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In vitro viability of bovine semen cryopreserved with addition of vitamin c and trolox to the extender medium

Viabilidade in vitro de sêmen bovino criopreservado com adição de vitamina c e trolox ao meio extensor

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Highlights

Antioxidant addition in sperm kinetics.
Improvement of post-thaw sperm parameters with addition of antioxidant.
Association of Trolox and Vitamin C maintain high quality and viability of sperm bovin.

Abstract

A cryopreservation of bovine semen contributes significantly to the milk and meat production chains, improving the efficiency of herds and, consequently, their profitability. In this context, different methods and additives are used for this process to improve sperm resistance to cryopreservation. The objective of this work was to evaluate the use of ascorbic acid (vitamin C) and Trolox (synthetic vitamin E) as antioxidants in the semen extender medium to preserve bull semen viability in the freezing-thawing process. Eight sires, five *Bos taurus indicus* and three *Bos taurus taurus,* were selected and subjected to three semen collections at 48-hour intervals. The ejaculates were subjected to a previous analysis

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of motility, vigor, and concentration, then put into 0.25 mL straws at a rate of 25×10^6 of viable sperm, and distributed into four groups: Group I (extended semen); Group II (semen extended with 250 µM mL^{-1} of Trolox); Group III (semen extended with 0.60 mg mL^{-1} of vitamin C); and Group IV (semen extended with 250 µM mL^{-1} of Trolox and 0.60 mg mL^{-1} of vitamin C); subsequently, they were subjected to cryopreservation. The movements and kinetics of the thawed sperm was analyzed using Computer-Assisted Semen Analysis (CASA), which showed no difference (p<0.05) between the treatments. The evaluation of sperm viability by flow cytometry and lipid peroxidation by Thiobarbituric Acid Reactive Substances (TBARS) also showed no differences (p<0.05) between the antioxidants used in the extended semen. The semen of group GIV presented 35.08% motility, evaluated by Thermal Resistance Test (TRT); and 49.41% plasma membrane integrity, quantified by Hypo-Osmotic Swelling Test (HOST). These values were higher (p<0.05) than those found for the semen of groups GI (29.83% and 42%), GII (30.66% and 43.85%), and GIII (32.41% and 46.68%), respectively, which presented no differences from each other (p>0.05). The interaction between Trolox and vitamin C generated significant results for sperm motility after thawing when evaluated through TRT, and sperm viability when evaluated through HOST, and were higher than those found in the control group and in the group with addition of vitamin C.
Key words: Antioxidants. Oxidative stress. Plasma membrane. Sperm kinetics. Sperm.

Resumo

A criopreservação do sêmen bovino contribui para as cadeias produtivas do leite e da carne, melhorando a eficiência dos rebanhos e, consequentemente, sua lucratividade. Neste contexto, diferentes métodos e aditivos são utilizados para melhorar a resistência espermática à criopreservação. O objetivo deste trabalho foi avaliar o uso de ácido ascórbico (vitamina C) e Trolox (vitamina E sintética) como antioxidantes no meio diluidor de sêmen para preservar a viabilidade do sêmen no processo de congelamento-descongelamento. Oito touros, cinco *Bos taurus indicus* e três *Bos taurus taurus,* foram selecionados e submetidos a três coletas de sêmen em intervalos de 48 horas. Os ejaculados foram submetidos a uma análise prévia de motilidade, vigor e concentração, a seguir colocados em palhetas de 0,25 mL na proporção de 25×10^6 de espermatozoides viáveis, e distribuídos em quatro grupos: GI (sêmen diluído sem adição de vitaminas), GII (sêmen diluído com 250 µmoL mL^{-1} de Trolox), GIII (sêmen diluído com 0,60 mg mL^{-1} de vitamina C) e GIV (sêmen diluído com 0,60 mg mL^{-1} de vitamina C + 250 µmoL mL^{-1} de Trolox); posteriormente, foram submetidos à criopreservação. Os movimentos e a cinética dos espermatozoides descongelados foram analisados por Computer-Assisted Semen Analysis (CASA), não havendo diferença (p<0,05) entre os tratamentos. A avaliação da viabilidade espermática por citometria de fluxo e peroxidação lipídica por Substâncias Reativas ao Ácido Tiobarbitúrico (TBARS) também não mostrou diferenças (p<0,05) entre os antioxidantes utilizados no sêmen diluído. O sêmen do grupo GIV apresentou 35,08% de motilidade, avaliada pelo Teste de Resistência Térmica (TRT); e 49,41% de integridade da membrana plasmática, quantificada pelo Teste de Inchaço Hiposmótico (HOST). Esses valores foram superiores (p<0,05) aos encontrados para o sêmen dos grupos GI (29,83% e 42%), GII (30,66% e 43,85%) e GIII (32,41% e 46,68%) que não apresentaram diferenças entre si (p>0,05). A interação entre Trolox e vitamina C gerou resultados significativos para a motilidade espermática após o descongelamento quando avaliada pelo TRT, e a viabilidade espermática quando avaliada pelo HOST, sendo superiores aos encontrados no grupo controle e no grupo com adição de vitamina C.

