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Cooling of equine semen at 16 °C for 36 h with the addition of cysteine in different concentrations

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Summary: Equine semen manipulation during the cooling process reduces sperm viability and fertility in consequence to, among others, membrane lipid peroxidation, because of the high content of polyunsaturated fatty acids, which makes cells highly susceptible to free radicals and reactive oxygen species. The objective ofthe present study was to evaluate the effect of in vitro addition of cysteine in four concentrations (0, 1, 1.5, and 2.5 mM) for cooling spermatozoa of 12 stallions at 16 °C for 36 h. Evaluated variables were total motility, strength, viability and plasmatic and acrosomal membrane integrity at four different time points (0, 12, 24 and 36 h). With the exception of acrosomal integrity, it was verified a reduction in total motility, strength and plasmatic membrane integrity in all samples, during cooling. In the evaluations at 36 h of cold storage, total motility (mot) and viability (viab) were higher in groups treated with 1 mM (mot:46,5 ± 6,1/viab:76,5 ± 6,9) and 1.5 mM (mot:46,0 ± 4,6/viab:76,9 ± 3,7) cysteine, respectively, compared to control (mot:35,5 ± 18,4/viab:68,1 ± 13,4) and 2.5mM (mot:39,7 ± 12,4/viab:66 ± 17,2) ($P < 0.05$). As for strength (str) and plasmatic membrane integrity (plasm), 1 mM cysteine (str:3.6 ± 0.5/plasm:57.2 ± 9.5) showed better results compared to control (str:3.2 ± 1.1/plasm:54.1 ± 11.8), 1.5 mM (str:3.5 ± 0.6/plasm:52.2 ± 13.3) and 2.5 mM (str:3.2 ± 1.1/plasm:55.8 ± 12.5) ($P < 0.05$). Regarding acrosomal membrane integrity, in general, there was no loss of integrity (70.5 ± 10.4; 69.4 ± 4.4; 68.0 ± 7.2 and 70.3 ± 0.5), control, 1 mM, 1.5 mM and 2.5 mM respectively. The concentration of 1mM cysteine was more efficient for the protection of sperm cells in the commercial system of passive cooling at 16 °C for 36 h, with higher values for total motility, strength, viability and plasmatic membrane integrity.

Keywords: antioxidant / cooled semen / genetic resources / spermatozoon / stallion

Die Kühlung von Sperma über 36 Std. auf 16°C mit Zusatz von Cystein in verschiedenen Konzentrationen

Die Manipulation von Pferdesamen während des Kühlprozesses reduziert deren Lebensfähigkeit und Fruchtbarkeit. Dies geschieht unter Anderem wegen der membranen Lipidperoxidation sowie des hohen Gehalts an mehrfach ungesättigten Fettsäuren, der die Zellen sehr anfällig für freie Radikale und reaktive Sauerstoff-Spezies macht. Das Ziel der vorliegenden Studie ist es, die Wirkung von künstlicher Zugabe von Cystein in vier Konzentrationen (0, 1, 1,5 und 2,5 mM) zu Kühlsperma von 12 Hengsten bei 16 °C für 36 Stunden zu beurteilen. Ausgewertete Variablen waren Motilität, Lebensfähigkeit sowie plasmatische und akrosomale Membran-Integrität zu vier verschiedenen Zeitpunkten. Mit Ausnahme der akrosomalen Integrität wurde eine Reduktion der Motilität, der plasmatischen Membran-Integrität während der Abkühlung in allen Proben festgestellt. Die Bewertung nach 36 Stunden Kühlung ergab, dass die gesamte Motilität (Mot) und Lebensfähigkeit (Lbf) jeweils höher in der mit 1 mM Cystein behandelten Gruppe (Mot:46,5 ± 6,1/Lbf:76,5 ± 6,9) und der mit 1,5 mM Cystein behandelten Gruppe (Mot:46,0 ± 4,6/Lbf:76,9 ± 3,7) waren, im Vergleich zur Kontrollgruppe (Mot:35,5 ± 18,4/Lbf:68,1 ± 13,4) und der mit 2,5 mM Cystein behandelten Gruppe (Mot:39,7 ± 12,4/Lbf:66 ± 17,2) ($P < 0,05$). Auch für Stärke (Stk) und plasmatische Membranintegrität (plasm MI) wies die Gruppe mit 1mM Cystein höhere Werte auf (str:3,6 ± 0,5/plasm MI:57,2 ± 9,5) im Vergleich zur Kontrollgruppe (Stk:3,2 ± 1,1/plasm MI:54,1 ± 11,8), zur 1,5 mM Gruppe (Stk:3,5 ± 0,6/plasm MI:52,2 ± 13,3) und der 2,5 mM-Gruppe (Stk:3,2 ± 1,1/plasm MI:55,8 ± 12,5) ($P < 0,05$). In Bezug auf die Membranintegrität des Akrosoms gab es im Allgemeinen keinen Integritätsverlust (70,5 ± 10,4; 69,4 ± 4,4; 68,0 ± 7,2 und 70,3 ± 0,5; jeweils Kontrollgruppe, 1 mM, 1,5 mM und 2,5 mM). Die Konzentration von 1mM Cystein war am effektivsten als Schutz der Samenzellen im kommerziellen System des passiven Kühlens auf 16 °C über 36 Stunden, mit höheren Werten für Motilität, Lebensfähigkeit sowie plasmatische Membranintegrität.

