http://dx.doi.org/10.1590/1678-4162-11293

Arq. Bras. Med. Vet. Zootec., v.72, n.1, p.9-17, 2020

S.C.C. Pinto
https://orcid.org/0000-0002-4220-3186
D.S. Almeida
https://orcid.org/0000-0002-5169-9320
M.B.R. Alves
https://orcid.org/0000-0002-9480-6608
S.A. Florez-Rodriguez
https://orcid.org/0000-0002-8200-2119
G.S. Abreu Júnior
https://orcid.org/0000-0001-9373-7295
E.C.C. Celeghini
https://orcid.org/0000-0003-1531-5497
L.M. Laskoki
https://orcid.org/0000-0003-0888-769X
F.A. Souza
https://orcid.org/0000-0002-9474-9404

Does supplementation of vitamin C, reduced glutathione or their association in semen extender reduce oxidative stress in bovine frozen semen?

[*A suplementação de vitamina C, glutationa reduzida ou sua associação no diluidor de sêmen reduz o estresse oxidativo no sêmen congelado bovino?]*

S.C.C. *Pinto*[1], D.S. *Almeida*[2], M.B.R. *Alves*[1], S.A. *Florez-Rodriguez*[1], G.S. *Abreu Júnior*[2], N. *Britto e Alves*[2], E.C.C. *Celeghini*[3], L.M. *Laskoki*[4], F.A. *Souza*[4*]

[1]Aluna de pós-graduação – Universidade de São Paulo – Pirassununga, SP
[2]Aluno de pós-graduação – Universidade Estadual do Maranhão – São Luís, MA
[3]Universidade de São Paulo – Pirassununga, SP
[4]Universidade Federal do Acre – Rio Branco, AC

ABSTRACT

The aim of this study was to evaluate the addition of vitamin C, reduced glutathione and the association thereof to the bovine semen cryopreservation extender. The ejaculate from nine bulls were divided into four fractions, each corresponding to a treatment, namely: control group–semen diluted with Tris-yolk extender; vitamin C group–semen diluted in Tris-yolk extender supplemented with vitamin C (2.5mmol/mL); glutathione group-semen diluted in Tris-yolk extender supplemented with reduced glutathione (2.5mmol/mL) and associated group-semen diluted in Tris-yolk extender supplemented with vitamin C (1.25mmol/mL) and reduced glutathione (1.25mmol/mL). Afterwards, the semen was packed into French straws and submitted to cryopreservation using automated equipment. After cryopreservation, the semen was thawed and evaluated considering sperm motility, morphology, plasma membrane, acrosome, mitochondrial potential and oxidative stress, as well as the thermo resistance test. Extender's supplementation with the association of vitamin C and reduced glutathione showed benefic effects on sperm motility and preservation of plasma and acrosomal membranes during semen cryopreservation, being also the group that showed higher values of reactive oxygen species. Thus, the association of both antioxidants contributed to the preservation of sperm cells in every analyzed characteristic, suggesting its use on bovine semen cryopreservation.

Keywords: reactive oxygen species, sperm membranes, spermatozoa

RESUMO

O objetivo deste estudo foi avaliar a adição de vitamina C, glutationa reduzida e sua associação ao diluidor de criopreservação de sêmen bovino. O ejaculado de nove touros foi dividido em quatro frações, cada uma correspondendo a um tratamento, a saber: grupo controle – sêmen diluído em Tris-gema; grupo vitamina C – sêmen diluído em Tris-gema, suplementado com vitamina C (2,5mmol/mL); grupo glutationa – sêmen diluído em Tris-gema, suplementado com glutationa reduzida (2,5mmol/mL) e grupo sêmen associado – diluído em Tris-gema, suplementado com vitamina C (1,25mmol/mL) e glutationa reduzida (1,25mmol/mL). Posteriormente, o sêmen foi envasado em palhetas francesas e submetido à criopreservação por meio de equipamento automatizado. Após a criopreservação, o sêmen foi descongelado e avaliado quanto à motilidade espermática, à morfologia, à membrana plasmática, ao acrossoma, ao potencial mitocondrial e ao estresse oxidativo, bem como pelo teste de resistência térmica. A suplementação de extensor com a associação de vitamina C e glutationa reduzida mostrou efeitos benéficos sobre a motilidade espermática e a preservação das membranas plasmática e acrossomal

Recebido em 8 de fevereiro de 2019
Aceito em 2 de agosto de 2019
*Autor para correspondência (*corresponding author*)
E-mail: femedvet@yahoo.com.br

durante a criopreservação de sêmen, sendo também o grupo que apresentou maiores valores de espécies reativas de oxigênio. Assim, a associação de ambos os antioxidantes contribuiu para a preservação dos espermatozóides em todas as características analisadas, sugerindo sua utilização na criopreservação de sêmen bovino.