Palavras-chave: Antioxidantes. Cinética Espermática. Espermatozoide. Estresse oxidativo. Membrana plasmática.

Introduction

Reproductive biotechnologies maximize the production potential of bovine herds, and bovine semen cryopreservation is the most used among them, especially artificial insemination, as it facilitates the marketing and transport of genetic materials, assists in the control of sexually transmissible diseases, and generates expressive genetic gains when correctly used (Milazzotto et al., 2008; E. C. B. Silva & Guerra, 2011; Tironi et al., 2019). However, refrigeration, freezing, and thawing processes damage the sperm, affecting the integrity of plasma membrane, mitochondria, acrosome, and DNA, decreasing sperm viability (Gürler et al., 2016).

Semen quality is dependent on the integrity of sperm cell structure and physiological function (Arruda et al., 2011). Spermatozoa subjected to cryopreservation can be damaged at different levels, compromising the energy metabolism and generating high production of reactive oxygen species (ROS), thus decreasing sperm kinetics and increasing DNA fragmentation (Schober et al., 2007; W. Oliveira et al., 2018).

In vivo ROS are spontaneously produced, as well as nitric oxide, superoxide anion, and hydrogen peroxide; small amounts of these compounds are involved in the sperm capacitation process and can improve tyrosine phosphorylation cascades (Gürler et al., 2016; Aitken, 2017; W. Oliveira et al., 2018).

However, continuous productions of ROS can overload the limited antioxidant defenses of gametes, inducing oxidative stress and hindering sperm cells by causing lipid peroxidation of the sperm plasma membrane. These processes disrupt and increase the permeability of the cell membrane, favoring the emergence of irreparable damages to DNA and mitochondria, compromising the sperm motility, vigor, and fertilizing capacity (Agarwal et al., 2014; Gürler et al., 2016; Aitken, 2017; L. F. M. C. Silva et al., 2017).

Agarwal and Sekhon (2011), reported that excessive production of ROS in cryopreserved semen are probably due to the dilutions used, as seminal plasma contains the largest part of antioxidants that can protect the sperm. The antioxidant capacity of the seminal plasma is represented by the sum of enzymes with anti-ROS potential (superoxide dismutase, catalase, and glutathione peroxidase); substances with low molecular weights, such as α-tocopherol (vitamin E), β-carotene, acid ascorbic (vitamin C), and urate; and chelate metals (transferrin, lactoferrin, and ceruloplasmin) (Mahfouz et al., 2009).

In this context, the objective of this work was to evaluate the effects of adding the antioxidants ascorbic acid (vitamin C) and Trolox (synthetic vitamin E) to the extender medium for frozen-thawed semen bull semen.

Materials and Methods

This study was approved by the Ethics Committee for the Use of Animals, Universidade Federal da Fronteira Sul (CEUA/UFFS; Protocol 23205.001354/2018-16).

Selection and collection of semen from the experimental animals

Eight healthy sires were selected, which were five Bos taurus indicus and three Bos taurus taurus presenting fertility confirmed by andrological evaluation, adequate body condition, sexual maturity, and similar handling and feeding. These sires were bred and adapted to the routine of a breeding company (Alta Genetics do Brasil Ltda., Uberaba, Minas Gerais, Brazil).

The animals were subjected to three semen collections at 48-hour intervals. The semen was collected by using an artificial vagina, according to the Brazilian College of Animal Reproduction (Colégio Brasileiro de Reprodução Animal [CBRA], 2013) in a 15-mL Falcon tube wrapped in leather to avoid thermal shock and light incidence, and subsequently sent to a laboratory for fresh evaluations, dilution, and cryopreservation.

Experimental groups

The fresh semen was subjected to evaluation and validation of minimum requirements for cryopreservation (CBRA, 2013). Each semen sample was divided into four treatments and placed in 5-mL cryotubes, namely: Group I (extended semen); Group II (semen extended with 250 µM mL^{-1} of Trolox); Group III (semen extended with 0.60 mg mL^{-1} of vitamin C); and Group IV (semen extended with 250 µM mL^{-1} of Trolox and 0.60 mg mL^{-1} of vitamin C).