Schlüsselwörter: Antioxidans / gekühlte Samen / genetische Ressourcen / Spermium /Hengst / Reproduktion

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Introduction

The transportation of equine semen was first described in arabian texts in 1322, when supposedly an Arabian chief stole a stallion's semen from a rival and inseminated a mare, resulting in a live foal. The first scientific report of the use of artificial insemination was in 1776, by Lazaro Spallanzani, who initially worked with dogs and latter with horses, including cooling of semen (*Brinsko* and *Varner* 1992).

Cooled semen has been widely used in equine industry. The procedure is relatively simple and can be performed in every farm (*Crockett* et al. 2001). Artificial insemination, in horses, is widely practiced all over the world and most commonly

after cooling and transporting semen (*Loomis* 2001). The countries that mostly use artificial insemination with cooled and transported equine semen are the United States of America and Brazil (*Papa* et al. 2005).

Cooled equine semen has been studied to maintain the fertilizing ability for a longer period of time, so that the farmer can choose to transport it when suitable (*Heckenbichler* et al. 2011). The advantages from using cooled semen are the reduction of costs with transport and animal shelter, stressful situations and risks of accidents and diseases (*Brinsko* and *Varner* 1992).

Equine semen manipulation during the cooling process reduces sperm viability and fertility in consequence, among other problems, to membrane lipid peroxidation, due to the high content of polyunsaturated fatty acids, which makes cells highly susceptible to free radicals and reactive oxygen species (ROS) (*Cocchia* et al. 2011).

Oxidation caused by the generation of ROS can be reduced by adding antioxidants to seminal plasma and freezing extenders. But there is controversy regarding the concentration of the antioxidant used for preservation of fresh and frozen semen as well as its specific mechanism of action (*Baumber* et al. 2005).

The antioxidant defense system present in mammalian semen consists of an enzymatic and a non-enzymatic system, with the last being mainly represented by gluthatione. The basic function of gluthatione is to interact with other systems to prevent the action of ROS. This function helps to counteract the oxidative stress of sperm cells, which can result in lipid peroxidation of the plasmatic membrane, irreversible motility loss, extravasation of intracellular enzymes and damage to the chromatin (*Aitken* 1999).

One of the antioxidants incorporated to the semen of some species is cysteine, which is a precursor in gluthatione biosynthesis. Cysteine has a low molecular weight (121.16) and is a non-enzymatic antioxidant, which prevents lipid peroxidation, having the ability to penetrate cellular membranes (*Bilodeau* et al. 2001).

The lack of information about in vitro fertilization and controversial results regarding the effect of antioxidants added to cooled equine semen indicate the need for more studies. Thus, the objective of the present study was to verify the in vitro effect of the addition of cysteine in four different concentrations to equine spermatozoa by the evaluation of total motility, strength, viability and acrosomal and plasmatic membrane integrity during the cooling process at 16°C/36h.

