrity, which may suggest that in non-homogeneous freezing rates, this concentration can preserve some spermatic features.

The protective effect on the progressive motility, integrity of DNA and stabilization of membranes using glutathione at a 10 mM concentration added to the freezing media was reported by Baumber *et al*. (2003), although there were no differences between the samples treated and control. In this experiment, samples treated with this glutathione concentration (10 mM) showed higher negative effect on spermatozoa. In a similar way to the findings of Baumber *et al*. (2003), treatment 1 (control) showed higher stability for motility and plasmatic membrane integrity, except for treatment 2 (MS-2.5 mM), suggesting that this protocol can be an alternative for the study of stallions that have spermatozoa with low tolerance to cooling procedures and/or cryopreservation.

There is no consensus in the literature for the preventive effect of antioxidative substances added to extenders. Some studies cite positive effects, while others state that there is no benefit in adding this substance (Ball *et al*., 2001; Baumber *et al*., 2005; Gadea *et al*., 2007; Maia and Bicudo, 2009; Silva *et al*., 2009, Oliveira *et al*., 2013). Discrepancies within species may be due to variations in age, animal breed, diluent components, semen conservation procedures, doses and combinations of antioxidatives.

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. Further studies are being conducted with decreased glutathione concentrations and its comparison with other antioxidants, as well as fertility tests on artificial insemination of mares.

In conclusion, this experiment demonstrated that for manual freezing protocols, the addition of 2 mM glutathione to the freezing extender increased total motility, viability, and plasmatic membrane integrity of stallion sperm. Glutathione concentrations above 2.5 mM were deleterious to spermatozoa in both protocols.

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