Palavras-chave: espécies reativas de oxigênio, membranas espermáticas, espermatozoides

INTRODUCTION

The use of cryopreserved semen allows the choice of best breeders that meet farm needs, being indispensable in artificial insemination, and *in vivo* and *in vitro* embryo transfer programs (Leite *et al.*, 2011). An adequate process of semen cryopreservation ensures the success of the reproductive techniques (Duarte-Junior *et al.*, 2015). Despite broadly used, semen cryopreservation is a process that causes great stress to the spermatozoa, which can occur during cooling, freezing and thawing, thus diminishing sperm quality compared to fresh semen (Maia and Bicudo, 2009).

Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the protective action of the antioxidant system, responsible for its neutralization and removal (Jedrzejowska *et al.*, 2013). All radicals and non-radicals from oxygen are considered ROS, which has high electron reactivity and instability. The ROS can react with a great number of compounds, acting as donors or receivers of electron (Agarwal *et al.*, 2005), and they are considered the main prompters of damage to living organisms (Bernard and Krause, 2007). Main ROS includes oxygen ions, free radicals and peroxide, and are naturally formed as sub-products of oxygen metabolism and play an important role in cellular signalization (Paparella *et al.*, 2015).

To avoid oxidative stress, the cell has a defense system compound by antioxidants, which are divided into enzymatic and non-enzymatic substances with low molecular weight (Halliwell and Gutteridge, 1999). However, during semen cryopreservation, antioxidant concentrations are reduced during the dilution stage, which results in the imbalance and, consequently, in cellular and oxidative stress (Bilodeau *et al.*, 2000).

One of the main enzymatic antioxidants is the glutathione, as peroxidase and reduced forms, which is responsible for the reconstruction of thiol groups (-SH) in proteins that can be eliminated during the oxidative stress, and protects cellular membranes from lipids oxidation, thus preventing the formation of free oxygen (Lenzi *et al.*, 1994). As for the non-enzymatic antioxidants, vitamin C is one of the main substances (Jedrzejowska *et al.*, 2013). The antioxidant function of Vitamin C is the inhibition of lipid peroxidation by the action of ions Fe^{2}+ and Cu^{1} (Halliwell and Gutteridge, 1999).

Studies performed by Turcer *et al.* (2010) and Gadea *et al.* (2004) demonstrated that supplementation of extenders with vitamin C and reduced glutathione has shown inconstant results on the preservation of sperm characteristics after cryopreservation. In addition, there is no study comparing the combination of these antioxidants in bull's semen. Therefore, this study aimed to determine the effects of the addition of vitamin C, reduced glutathione, or the combination thereof to the cryopreservation semen extender on the bovine sperm motility, vigor, integrity of plasma and acrosome membranes, mitochondrial function and oxidative stress.

MATERIAL AND METHODS

This study was approved by the Ethical Committee on Animal Use (CEUA) at the Federal University of Acre, under the registration number 50/2015. Nine Nellore bulls, average age of 2 years old and mean weight of 545kg, were used. Semen collection was done using an electro ejaculator (Boijector®), and fresh semen was evaluated according to volume (mL), concentration (spermatozoa/mL), motility (%), vigor (1-5 score), and sperm morphology (%). For the cryopreservation, the ejaculates used showed a minimum of 70% of sperm motility and maximum of 30% of abnormal sperm morphology.

Each ejaculate was divided into four equal fractions, corresponding to the treatments: 1) Control Group (CON) – only Tris-yolk extender;

2) Vitamin C Group (VTC) – Tris-yolk extender supplemented with Vitamin C (2.5mmol/mL); 3) Reduced Glutathione Group (GSH) – Tris-yolk extender supplemented with reduced glutathione (2.5mmol/mL); and 4) Associated Group (ASS) – Tris-yolk extender supplemented with vitamin C (1.25mmol/mL) and reduced glutathione (1.25mmol/mL).

After the dilution, according to each group, semen was packed into 0.25mL French straws, at the concentration of $50x10^6$ sperm cells/mL, and frozen using a programmable equipment, TK 3000® (TK Equipment for Reproduction). The cooling rate was 0.5°C/min until reaching 5°C, remaining for 2 hours in stacking, followed by a negative curve 15°C/min of five at -80°C, then 10°C/min until reaching -120°C. The final step of the process was to immerse the straws in liquid nitrogen (-196ºC). After cryopreservation, the straws were kept in canisters, in a cryogenic container.