Materials and methods

Semen collection and processing

Stallions' semen was collected once a day for 7 days to stabilize extragonadal reserve and daily sperm output. Afterwards, ejaculates were collected three times per week from 12 fertile light-horse stallions between the ages of 4 and 8 years, using the Colorado model (Equine artificial vagina; ARS, Chino-CA, USA), 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.

Addition of antioxidant and cooling

The sperm-rich fraction (gel-free) was centrifuged at 600×g for 10 minutes, and sperm pellets were resuspended in a skim milk-glucose extender with amikacin (Botu-sêmen, Botupharma, Botucatu/SP/Brazil) containing 0mM (control), 1mM, 1.5mM and 2.5mM cysteine (C7352, Sigma Chemical CO, USA), in a final concentration of 50×10^6 spermatozoa/mL. The pH was measured and did not differ from the control group. For semen transportation boxes were used (Maxsêmen Express®), one for each group. These boxes are a passive refrigeration commercial system that keeps semen at 16°C. Four centrifuged tubes were distributed in each box together with a recipient filled with water to complete 100mL

Table 1 – Effect of different cysteine concentrations (treatment) in equine cooled semen on total motility (0-100%) and strength (0-5) during the different storage time (0, 12, 24 and 36h) of cooling at 16°C. / *Wirkung der verschiedenen Cystein-Konzentrationen (Behandlung) bei gekühltem Pferdesperma auf die Gesamtmotilität (0-100%) und Stärke (0-5), während den unterschiedlichen Lagerungszeiten (0, 12, 24 und 36 St) bei Kühlung auf 16°C.*

	Storage time (h)			
Treatment	0	12	24	36
	Motility %			
0	64,8 ± 6.0^{ab1}	53.2±8.3^{a2}	53.5 ± 7.2^{a2}	35.5 ±18.4^{a3}
1mM	67.2 ± 6.1^{a1}	56.5±8.7^{b2}	54.0 ± 4.2^{a2}	46.5 ± 6.1^{b3}
1.5mM	63.7 ± 4.9^{b1}	51.8 ± 8.8^{a2}	50.4 ±10.9^{a2}	46.0 ± 4.6^{b3}
2.5mM	64.6 ± 4.9^{a1}	53.9±4.7^{a2}	52.7 ± 3.8^{a2}	39.7 ±12.4^{a3}
	Strength (0-5)			
0	4.2 ±0.4^{a1}	3.8 ± 0.5^{a2}	3.6 ± 0.5^{ab2}	3.2 ± 1.1^{a3}
1mM	4.2 ± 0.5^{ab1}	3.8 ± 0.4^{a2}	3.7 ± 0.5^{a2}	3.6 ± 0.5^{b2}
1.5mM	4.0 ± 0.7^{b1}	3.8 ± 0.4$^{a}1^{2}$	3.5 ± 0.5^{b2}	3.5 ± 0.6^{ab2}
2.5mM	4.1 ± 0.5^{ab1}	3.8 ± 0.8^{a12}	3.7 ± 0.5^{ab2}	3.2 ± 1.1^{a3}

Different letters and numbers in the same row are indicative of difference ($P<0.05$)

per box. This volume is the amount necessary for the correct refrigeration curve.

Semen analysis

Motility, strength, viability and acrosomal and plasmatic membrane integrity were analyzed at 0, 12, 24 and 36h after refrigeration. For evaluation, samples were removed from the boxes and kept in a water bath at 37°C for ten minutes for stabilization. To evaluate total motility (0–100%) and strength (0–5), a 10μL drop of semen was placed between a previously heated (37°C) slide and coverslip visualized under a contrast phase optical microscope at 200 magnification. Strenght was expressed as the speed at which the spermatozoa move in the field. The result was expressed on a scale of 0–5, 0 being no motion and 5 intense speed.

To evaluate viability, the supravital eosin-nigrosin staining technique (LIVE/DEAD) was used, where equal volumes (20μL) 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 considered viable (*Dott* and *Foster* 1972).