The substances used were: a commercial extender medium (Triladyl®; MiniTube, Boulder, USA); vitamin C, Acid L-ascorbic (SIGMA-ALDRICH, code A92902); and Trolox, a synthetic vitamin E, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (SIGMA-ALDRICH, code 238813).

After the specific dilutions in each group, the semen was cooled at 4 °C for four hours, put into 0.25-mL straws (MiniTube®) containing 25 million viable sperm each, sealed in a straw sealer (Neovet®), and subjected to cryopreservation in a freezing machine (Digitcool®; IMV Technologies, Campinas, Brazil), where they remained under nitrogen vapor for 20 minutes until reaching -140 °C, and then submerged into liquid nitrogen until reaching -196 °C.

Twelve samples of each animal were cryopreserved for each treatment, with three replications for each collection, totaling 1.152 semen samples; they were placed in racks, which were identified according to the treatments and stored in a cryogenic cylinder.

Fresh semen evaluation

The fresh semen was maintained in a water bath at 37 °C and subjected to macroscopic (volume, color, and physical aspect) and microscopic (motility, vigor, and morphology) evaluations (CBRA, 2013). The sperm concentration was evaluated using a spectrophotometer (Genesys 20; Thermo Scientific, Waltham, USA): 50 µL of semen

was pipetted and 7 mL of a formol and physiological serum solution was added, and readings were carried out for 30 seconds at a wavelength of 550 nm.

Hypo Osmotic Swelling Test (HOST) was carried out to evaluate the functional integrity of the plasma membrane and quantify the percentage of intact sperm (Jeyendran et al., 1984).

A fresh semen sample was incubated in a water bath at 37 °C for 5 hours to evaluate progressive motility and vigor of spermatozoa and to validate the Thermal Resistance Test (TRT), following the guidelines of the Brazilian College of Animal Reproduction (CBRA, 2013).

Evaluation of cooled semen (pre-freezing)

The semen was refrigerated at 4 °C for five hours before freezing; a sample of each experimental group was subjected to HOST and TRT, according to the methods described by Jeyendran et al. (1984) and the CBRA (2013)), respectively.

Post-thawing semen evaluation

After freezing and thawing, semen samples of all experimental groups were evaluated for sperm viability, movement, trajectory, and concentration, lipid peroxidation index, and membrane functional integrity, and subjected to TRT.

The sperm viability was evaluated using flow cytometry; propidium iodide and SYBR-14 fluorescent probes were diluted in a phosphate-buffered saline solution and refrigerated protected from light. The semen was thawed and incubated with a dye solution at 37 °C for 10 minutes; 10 µL of the chilled solution was then pipetted into a microtube and placed in a cytometer (CyFlow SL; Partec, Goerlitz, Germany) for reading to assess the sperm plasma membrane integrity, considering 5,000 cells per sample.

The sperm concentration, motility, and trajectory were evaluated using Computer-Assisted Semen Analysis (AndroVision® CASA Software; MiniTube, Boulder, USA). After thawing, the content of each semen straw was placed in an Eppendorf preheated to 37 °C and 3 µL was pipetted to one of the four chambers of a specific slide for readings in the device, which was coupled to a camera to capture images of spermatozoa, using a 200x objective. The software coupled to the device enabled the reconstruction of the sperm trajectory over time and the calculation of the parameters: linear velocity, curvilinear velocity, amplitude of lateral head displacement, linearity, and mean trajectory velocity, as well as motility and progressive motility.

The lipid peroxidation index was evaluated through Thiobarbituric Acid Reactive Substances (TBARS), as proposed by Ohkawa et al. (1979); TBARS concentrations were determined using the value of 1.56×105M mL-1 as molar extinction coefficient of MDA (Buege & Aust, 1978) and lipid peroxidation index described as nanograms of TBARS per 106 sperm.

The membrane functional integrity was evaluated by HOST (Jeyendran et al., 1984) and TRT, as proposed by the the Brazilian College of Animal Reproduction (CBRA, 2013).

Statistical analysis

A completely randomized design was used; the data were subjected to analysis of variance by the proc MIXED procedure, using the mathematical model:

$$Y_{ijk} = \mu + \beta_i + T_j + (\beta*T)_{ij} + \delta_k + \varepsilon_{ijk},$$

where Y_{ij} is the dependent variables; μ is the overall mean of the observations; β_i is the effect of the animal's breed; T_i is the effect of the treatment used; $(\beta*T)_{ij}$ is the effect of the interaction between the i-th breed and the j-th treatment; δ_k is the effect of the collection day; and ε_{ij} is the random residual error.