For post-cryopreservation semen evaluation, two straws from each treatment and bull were thawed in a water bath at 37ºC for 30 seconds, then placed into a 1.5mL centrifuge tube and homogenized. For analyses of sperm motility (%) and vigor (1 to 5 score, where 1 is the slowest spermatozoa movement and 5 the maximum rectilinear and high speed of sperm), 10µL of semen was placed between pre-warmed slide and cover slip, and evaluated under phase contrast microscopy (Nikon, Eclipse 80i Model), at a magnification of 100x.

The rapid thermo resistance test (RTT) was performed using a test adapted from the proposed by Dimitropoulos (1967). Thereafter, 0.25mL French straws were thawed in a 37ºC water bath for 30 seconds, transferred to a 45ºC water bath and kept for 30 minutes, thus sperm motility and vigor were assessed every 10 minutes.

For sperm morphology, 100µL aliquot of semen was placed in a micro tube and 50µL of 4% buffered saline formalin was added, homogenized and stored at 5ºC for assessment. Then, 3µL of the diluted sample was placed between a slide and coverslip to count 200 cells, being evaluated by differential interference contrast microscopy (DIC), under 1000x magnification. Abnormal spermatozoa were classified as major and minor defects, according to Blom (1973).

Sperm membranes were evaluated using techniques described by Celeghini *et al.* (2007). Briefly, a sample of the semen was diluted in TALP sperm media to obtain a concentration of $10x10^6$ spermatozoa/mL. After the dilution, 2µL of Hoechst 33342 (H342, 0.5mg/mL in DPBS), 3µL of propidium iodide (PI, 0.5mg/mL in DPBS), 6µL of tetrachloro-tetraethylbenzimidazolocarbocyanine iodide (JC-1, 153µM in DMSO) and 20µL of fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin (FITC-PSA, 100µg/mL in DPBS) were added in 150µL of the diluted semen. The samples were incubated for 8 minutes at 37ºC, in the dark. After incubation, a 4µL drop was used to prepare the wet chamber, between the pre heated slide and coverslip (37ºC), and the evaluation was done under epifluorescence microscopy (Nikon, Eclipse 80i Model) in a triple filter (D/F/R, C58420) presenting the groups UV-2E/C (340-380nm excitation and 435-485nm emission), B-2E/C (465-495nm excitation and 515-555nm emission) and G-2E/C (540-525nm excitation and 605-655nm emission), under 1000x magnification.

Two hundred cells were counted and classified according to Celeghini *et al.* (2007), in IPIAH (intact plasma membrane, intact acrosomal membrane and high potential of mitochondrial membrane), IPIAL (intact plasma membrane, intact acrosomal membrane and low potential of mitochondrial membrane), IPDAH (intact plasma membrane, damaged acrosomal membrane and high potential of mitochondrial membrane), IPDAL (intact plasma membrane, damaged acrosomal membrane and low potential of mitochondrial membrane), DPIAH (damaged plasma membrane, intact acrosomal membrane and high potential of mitochondrial membrane), DPIAL (damaged plasma membrane, intact acrosomal membrane and low potential of mitochondrial membrane), DPDAH (damaged plasma membrane, damaged acrosomal membrane and high potential of mitochondrial membrane), and DPDAL (damaged plasma membrane, damaged acrosomal membrane and low potential of mitochondrial membrane). For the results, the percentage of cells for IPIAH, intact plasma membrane (PI), intact acrosome

(AI) and high potential of mitochondrial membrane (HP), were considered.

To assess the oxidative stress, 50µL of semen diluted in TALP media sperm ($20x10^6$ spermatozoa/mL) was used. 1µL of H33342 (2.5mg/mL in DPBS) and 2µL of CellROX (1mmol in DMSO) was added before it was incubated at 37ºC for 30 minutes. Afterwards evaluation was conducted under epifluorescence microscope (Nikon, Eclipse 80i Model) in a triple filter (D/F/R, C58420) presenting the groups UV-2E/C (340-380nm excitation and 435-485nm emission), B-2E/C (465-495nm excitation and 515-555nm emission) and G-2E/C (540-525nm excitation and 605-655nm emission), at magnification of 1.000x, according to Alves *et al.* (2015), adapted to the bovine species.

The experiment was conducted in randomized blocks, where the parametric variables were tested by ANOVA, comparing means thru Tukey's test. All variables passed the Shapiro-Wilk and Lilliefors normality tests. The variables with subjective variables or that did not meet normality after transformation were analyzed as non-parametric, and ranked data was compared by the Friedamn's test. Dichotomous variables were evaluated by Fisher's exact test. The interval of confidence used was 95% and the Bio Estat 5.0 program was used for data analysis.