To evaluate sperm plasmatic membrane integrity the hypo-osmotic swelling test (HOST) was used by incubating 100 microliters of post-thawed semen in 1.0mL of a hypo-osmotic (100mOsm/L) sucrose solution in water bath at 37°C for 30 minutes. After this time, 20 microliters of this solution (post-thaw semen + hypo-osmotic solution) was analyzed in a humid chamber, using phase contrast microscopy at ×1,000 magnification. A total of 200 spermatozoa were counted, and those considered swollen (coiled) were determined as having 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 0.2% trypan blue (20mL; 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 (*Kútvölgyi* et al. 2006). Evaluation was performed at 1,000× magnification, and 200 spermatozoa were counted and classified as: live – acrosomes stained in pink or purple and post-acrosomal regions unstained; dead – in which they were stained in blue at the postacrosomal region and acrosomes were stained in purple or pink; true acrosome reaction – acrosomes and postacrosomal regions were unstained; and false acrosome reaction – in which acrosomes were unstained and post-acrosomal regions were stained in blue (*Didion* et al. 1989).

Statistical analysis

Experimental design was completely randomized. Data analysis was performed using Univariate (SAS 2000) 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, a nonparametric test, the Kruskal-Wallis H test was used (*Fitch* 2006).

Results

Table 1 shows the results for total motility and strength. Total motility was higher with 1mM cysteine treatment after 12h of cooling (56.6 ± 8.7) and with 1 (46.5 ± 6.1) and 1.5mM (46 ± 4.6) cysteine treatments after 36h of cooling when compared to the other treatments ($P < 0.05$). The highest value for strength was observed with 1mM cysteine treatment after 36h of cooling.

Table 2 shows the results for sperm viability and storage time. The highest value was observed with 1mM (76.5 ± 6.9) and 1.5mM (76.9 ± 3.7) cysteine compared to control (68.1 ± 13.4) and 2.5mM (66 ± 17.2) cysteine after 36h of cooling ($P < 0.05$)..

Plasmatic membrane integrity data from spermatozoa submitted to cooling are presented in Table 3. Table 4 depicts acrosome membrane integrity and storage time, where no difference can be observed among treatments ($P > 0.05$).It can be verified a reduction of all variables analyzed from 0 to 36h, in all samples.

Discussion

In the present study, cooling semen for 36h was chosen to challenge spermatozoa regarding the generation of free radi-

Table 2 – Effect of different cysteine concentrations (treatment) in equine cooled semen on sperm viability during the different storage time (0, 12, 24 and 36h) of cooling at 16°C. / *Wirkung verschiedener Cystein-Konzentrationen (Behandlung) bei gekühltem Pferdesperma auf die Lebensfähigkeit des Spermas während den unterschiedlichen Lagerungszeiten (0, 12, 24 und 36 St) bei Kühlung auf 16°C.*

	Storage time (h)			
Treatment	0	12	24	36
	Viability (%)			
0	84.2 ± 3.3^{ab1}	84.3 ± 2.7^{a1}	75.5 ± 9.7^{ab2}	68.1 ±13.4^{a3}
1mM	83.1 ± 4.2^{a1}	83.7 ± 2.9^{a1}	76.0 ± 5.8^{ab2}	76.5 ± 6.9^{b2}
1.5mM	85.5 ± 2.6^{b1}	83.1 ± 3.2^{a1}	78.5 ± 10.5^{a2}	76.9 ± 3.7^{b2}
2.5mM	82.7 ± 3.5^{a1}	83.5 ± 3.6^{a1}	74.8 ± 7.3^{b2}	66.0 ± 17.2^{a3}

Different letters and numbers in the same row are indicative of difference ($P<0.05$)

cals and evaluate the effect of the addition of the antioxidant used and also mimic the time needed to ship cooled semen to distant places. The refrigeration system chosen was a passive method at 16°C, due to low cost, easy access to farmers and veterinarians and is the most used for the transportation of semen in Brazil. The hypothesis that the addition of antioxidants to equine cooled semen would prolong sperm viability was tested by *Aurich* et al. (1997) (ascorbic acid) and *Bruemmer* et al. (2002) (pyruvate), which verified that membrane integrity, motility and fertility were maintained; but *Ball* et al. (2001) did not observe differences using different antioxidants and different concentrations in equine semen kept at 5°C for 96 hours.