Different structures of covariance were tested for each variable analyzed; the structure that presented the lowest Akaike Information Criterion (AIC) was used for the respective variable. The variables were tested for normality by the Kolmogorov-Smirnov test. Means classified by the F test and parameters with significant effect of the treatments or interaction between breed and treatment were compared by the t test at 5% significance level.

The sperm quality relative to the thermal treatment was evaluated by subjecting the data to analysis of variance by the proc MIXED procedure, using the mathematical model:

$$Y_{ij} = \mu + T_j + \delta_j + \varepsilon_{ij}$$

where Y_{ij} is the dependent variable; μ is the overall mean of the observations; β_i is the effect of the animal' breed; T_i is the effect of the thermal treatment; δ_k is the effect of the collection day; and ε_{ij} is the random residual error.

Different structures of covariance were tested for each variable analyzed; the structure that presented the lowest AIC was used for the respective variable. The variables were tested for normality by the Kolmogorov-Smirnov test. The means classified by the F test and the parameters with significant effect of the thermal treatment were compared by the t test at 5% significance level.

All statistical analyses were carried out using the statistical package SAS 8.2 (Statistical Analysis System).

Results and Discussion

The interaction between the additives added to the semen extender medium and the genetic groups was not significant ($p>0.05$). The effect of thermal changes on sperm quality during the cryopreservation process at different times (37 °C for fresh, 4 °C for cold, and -196 °C for cryopreserved semen) showed that the thermal stress causes injuries ($p<0.05$), decreasing the sperm viability (HOST), motility, and vigor (TRT) ($p<0.05$) (Figure 1).

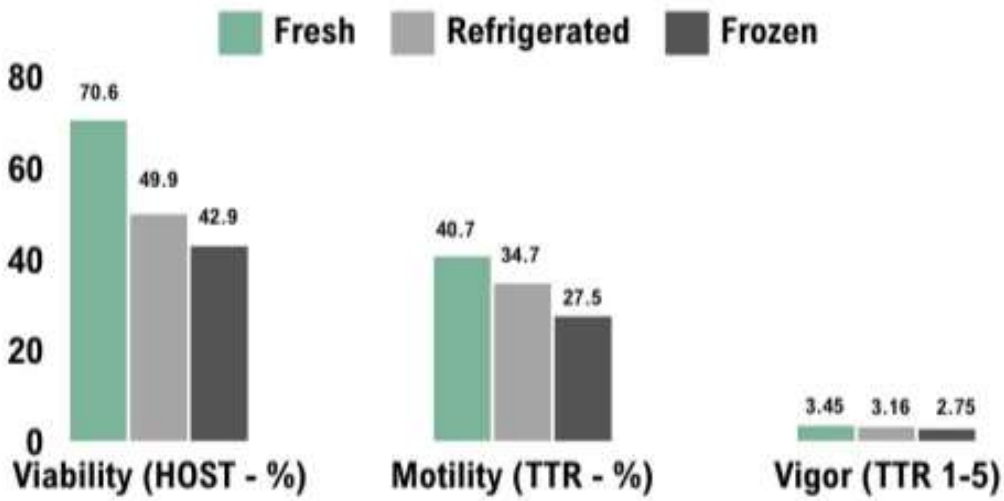

Figure 1. Semen viability by the hypo osmotic swelling test (HOST), and motility and vigor by the Thermal Resistance Test (TRT) in fresh, refrigerated, and frozen bovine semen in extender medium enriched with vitamin C, Trolox, and the combination of both.
Values followed by different letters in the same variable are different ($p<0.05$).

The sperm fertility, estimated by the sperm vigor and motility in the TRT, showed that the combination of vitamin C and Trolox presented a better result for motility ($p<0.05$) than the control treatment and the treatment with addition of vitamin C: 32.14%, 33.07%, and 37.72% sperm motility, respectively (Table 1).

The sperm viability evaluated through plasma membrane integrity by HOST showed that the combination of vitamin C and Trolox presented better results ($p<0.05$) (49,41%) than the treatments with addition of only vitamin C and those with no addition of antioxidant, which presented similar results: 43.85% and 42%, respectively. The addition of vitamin C also had no effect on sperm viability when compared to addition of Trolox ($p>0.05$): 43.85% and 46.88%, respectively (Table 1).