RESULTS

Mean values for progressive motility before semen cryopreservation processes were 81.1±3.33% and 3.0±0.0 for vigor. Means and standard deviation for sperm motility of bovine cryopreserved semen without supplementation (control) and supplemented with Vitamin C, reduced glutathione and association of vitamin C + reduced glutathione were 35.00±2.65b, 40.00±2.79ab, 42.22±2.51ab and 44.44±3.11a, respectively. Higher sperm progressive motility was observed for the ASS group when compared to the control group (P< 0.05); however, no difference was observed for other treatments.

There was no difference (P> 0.05) in sperm vigor among semen extenders. The mean values for CON, VTC, GSH and ASS groups were, respectively, 2.11±0.33, 2.00±0.00, 2.00±0.00 and 2.11±0.33. When sperm motility was assessed after the RTT test, within each post thawing time, there was no statistical difference between treatments in relation to the CON group (P> 0.05). (Table 1).

Regarding major defects, the ASS group presented lower percentage of sperm defects when compared to thtr GSH group (P< 0.05); however, this did not differ from the other groups. Evaluated separately, the antioxidants had no influence on major sperm defects percentage after the process of cryopreservation (P> 0.05). Minor defects had similar response, as an interaction between antioxidants or the main effect of each substance was not observed (Table 2).

Table 1. Mean and standard deviation for sperm motility after rapid thermal resistance (RTT) evaluation time in bovine cryopreserved semen without supplementation and supplemented with Vitamin C, reduced glutathione and association of vitamin C + reduced glutathione (Association)

Treatment	Evaluation time after RTT		
	10min	20min	30min
Control	36.66±10.30	24.44±8.81	2.22±4.40
Vitamin C	43.33±7.90	30.00±10.00	8.88±12.69
Reduced Glutathione	42.77±8.33	31.66±15.81	6.66±8.66
Association	40.55±9.16	30.00±11.18	13.33±12.24

There was no statistical difference (P> 0.05) between treatments when the means were compared by Friedman's test.

Table 2. Mean and standard deviation for sperm morphology in bovine cryopreserved semen without supplementation and supplemented with Vitamin C, reduced glutathione (GSH) and association of vitamin C + reduced glutathione (Association)

Treatment	Sperm Morphology (%)	
	Major Defect	Minor Defect
Control	6.27±0.60[ab]	3.22±0.75[a]
Vitamin C	6.5±0.63[ab]	2.72±0.41[a]
Reduced Glutathione	6.66±0.81[b]	2.88±0.64[a]
Association	4.88+0.55[a]	2.11±0.32[a]

[a,b] different letters in column represent statistical difference by the Tukey test (P< 0.05).

Mean and standard deviation of integrity of plasma and acrosomal membranes of bovine cryopreserved semen without supplementation and supplemented with Vitamin C, reduced glutathione and association of vitamin C + reduced glutathione were 22.55±4.19b, 30.66±4.18ab, 27.22±5.10ab and 37.77±2.29a, respectively. For integrity of the plasma and acrosomal membranes, reduced cell damage was observed in the ASS group compared to the CON group (P< 0.05).

When evaluated separately for plasma membranes, as for the acrosomal integrity and mitochondrial potential categories, it was observed that there was no statistical difference (P> 0.05) for the supplementation of the antioxidants used experimentally (Table 3).

Interestingly, although ASS had shown better results for sperm motility, major defects and integrity of plasma and acrosomal membranes, a higher oxidative stress was observed in the semen from the ASS group (P< 0.05) compared to CON, VTC and GSH groups (Figure 1).

Table 3. Mean and standard deviation of for the acrosomal integrity (AI) and mitochondrial potential (HP) categories of bovine cryopreserved semen without supplementation (Control) and supplemented with Vitamin C, Reduced glutathione and association of vitamin C + reduced glutathione (Association)

Membranes	Treatments			
	Control	Vitamin C	Reduced Glutathione	Association
HP (%)	77.77±28.20	81.22± 24.48	77.22±26.49	89.00±9.09
AI (%)	38.66±13.45	41.44±16.82	42.11±11.87	48.55±6. 83

In line; there was no statistical difference (P> 0.05) when the means were compared by the Tukey test.