Regardless of the treatment, there was a drop in motility during the evaluation period, especially after 24 h of cooling that was observed in all treatments after 24 h of storage at 16°C. This drop is a consequence of cold shock, when spermatozoa are submitted to temperatures below 20°C. These alterations can be characterized by abnormal movement and rapid drop in motility, membrane damage, metabolism reduction, loss of enzymes and other intracellular components (*Aurich* 2005) and lipid peroxidation in the presence of oxygen ions. Sperm membrane contains great quantities of unsaturated fatty acids, particularly susceptible to peroxidation, with subsequent loss of membrane integrity and cellular function (*Aurich* et al. 1997).

Cysteine concentrations of 1.5 and 2.5 mM demonstrated the best results for motility at 36 hours after cooling. This difference in comparison to control can be explained by the mechanism of action of antioxidants, because they retard oxidation by inhibiting the production of free radicals and ROS (*Ball* et al. 2001). In special to cysteine, a non-enzymatic antioxidant that prevents lipid peroxidation and a precursor of glutathione biosynthesis, which increases the levels of reduced glutathione (GSH) (*Bilodeau* et al. 2001). Reduced glutathione is capable to act directly in ROS (*Luberda* 2005) and is a co-factor for glutathione peroxidase, which catalyzes the reduction of hydrogen peroxide and hydroperoxides, protecting the cell against oxidative stress. These differences in motility were not verified by *Reghini* et al. (2010), who evaluated the effect of the incorporation of N-acetylcysteine in different concentrations to the extender medium for cooling equine semen for 24 hours at 5 and 15°C, and for *Pagl* et al. (2006), who used the same compounds added to equine semen kept at 5°C for 72 hours.

During the passage through the female genital tract, spermatozoa are exposed to ROS secreted by leucocytes. However, inside the uterus, the anaerobic condition reduces the potential damage of reactive species (*Foote* et al. 2002). ROS production is induced when semen is processed after ejaculation, especially due to contamination with leucocytes and the presence of spermatozoa with excess residual cytoplasm (*Brouwers* et al. 2005). The spermatic cell also produces intracellular ROS due to flagellar activity (*Gavella* and *Lipovac* 1992). While a slight peroxidation seems to be a physiological mechanism promoting sperm cell capacitation, the excess causes damage to the membrane and results in loss of motility and fertilizing capacity (*Aitken* and *Baker* 2004).

Regarding sperm viability evaluated by supravital staining, there was a decrease in all groups after 36 hours at 16°C. Percentage values started to decline 24 h after of cooling. For plasmatic membrane integrity evaluated by hypoosmotic test, even though cysteine showed higher values at 24 and 36 hours after cooling, there was no difference in comparison to control ($P > 0.05$), as also observed by *Reghini* et al. (2010) and *Pagl* et al. (2006). According to *Bedford* et al (2000) the passive refrigeration process induces acrosome lesions and in

Table 3 – Effect of different cysteine concentrations (treatment) in equine cooled semen on plasmatic membrane integrity during the different storage time (0, 12, 24 and 36h) of cooling at 16°C. / *Wirkung verschiedener Cystein-Konzentrationen (Behandlung) bei gekühltem Pferdesperma auf die plasmatische Membranintegrität während den unterschiedlichen Lagerungszeiten (0, 12, 24 und 36 St) bei Kühlung auf 16°C.*

	Storage time (h)			
Treatment	0	12	24	36
	Plasmatic membrane integrity (%)			
0	65.2 ± 4.8^{a1}	62.8 ± 6.1^{a1}	56.2 ± 14.5^{a2}	54.1 ± 11.8^{ab2}
1mM	65.2 ± 4.1^{a1}	56.4 ± 9.2^{bc2}	59.5 ± 20.3^{a2}	57.2 ± 9.5^{a2}
1.5mM	64.9 ± 4.7^{a1}	54.7 ± 10.1^{b2}	44.5 ± 7.7^{b3}	52.2 ± 13.3^{b2}
2.5mM	61.6 ± 9.5^{b1}	58.8 ± 5.9^{c13}	45.6 ± 15.2^{b2}	55.8 ± 12.5^{ab3}

Different letters and numbers in the same row are indicative of difference ($P<0.05$)