The treatments showed no significant difference ($p<0.05$) for the parameters: total motility (%), progressive motility (%), curvilinear velocity (μm s^{-1}), linear progressive velocity (μm s^{-1}), mean trajectory velocity (μm s^{-1}), amplitude of lateral head displacement (μm), cross beat frequency (Hz), straightness (%), linearity (%), and oscillatory movement (%) when evaluated by Computer-Assisted Semen Analysis (CASA) (Table 2).

The lipid peroxidation resistance index, evaluated by the TBARS test to predict the antioxidant potential of the substances, showed similar results for the treatments ($p<0.05$) only between the genetic groups, denoting the superiority of *Bos taurus taurus* (Figure 2).

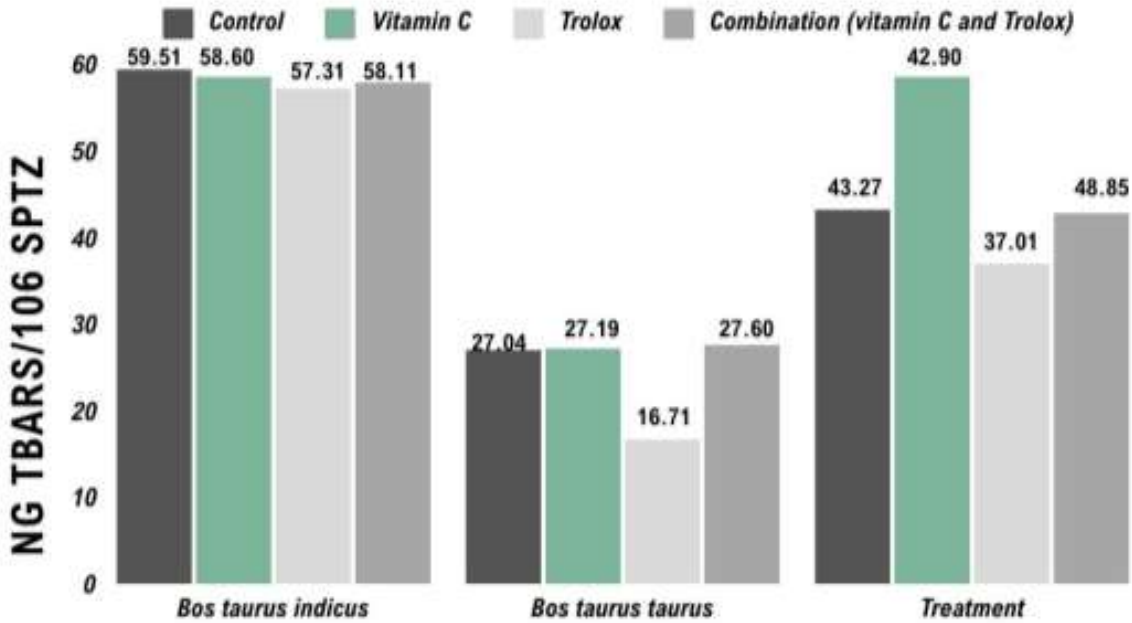

Figure 2. Lipid peroxidation resistance index by test of Thiobarbituric Acid Reactive Substances (TBARS) in bovine sperm cryopreserved with addition of vitamin C, Trolox, and the combination of both in the semen extender medium.
Values followed by different letters in the same variable are different (p<0.05).

The sperm kinetics after thawing presented no difference (p>0.05) between *Bos taurus indicus* and *Bos taurus taurus*, despite of the high contents of reactive oxygen species in the semen of Bos taurus indicus animals.

The cooled semen presented no significant difference (p>0.05) in sperm quality when compared to the fresh semen, confirming the results of other studies reporting that sperm refrigeration for up to 24 hours causes less damages to sperm cells when compared to cryopreservation, mainly when using glycerol in the extender for semen processing (Borges-Silva et al., 2015; Papa et al., 2015).

The qualitative parameters of the cryopreserved semen decreased when compared to the fresh or cooled semen, probably due to exposure to extreme temperatures, which damages the cell despite the cryoprotectant medium. However, the use of frozen semen is indispensable and a facilitator for semen marketing and transport, stimulating the search for new substances that can protect the sperm when exposed to such critical conditions (W. Oliveira et al., 2018; E. C. B. Silva & Guerra, 2011).

The motility results found by the TRT were similar to those described by Beheshti et al. (2011), who added vitamin E to the semen extender and highlighted its potential to prevent fast motility in buffalo semen. Batool et al. (2012) added vitamins E and C at different rates to bovine semen extender and found increases in sperm motility and viability for the rate of 0.5 mM vitamin E, with no difference for the same rate of vitamin C.