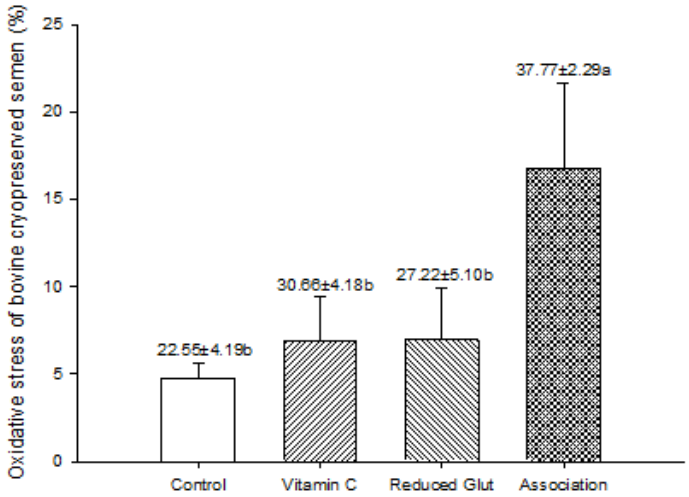

Figure 1. Mean and standard deviation for oxidative stress of bovine cryopreserved semen without supplementation and supplemented with Vitamin C, reduced glutathione (GSH) and association of vitamin C + reduced glutathione (Association). Different letters differ by Fisher's Exact test (P< 0.05).

DISCUSSION

In this study, the effect of reduced glutathione and vitamin C, isolated or combined, were evaluated on sperm motility, vigor, morphology, integrity of plasma and acrosomal membranes and oxidative stress in extender of bovine frozen semen. Reduced glutathione and vitamin C, when single added to the extender, did not affect the progressive motility after cryopreservation, contrasting with what was found by Zhao *et al.* (2015) in bovine semen supplemented with vitamin C, as well as Ansari *et al.* (2012) in buffalo semen, Oliveira *et al.* (2013) in equine semen and Ogata *et al.* (2015) in canine semen supplemented with reduced glutathione.

These previous studies evidenced positive effects of supplementation with vitamin C and reduced glutathione on post thawing sperm characteristics. This divergence in the results could be explained by the difference of the antioxidant concentration experimentally used. The absence of positive response when glutathione was used in the present study can be the result of the time that this antioxidant was added in the semen extender. Gadea *et al.* (2004) affirm that reduced glutathione would have better response if added to the semen post thawing, since it would increase intracellular glutathione to be used by glutathione peroxidase in order to avoid the damage caused by lipid peroxidation, as during semen cryopreservation there is a decrease of this antioxidant. Nevertheless, reduced glutathione was added during dilution, before the semen cryopreservation process. As for vitamin C, when compared to the other treatments, had the smaller percentage of sperm motility, this decrease in sperm motility can be explained due to a decrease in the pH leading to an irreversible decrease in sperm motility, corroborating the findings of Aurich *et al.* (1997), with the suppression of its effect on axoneme integrity and mitochondria, as evidenced by Memon *et al.* (2012).

On the other hand, the association between vitamin C and reduced glutathione, resulted in a better preservation on sperm progressive motility, which together is believed to favor the decrease of hydrogen peroxide, which in relation to other ROS, is easier to pass through the plasma membrane (Baumber *et al.*, 2000), destabilizing by lipid peroxidation. Benefic effects of combined supplementation of reduced glutathione and vitamin C were also observed in swine semen by Giaretta *et al.* (2015), who observed positive effects on the following sperm characteristics: viability, motility and nuclear-proteic structure on hydrogen peroxide.

Sperm motility during RTT time decreased in all groups, which conflicts with the results found by Bilodeau *et al.* (2000), which obtained positive results on semen incubation at 38ºC for 6 hours, and Peixoto *et al.* (2013), in a study with canine semen incubated at 37ºC for 60 minutes using reduced glutathione. However, these researchers applied the normal temperature of the female reproductive tract, which differed from the present study, where semen was incubated at 45ºC.

Despite the distinct evaluations between previous and the present study, there was a great decrease on sperm motility after 30 minutes, differing from that found in the literature, and Gonçalves *et al.* (2011) in a study with buffalo semen supplemented with vitamin C (2.5mmol/mL) and pentoxifylline, after RTT, stated significant preservation on sperm motility parameters. On the other hand, Peixoto *et al.* (2008) in a study using a supplementation of 600mmol/mL of vitamin C in semen extender, observed that this antioxidant did not help the maintenance of sperm motility, and neither has the capacity to avoid ROS effects. This decrease in sperm motility within the time is expected, according to some authors, as related by Peixoto *et al.* (2013), who observed a decrease in sperm motility and vigor after 45 minutes in canine semen supplemented with reduced glutathione (2 and 5mmol/mL) and trolox (100 and 200U/mL). Despite this decrease, the association between vitamin C and glutathione increased the preservation of sperm cells, reaffirming the benefic response of the combined action of these antioxidants.

Sperm vigor was not influenced by the supplementation of the antioxidants; however, sperm vigor reduced after the semen cryopreservation process. It was expected that the addition of these antioxidants in the extender would preserve the vigor, as found by Monteiro *et al.* (2009) in canine semen supplemented with reduced glutathione and vitamin C, this study showed that the antioxidants preserved sperm

vigor until 120 minutes. The reduction in the thawed semen can be explained by the normal consumption of substrates, intra and extracellular, as well as ATP metabolization during cryopreservation (KAMP *et al.*, 2003), because when compared to semen *in natura*, vigor decrease was not so accentuated after cryopreservation.