Table 4 – Effect of different cysteine concentrations (treatment) in equine cooled semen on acrosomal membrane integrity during the different storage time (0, 12, 24 and 36h) of cooling at 16°C. / *Wirkung verschiedener Cystein-Konzentrationen (Behandlung) bei gekühltem Pferdesperma auf die akrosomale Membranintegrität während den unterschiedlichen Lagerungszeiten (0, 12, 24 und 36 St) bei Kühlung auf 16°C.*

	Storage time (h)			
Treatment	0	12	24	36
	Acrosomal membrane integrity (%)			
0	75.2 ± 8.9^{a1}	73.3 ± 7.6^{a12}	72.1 ± 9.6^{a12}	70.5 ± 10.4^{a2}
1mM	73.9 ± 3.5^{a1}	74.1 ± 5.7^{a1}	69.7 ± 5.2^{a2}	69.4 ± 4.4^{ab2}
1.5mM	71.5 ± 5.6^{a1}	$71.4 \pm 10.2a^{1}$	70.5 ± 3.6^{a1}	68.0 ± 7.2^{b2}
2.5mM	75.4 ± 1.3^{a1}	72.9 ± 7.3^{a12}	71.6 ± 10.1^{a2}	70.3 ± 0.5^{a2}

Different letters and numbers in the same row are indicative of difference ($P<0.05$)

the present study none of the cysteine concentrations tested was capable to preserve high percentages of integral acrosomes. However, it is important to state that this method is highly subjective to slide quality and number of evaluated parameters at evaluation. *Baumber* et al. (2000) verified that the generation of ROS did not show significant effects in viability or acrosomal integrity, or in mitochondrial membrane potential or lipoperoxidation levels and concluded that sperm motility is the best indicator of oxidative stress in this species.

A temperature reduction from 38°C to 30°C in semen samples containing cells with unstable membranes due to capacitation is already sufficient to unleash acrosome reaction (*Gadella* et al. 2001). Therefore, it is important to be aware of potential damage that extenders and the cooling curve can cause to acrosome.

There is no consensus in the literature regarding the protective effect of antioxidant substances added to the extender, since some studies report positive effects and others report that beneficial effects were not observed. This controversy is certainly due to age and animal breed, extender composition, semen conservation method, antioxidant dose and combination, among others. Optimized assays will certainly provide consistency in results and which antioxidant and its concentration to be used to prevent oxidative damage caused by ROS to spermatozoa.

Cysteine added to the extender for the maintenance of cooled equine spermatozoa at 16°C can reduce the losses of both motility and sperm viability, especially after cooling. The damages that occur on the sperm cell during the cooling process cannot be individually overcome due to several factors involved in this process. This way, more research should be done to allow the development of protocols that can minimize these effects, as well as, the development of optimized methods to evaluate sperm viability after cooling to improve fertility rates in artificial insemination programs. Nevertheless, additional studies are needed to evaluate cysteine dose response and determine an optimal 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 cooled semen.

Conclusion

The addition of 1.0 mM cysteine can reduce the losses of both motility and sperm viability, especially after 24 hours of cooling at 16°C. The cysteine concentration of 1.0 mM was more efficient to protect the sperm cell in the commercial passive refrigeration system at 16°C for 36 h, with higher motility and viability values.

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Erweiterte Zusammenfassung

Die Manipulation von Pferdesamen bei der Kühlung reduziert die Lebensfähigkeit und Fruchtbarkeit des Spermas, woraus neben weiteren Problemen die Lipidperoxidation der Membran resultiert. Der hohe Gehalt an mehrfach ungesättigten Fettsäuren macht die Zellen sehr anfällig für freie Radikale und reaktive Sauerstoffarten (EROS). Die oxidative Wirkung, verursacht durch die Generierung von EROS, kann verringert werden durch die Zugabe von Antioxidantien ins Samenplasma oder in den Verdünner während des Gefriervorgangs. Die Ergebnisse sind widersprüchlich hinsichtlich der Lebensfähigkeitserhaltung der Spermien im frischen und eingefrorenen Samen aufgrund der Art und Konzentration der verwendeten Antioxidantien sowie des spezifischen Wirkmechanismus. Eines der Antioxidantien, das im Samen einiger Arten eingebettet ist, ist das Cystein, der Vorläufer der Biosynthese von Glutathion. Cystein hat ein niedriges Molekulargewicht und ist ein nicht enzymatischer Antioxidans, das die Lipidperoxidation verhindert mittels der Fähigkeit, in die Zellmembran einzudringen.