Gangwar et al. (2015) found that vitamin C at the rate of 56.78 µM can maintain sperm motility when compared to groups with no addition of antioxidant in sheep semen extender. In addition, W. L. Souza et al. (2017) reported improvements in sheep sperm quality, with protection of gametes from cryoinjury and in vitro fertilizing potential after cryopreservation when using vitamins E and C with melatonin. These results denote the capacity of vitamin E and C to protect sperm of different species.

The sperm integrity results were similar to those found by Wahjuningsih and Rachmawati (2012), who evaluated, through HOST, the addition of vitamin E to the goat semen and found that the rate of 0.4g 100 mL^{-1} protected the plasma membrane. In addition, Khellouf et al. (2018) found that the use of vitamin E combined with cholesterol can maintain bovine sperm viability after cryopreservation.

Table 1

CT: control treatment; VC: treatment with addition of vitamin C; INT: treatment with addition of vitamin C and Trolox; VE: treatment with addition of Trolox; SEM: standard error of the mean. Means followed by the different letters in the same row are different (p<0.05). Mean values obtained by Computer-Assisted Semen Analysis (CASA) for movement and kinetics of bovine sperm cryopreserved with addition of vitamin C, Trolox, and the combination of both in the semen extender medium

Treatment/ Parameter	*Bos taurus indicus*				*Bos taurus taurus*				SEM	Treatment				SEM	*P* value		
	CT	VC	INT	VE	CT	VC	INT	VE		CT	VC	INT	VE		Group Genetic	Treatment	Genetic Group × Treatment
Viability (HOST - %)	44.7	45.9	53.1	50.6	56.9	60.1	63.1	61.5	2.93	50.8c	53.0^{bc}	58.07^{a}	56.05^{ab}	2.82	0.0079	0.0013	0.5885
Vigor (TRT – 1-5)	3	3.6	3.13	3.23	2.78	3.05	3.61	3.11	0.18	2.89^{bc}	3.06^{ab}	3.37^{a}	3.17^{ab}	0.16	0.7609	0.0464	0.1077
Motility (TRT - %)	31.3	32	35	34.7	32.9	34.6	40.4	33.4	2.79	32.14^{b}	33.7^{b}	37.72^{a}	34.1^{ab}	2.1	0.2139	0.0713	0.3666

Semen parameters after cryopreservation of bull semen using vitamin C, Trolox, and the combination of both in the semen extender medium, evaluated by hypo osmotic swelling test (HOST) and Thermal Resistance Test (TRT).

Table 2

CT: control treatment; VC: treatment with addition of vitamin C; INT: treatment with addition of vitamin C and Trolox; VE: treatment with addition of Trolox. SEM: standard error of the mean; TM: total motility; PM: progressive motility; CV: curvilinear velocity; PLV: progressive linear velocity; MTS: mean trajectory velocity; SHBF: sperm head beat frequency; ALH: amplitude of lateral head displacement; STR: straightness; LIN: linearity; COI: cell oscillation index

Treatment/ Parameter	*Bos taurus indicus*				*Bos taurus taurus*				SEM	Treatment				SEM	*P* value		
	CT	VC	INT	VE	CT	VC	INT	VE		CT	VC	INT	VE		Group Genetic	Treatment	Genetic Group × Treatment
TM (%)	26.91	23.59	21.52	24.75	31.2	30.3	21.2	30.4	2.75	29	26.9	21.3	27.6	3.15	0.2467	0.2494	0.7683
PM (%)	18.2	17.14	15.7	17.19	22.38	23.08	16.33	22.76	1.36	20.33	20.11	16.02	19.97	1.93	0.0305	0.3041	0.7362
CV [µm s^{-1}]	40.69	37.54	36.93	40.08	45.00	49.73	37.72	45.41	2.04	42.85	43.64	37.32	42.75	2.89	0.0281	0.2750	0.4520
PLV [µm s^{-1}]	15.30	15.08	14.16	15.25	18.92	19.32	14.66	17.84	1.68	17.11	17.2	14.41	16.54	1.06	0.0110	0.2200	0.6200
MTS [µm s^{-1}]	20.77	19.61	19.06	20.60	23.76	25.75	19.65	23.13	0.98	22.26	22.68	19.35	21.87	1.39	0.0170	0.240	0.476
SHBF [Hz]	5.11	4.86	4.40	4.91	6.23	5.69	4.72	6.62	0.38	5.67	5.28	4.56	5.77	0.53	0.0200	0.1790	0.5692
ALH [µm]	0.51	0.52	0.47	0.50	0.56	0.14	0.51	0.59	0.27	0.53	0.58	0.49	0.54	0.38	0.045	0.413	0.843
STR	0.58	0.57	0.59	0.57	0.61	0.58	0.60	0.63	0.01	0.59	0.58	0.52	0.60	0.01	0.0200	0.5570	0.4860
LIN	0.30	0.31	0.30	0.29	0.35	0.32	0.33	0.34	0.01	0.32	0.31	0.32	0.32	0.01	0.011	0.9140	0.7800
COI	0.45	0.43	0.46	0.45	0.46	0.46	0.47	0.47	0.01	0.47	0.45	0.46	0.46	0.01	0.0004	0.1710	0.9800