Antioxidants did not affect sperm morphology in the present study. Adrabi *et al.* (2008) and Akhter *et al.* (2011), in a study with buffalo, using vitamin C and vitamin E also did not see antioxidants benefic actions on sperm morphology. A study from Castilho *et al.* (2009) pointed that the ROS are responsible for metabolic alterations and an increase on intracellular compound liberation rate, for example, accentuated decrease of sperm motility and not on morphology. Perez *et al.* (2012) and Turcer *et al.* (2010), did not attest the effects of reduced glutathione supplementation on sperm defects (minors, majors and totals), in ovine semen supplemented with glutathione.

On integrity of the plasma and acrosomal membranes and for mitochondrial potential, it was seen that the antioxidants protected sperm cells, although the supplementation of vitamin C or reduced glutathione did not differ from the non-supplemented group. However, when the supplementation of this combination of antioxidants was evaluated, there was a greater protection on plasma and acrosomal membranes as well as on the mitochondrial potential after cryopreservation, evaluated numerically.

The antioxidants could have acted on lipid peroxidation that occurs during cryopreservation, thus this combination acted in synergy, having the reduced glutathione, due its characteristics, acted on disulphide bridges, and both acted on lipid peroxidation, as verified by Giaretta *et al.* (2015). Silva *et al.* (2011) demonstrated in their study that lower reduced glutathione concentrations (2.5 and 7mmoL), drill better protection to sperm cells, and Bilodeau *et al.* (2002) also observed that high concentrations of antioxidants in the bovine semen alter extender osmolality, leading to plasma membrane damage. Thus, the use of half concentration of the combined antioxidant provided better protection to plasma membrane after cryopreservation. Such results corroborate to other researches, such as Foote *et al.* (2002) in bulls, which also affirmed that lower antioxidant concentrations would give better protection to plasma and acrosomal membranes.

The evaluation of oxidative stress by CellRox® was adapted from Alves *et al.* (2015), and this is the first time that this methodology is used to evaluate the oxidative stress in bovine semen. The association of antioxidants resulted in higher oxidative stress compared to the other groups. However, the fluorescent probe used detects superoxide and hydroxyls radicals only in live cells (Grinberg *et al.*, 2013). Higher mensuration of ROS in the associated group, in this case, was due to higher percentage of mobile sperm, higher integrity of intact plasma membrane, intact acrosome and high potential of mitochondrial membrane, therefore higher number of living cells, with higher quantification of reactive species.

From another point of view, CellRox® probe detects the quantity of reactive species in the sample, finding increased ROS to be benefic or to mean that there was increase in peroxidation processes. Sperm naturally produce ROS, and this controlled production is important for sperm survival and functionality, having direct influence on fertility (Bilodeau *et al.*, 2000). Other benefic functions are: contribution to sperm motility and mitochondrial sheath stability (Jedrzejowska *et al.*, 2013). Therefore, the ASS group presented better motility, higher protection of the plasma membrane, which can be seen that the reactive species quantified by the probe could be beneficiating the sperm cells, and that CellRox® did quantify the reactive oxygen species present in the sample.

In conclusion, the supplementation with 2.5mmol/mL of vitamin C or reduced glutathione in semen extender before cryopreservation does not improve bovine sperm viability after thawing. However, the association of these antioxidants (1.25mmol/mL each) in the semen extender contributes to improve preservation of sperm characteristics during cryopreservation process and could be a potential strategy to improve the bovine semen fertility in artificial insemination and in vitro embryo production programs.

REFERENCES

ADRABI, S.M.H.; ASARI, M.S.; ULLAH, N., AFZAL, M. Effect of non-enzymatic antioxidants in extender on post-thaw quality of buffalo (bubalus bubalis) bull spermatozoa. *Pak. Vet. J*., v.28, p.159-162, 2008.

AGARWAL, A.; GUPTA, S.; SHARMA, R.K. Role of oxidative stress in female reproduction. *Reprod. Biol. Endocrinol*., v.3, p.28-49, 2005.

AKHTER, S.; RAKHA, B.A.; ANSARI, M.S.; MURTAZA, S. *et al.* Storage of nili-ravi buffalo (bubalus bubalis) semen in skim milk extender supplemented with ascorbic acid and α-tocopherol. *Pak. J. Zool.*, 43, 273-277, 2011.