Die Bewertung des In vitro-Effekts wurde der Samen von 12 Hengsten mit Cystein in vier Konzentrationen (0, 1, 1,5 und 2,5 mM) im Verdünnungsmittel versetzt und bei 16 °C für 36 h gekühlt. Die ausgewerteten Variablen waren Mortalität, Vitalität, Lebensfähigkeit und Integrität der Plasma-Membranen und Akrosomreaktion in vier verschiedenen Zeiten (0, 12, 24 und 36). Die Ejakulate der 12 Hengste wurden dreimal wöchentlich gesammelt. Das Sperma wurde zentrifugiert und das daraus resultierende Pellet in einer dünnen Basis von Magermilch Pulver wieder aufgelöst (0 mM-Control, 1 mM, 1,5 mM und 2,5 mM Cystein), mit einer Endkonzentration von 50×10^6 Spermium/mL. Der pH-Wert wurde gemessen und zeigte bei der Kontrolle keine Unterschiede. Zur Kühlung wurden vier kommerzielle Samen-Transportkisten verwendet, eine für jede Gruppe, die ine Kühlung auf 16 °C gewährleisten.

Mit Ausnahme der akrosomalen Integrität (Tabelle 4) wurde in allen Proben während der Abkühlung eine Reduktion der Motilität (Tabelle 1) und der plasmatischen Membran-Integrität (Tabelle 3) festgestellt. Die Bewertung nach 36 Stunden Kühlung ergab, dass die gesamte Motilität (Mot) (Tabelle 1) und Lebensfähigkeit (Lbf) (Tabelle 2) in der mit 1 mM Cystein behandelten Gruppe (Mot: $46{,}5 \pm 6{,}1$/Lbf:$76{,}5 \pm 6{,}9$) und der mit 1,5 mM Cystein behandelten Gruppe (Mot: $46{,}0 \pm 4{,}6$/Lbf:$76{,}9 \pm 3{,}7$) jeweils höher waren, im Vergleich zur Kontrollgruppe (Mot: $35{,}5 \pm 18{,}4$/Lbf: $68{,}1 \pm 13{,}4$) und der mit 2.5 mM Cystein behandelten Gruppe (Mot: $39{,}7 \pm 12{,}4$/Lbf:$66 \pm 17{,}2$) ($P < 0{,}05$). Auch für Stärke (Stk) (Tabelle 1) und plasmatische Membranintegrität (Tabelle 3) (plasm MI) wies die Gruppe mit 1 mM Cystein höhere Werte auf (str:$3{,}6 \pm 0{,}5$/plasm MI: $57{,}2 \pm 9{,}5$) im Vergleich zur Kontrollgruppe (Stk: $3{,}2 \pm 1{,}1$/plasm MI: $54{,}1 \pm 11{,}8$), zur 1,5 mM-Gruppe (Stk:$3{,}5 \pm 0{,}6$/plasm MI: $52{,}2 \pm 13{,}3$) und der 2,5 mM Gruppe (Stk:$3{,}2 \pm 1{,}1$/plasm MI: $55{,}8 \pm 12{,}5$) ($P < 0{,}05$). In Bezug auf die Membranintegrität des Akrosoms (Tabelle 4), gab es, im Allgemeinen, keinen Integritätsverlust ($70{,}5 \pm 10{,}4$; $69{,}4 \pm 4{,}4$; $68{,}0 \pm 7{,}2$ und $70{,}3 \pm 0{,}5$), jeweils Kontrollgruppe,1 mM, 1,5 mM und 2,5 mM.

Im Ergebnis war die Konzentration von 1 mM Cystein zum Schutz der Samenzellen im kommerziellen System des passiven Kühlens auf 16°C über 36 Stunden am effektivsten, mit jeweils höheren Werten für Motilität, Lebensfähigkeit sowie plasmatische Membran-Integrität.