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Moreover, Zhao et al. (2015) used vitamin C (1400 UI mL^{-1}) combined with vitamin E (0.12UI mL^{-1}) and found positive effects on sperm viability, motility, and acrosome integrity and increases in activity of antioxidant enzymes in the semen of Quinchan bovine. In addition, Arguello et al. (2017) pointed out the potential of oral treatments with vitamin E to improve the plasma membrane functionality and minimize damages to membrane caused by cryopreservation in sperm of Brangus bulls.

The sperm plasma membrane, evaluated by SYBIR-14 fluorescent probes and propidium iodide and read by flow cytometry, showed similar sperm viability (p<0.05) in all groups (Figure 3).

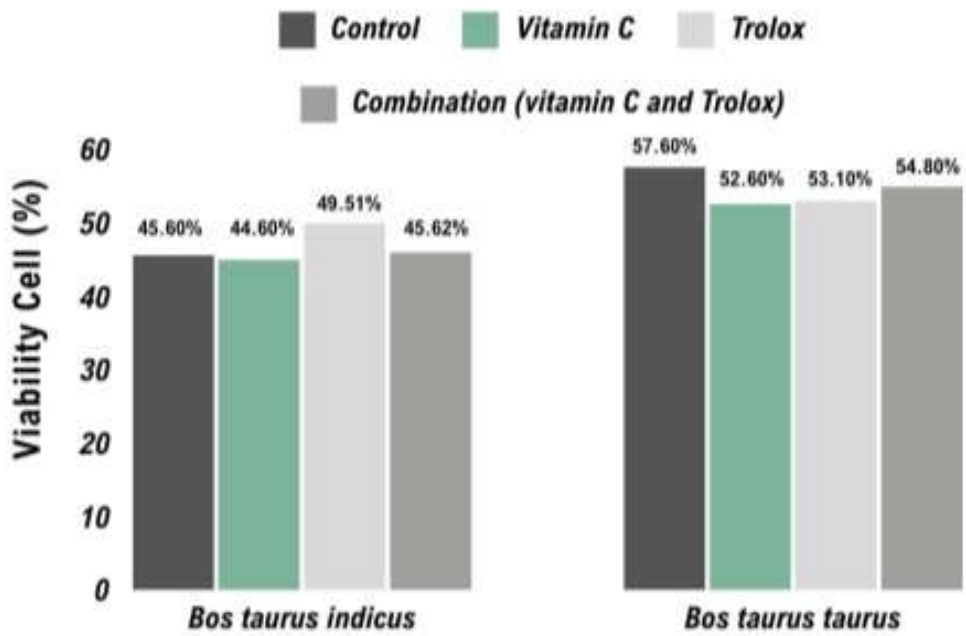

Figure 3. Plasma membrane evaluation using SYBIR-14 fluorescent probes and propidium iodide by flow cytometry for bovine sperm cryopreserved with addition of vitamin C, Trolox, and the combination of both in the semen extender medium.
Percentage values in the same variable without letters are similar (p<0.05).

The flow cytometry and fluorescent probes generated a high accuracy in evaluating the sperm plasma membrane integrity and allowed for the evaluation of thousands of cells in a few seconds, facilitating the identification of damages caused to cells, which is parameter used to predict pregnancy rates in cows (Sellem et al., 2015; L. Z. Oliveira et al., 2013).

Sperm kinetics is one of the most important attributes associated to fertilizing ability of spermatozoa, as the absence of motility prevents sperm cells to migrate to the female reproductive tract and meet the oocyte for fertilization (Bertol et al., 2014).

The results found for kinetics parameters evaluated by CASA can be connected to the effects of in vivo bull fertility. L. Z. Oliveira et al. (2013) evaluated the correlation between computerized analysis of bovine sperm characteristics and pregnancy rates in a fixed-time artificial insemination program and found that total motility, progressive motility, and sperm head beat frequency are the main predictors of fertility.