ALVES, M.R.B.; ARRUDA, R.P.; BATISSACO, L.; RODRIGUEZ-FLOREZ, S.A. *et al.* An efficient technique to detect sperm reactive oxygen species: the CellRox Deep Red® Fluorescent Probe. *Biochem. Physiol*., v.4, p.157, 2015.

ANSARI, M.; RAKBHA, B.A.; ANDRABI, S.M.H.; OLLHAN, N. *et al.* Glutathione-supplemented tris-citric acid extender improves the post-thaw quality and in vivo fertility of buffalo (Bubalus bubalis) bull spermatozoa. *Reprod. Biol*., v.12, p.271-276, 2012.

AURICH, J.E.; SCHONHERR, U.; HOPPE, H.; AURICH, C. Effects of antioxidants on motility and membrane integrity of chilled-stored stallion semen. *Theriogenology*, v.48, p.185-192, 1997.

BAUMBER, J.; BALL, B.A.; GRAVANCE, C.G.; MEDINA, V. *et al.* The effect of reactive oxygen species on equine sperm motility, viability, acrossomal integrity, mitochondrial membrane potential and membrane lipid peroxidation. *J. Indol.*, v.21, p.895-902, 2000.

BERNARD, K.; KRAUSE, K.H.; The NOX family of ROS-Generating NADPH oxidases: physiology and pathophysiology. *Physiol. Rev*., v.87, p.245-313, 2007.

BILODEAU, J.F.; BLANCHETTE, S.; CORMIER, N.; SIRAD, M.A. Reactive oxygen species-mediated loss of bovine sperm motility in egg yolk Tris extender: protection by pyruvate, metal chelators and bovine liver or oviductal fluid catalase. *Theriogenology*, v.57, p.1105-1122, 2002.

BILODEAU, J.F.; CHATEERJEE, S.; SIRRARD, M. Levels of antioxidant defenses are descreased in bovine spermatozoa after a cycle of freezing and twaning. *Mol. Reprod. Dev*., v.55, p.282-288, 2000.

BLOM, E. The ultrastructure of some characteristic sperm defects and a proposal for a new classification of the bulls permiogram. *Nord. Veterinaermed*., v.25, p.383-391, 1973.

CASTILHO, E.F.; GUIMARAES, J.D.; MARTINS, L.F.; PINHO, R.O. *et al.* Use of propolis and ascorbic acid on goat semen cryopreservation. *Rev. Bras. Zootec*., v.38, v.2335-2345, 2009.

CELEGHINI, E.C.C.; ARRUDA, R.P.; ANDRADE, A.F.C.; NASCIMENTO, J. *et al.* Practical techniques for bovine sperm simultaneous fluorimetric assessment of plasma, acrosomal and mitochondrial membranes. *Reprod. Domest. Anim*., v.42, p.479-488, 2007.

DIMITROPOULOS, E. La signification du test de la thermoresistence dans l'appreciation de valeur fécondante du sperme congelé. *Ann. Méd. Vét*., v.4, p.215-224, 1967.

DUARTE-JÚNIOR, M.F.; ZERVOUDAKLS, L.K.H.; ZERVOUDAKLS, J.T.; NICHI, M.; *et al.* Tocopherol evaluations at the freezing of bovine semen on the pregnancy rates after fixed time artificial insemination. *Rev. Bras. Ciênc. Vet*., v.22, P.114-118, 2015.

FOOTE, R.H.; BROCKETT, C.C.; KAPROTH, M. Motility and fertility of bull sperm in whole milk extender containing antioxidants. *Anim. Reprod. Sci*., v.71, p.13-23, 2002.

GADEA, J.; SELLÉS, E.; MARCO, M.A.; COY, P. *et al.* Decrease in glutathione content in boar sperm after cryopreservation. Effect of the addition of reduced glutathione to the freezing and thawing extenders. *Theriogenology*, v.62, p.690-701, 2004.

GIARETTA, E.; ESTRADA, E.; BUCCI, D.; SPINACI, M. *et al.* Combining reduced glutathione and ascorbic acid has supplementary beneficial effects on boar sperm cryotolerance. *Theriogenology*, v.83, p.399-407, 2015.

GONÇALVES, A.A.; CASTRO, S.R.S.; GARCIA, A.R.; SANTOS, A.X. *et al.* Effect of antioxidant substances on cryopreserved semen of buffalo (bubalus bubalis). In: ANNUAL MEETING OF THE BRAZILIAN SOCIETY OF EMBRYO TECHNOLOGY, 25., 2011. *Proceedings*… [s.l.]: SBTE, 2011. p.451.

GRINBERG, Y.Y.; DIBBERN, M.E.; LEVASSEUR, V.A.; KRAIG, R.P. Insulin-like growth factor-1 abrogates microglial oxidative stress and TNF-α responses to spreading depression. *J. Neurochem*., v.126, p.662-672, 2013.