Verstegen et al. (2002) found a correlation of curvilinear velocity and mean trajectory velocity with the fertilization process, as they increase after sperm capacitation. In addition, Mortimer and Maxwell (2004) found that progressive motility associated to a high amplitude of lateral head displacement is connected to the ability of sperm to penetrate the female cervical mucus.

Hu et al. (2010) evaluated the addition of different concentrations of vitamin C to the bovine semen extender and found increases in sperm motility, progressive linear velocity, linearity, mean trajectory velocity, cell oscillation index, and amplitude of lateral head displacement for the vitamin C rate of 4.5 mg mL^{-1}. Moreover, Hu et al. (2011) evaluated addition of different concentrations of vitamin E in the bovine semen extender and found positive results, mainly for progressive linear velocity and straightness in semen groups subjected to addition of 1.0 and 1.5 mg mL^{-1} of vitamin E; whereas the rates of 0.5 mg mL^{-1} and 2.0 mg mL^{-1} presented equal or worse results than that of the group with no addition of vitamin E. These results found for vitamins C and E were attributed to the antioxidant properties of the vitamins, but the studies pointed out the need for a better understanding of their functions in animal reproduction.

Similarly, Motemani et al. (2017) found higher total motility and progressive motility when using vitamin E at rates of 2.4 mM mL^{-1} and 4.8 mM mL^{-1} in the bovine semen extender (Bioxcell®; IMV Technologies), compared to groups with no addition of vitamin E, indicating that vivo fertility tests should be carried out to confirm the vitamin efficiency. However, Duarte et al. (2016) added vitamins C and E to the bovine semen extender at rates of 0.45 mg mL^{-1} and 10 mmol mL^{-1}, respectively, and found no improvement in the sperm motility and kinetics. These different results denote the need for searching more efficient rates, as these molecules have potential to protect sperm cells.

The superiority of *Bos taurus taurus* genetic group for lipid peroxidation (Figure 3) are consistent with the results found by Anchieta et al. (2005), who reported that semen from European breeds (B. taurus taurus) presents better freezability than zebu animals. Contrastingly, Nichi et al. (2006) reported that European bulls produce higher amounts of reactive oxygen species than Indian bulls (*B. taurus indicus*), increasing the amount of unviable sperm, differing from the present study.

High TBARS contents in the semen can hinder sperm quality; sperm lipid peroxidation is an important predictor of

fertility in bovine (L. Z. Oliveira et al., 2013). Tvrdá et al. (2015) added α-tocopherol in the bovine semen extender and found that it was effective to improve the percentage of motile and viable sperm and prevent intracellular overproduction of free radicals inside the sperm mitochondrial membrane; the α-tocopherol concentrations of 100 and 500 μM L^{-1} were the most effective to protect the bovine sperm against damages caused by reactive oxygen species.

Duarte et al. (2015) added 10 mM mL^{-1} of tocopherol to bovine semen extender and found no positive results for the oxidative evaluation and pregnancy indexes. In addition, Duarte et al. (2016) evaluated 10 mM mL^{-1} of tocopherol and 0.45 mg mL^{-1} of vitamin C and found no efficiency to decrease damages caused by cryopreservation and oxidative stress in bovine semen samples after thawing.

Motemani et al. (2017) also found no efficiency to reduce malondialdehyde production the main by-product of lipid peroxidation by the addition of different concentrations of α-tocopherol to the bovine semen extender and attributed these effects to reactions of α-tocopherol with low concentrations of malondialdehyde.

E. S. Souza et al. (2019) added 0.3 mg mL^{-1} of Trolox to the extender medium for cryopreservation of bovine semen and measured TBARS; they found deleterious effect on sperm cells, with no decrease in the lipid peroxidation level, but decreasing the percentage of sperm active mitochondria and, consequently, the semen quality. No significant improvement was found in the present study regarding the levels of substances reactive to thiobarbituric acid.

Conclusion

The addition of Trolox combined with vitamin C at the rates used improved the motility (evaluated by Thermal Resistance Test) and viability (evaluated by hypo osmotic swelling test) of post-thawed sperm, which were higher than those found for the semen groups with no addition of antioxidants or with addition of only vitamin C. Thus, Trolox and its association with Vitamin C have antioxidant potential to maintain high quality and viability of bovine semen. However, complementary studies should be carried out, adding the evaluation of the mechanisms of action of these substances on the sperm cell and the definition of more efficient rates, according to the genetic group used.

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