HALLIWELL, B.; GUTTERIDGE, J.M.C. *Free radicals in biology and medicine*. 3.ed. Oxford: Clarendon Press, 1999.

JEDRZEJOWSKA, R.W.; WOLSKI, J.K.; HILCZER, J.S. The role of oxidative stress and antioxidants in male fertility. *Cent. Eur. J. Urol*., v.66, p.60-67, 2013.

KAMP, G.; BUSSELMANN, G.; JONES, N.; WESNER, B. Energy metabolism and intracellular pH in boar spermatozoa. *Reproduction*. v.126, p.517-525, 2003.

LEITE, P.A.; SCHREDER, G.C.; ALMEIDA, C.L.R.; ZÚCCARI, C.E.S.N. *et al.* Cryopreservation of bovine semen. *UNOPAR Cient. Cienc. Biol. Saúde*, v.13, p.279-286, 2011.

LENZI, A.; PICARDO, M.; GANDINI, L.; LOMBARDO, F. *et al.* Gluthatione treatment of dyspermia: effect on the lipoperoxidation process. *Hum. Reprod.*, v.9, p.2044-2050, 1994.

MAIA, M.S.; BICUDO, S.D. Free radicals, antioxidant and sperm function in mammals: a review. *Rev. Bras. Reprod. Anim*., v.33, p.83-193, 2009.

MEMON, A.A.; WAHIDA, H.; ROSNINA, Y.; GOH, Y.M. *et al.* Effect of antioxidants on post thaw microscopic, oxidative stress parameter and fertility of boer goat spermatozoa in tris egg yolk glycerol extender. *Anim. Reprod. Sci*., v.136, p.55-60, 2012.

MONTEIRO, J.C.; GONÇALVES, J.S.A.; RODRIGUEZ, J.A.; LUCIO, C.F. Influence of ascorbic acid and glutathione antioxidants on frozen-thawed canine semen. *Reprod. Domest. Anim*., v.44, p.359-362, 2009.

OGATA, K.; SASAKI, A.; KATO, Y.; TAKEDA, A. *et al.* Glutathione supplementation to semen extender improves the quality of frozen-thawed canine spermatozoa for transcervical insemination. *J. Reprod. Develop*., v.61, p.116-122, 2015.

OLIVEIRA, R.A.; WOLF, C.A.; VIU, M.A.O.; GAMBARINI, M.L. Addition of glutathione to an extender for frozen equine semen. J. Equine Vet. Sci., v.33, p.1148-1152, 2013.

PAPARELLA, S.; ARAÚJO, S.S; ROSSI, G.; WIJAYASINGHE, M. Seed priming: state of the art and new perspectives. *Plant Cell Rep*., v.34, p.1281-1293, 2015.

PEIXOTO, A.L.V.A.; MONTEIRO, P.L.J.; CAMARA, D.R.; VALENÇA, R.M.B. *et al.* Incubation time effect after thawing on the viability of cryopreserved ovine sperm with tris-yolk supplemented with vitamin C and Trolox. *Ciênc. Vet. Tróp*., v.11, p.16-24, 2008.

PEIXOTO, P.C.V.A.; COLETO, Z.E.; MOURA, C.S.; ALMEIDA, F.C. *et al.* Effect of Trolox and Glutathione Reduced (Gsh) addition on the In Vitro viability of dog cryopreserved sperm. *Ciênc. Anim. Bras*., v.14, p.436-447, 2013.

PEREZ, E.G.; NICHI, M.; VIANA, C.H.; SILVA, R.O.C. *et al.* Effect of glutathione on the function and oxidative status of ovine cryopreserved sperm. *Braz. J. Vet. Res. Anim. Sci.*, v.49, p.262-268, 2012.

SILVA, S.V.; SOARES, A.T.; BATISTA, A.M.; ALMEIDA, F.C. *et al.* In vitro and in vivo evaluation of ram sperm frozen in tris egg-yolk and supplemented with superoxide dismutase and reduced glutathione. *Reprod. Domest. Anim*., v.46, p.874-881, 2011.

TURCER, P.B.; BUCAK, M.N.; BUYULEBLECI, S.; SARIOZKAN, S. *et al.* The effect of cysteine and glutathione on sperm and oxidative stress parameters of post-thawed bull semen. *Cryobiology*, v.61, p.303-307, 2010.

ZHAO, X.L.; CAO, S.; HU, J.H.; WANG, W.H. Protective effects of ascorbic acid and vitamin E on antioxidant enzyme activity of freeze-thawed semen of Qinchuan bulls. *Genet. Mol. Res*., v.1, p.2572-2581, 2015.