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Lipid peroxidation in bull semen influences sperm traits and oxidative potential of Percoll®-selected sperm

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Research Article

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Summary

Although bovine embryo *in vitro* production (IVP) is a common assisted reproductive technology, critical points warrant further study, including sperm traits and oxidative status of sperm for *in vitro* fertilization (IVF). Our aim was to evaluate whether the lipid peroxidation index of commercial bull semen is influenced by sperm traits and oxidative status of sperm populations selected using Percoll® gradient. Semen straws from 48 batches from 14 Nelore bulls were thawed individually, analyzed for motility and subjected to Percoll selection. After Percoll, the lipid peroxidation index of the extender was evaluated, whereas selected sperm were analyzed for motility, acrosome and membrane integrity, mitochondrial membrane potential, chromatin resistance and oxidative potential under IVF conditions. Batches were divided retrospectively in four groups according to lipid peroxidation index. Sperm from Group 4 with the lowest index of lipid peroxidation had, after Percoll selection, greater plasma membrane integrity (81.3%; $P = 0.004$), higher mitochondrial potential (81.1%; $P = 0.009$) and lower oxidative potential (135.3 ng thiobarbituric acid reactive substances (TBARS)/ml; $P = 0.026$) compared with Group 1 with highest lipid peroxidation index (74.3%, 73% and 213.1 ng TBARS/ml, respectively). Furthermore, we observed negative correlations for the lipid peroxidation index with motility, membrane integrity and mitochondrial potential, and positive correlations with oxidative potential. In conclusion, oxidative stress in semen straws, as determined using lipid peroxidation in the extender, is associated with sperm traits and their oxidative potential under IVF conditions. These results provided further insights regarding the importance of preventing oxidative stress during semen handling and cryopreservation, as this could affect sperm selected for IVF. Finally, Percoll selection did not completely remove sperm with oxidative markers.

Introduction

In vitro production (IVP) can produce many embryos over a short time interval, providing more embryos compared with *in vivo* production (Pontes *et al.*, 2009). Therefore, IVP is a robust tool to enhance genetic gain (Vieira *et al.*, 2016), contributing to the livestock economy (Blondin, 2017). In 2018, Brazil was responsible for 33.5% of global numbers of *in vitro* produced bovine embryos (Viana, 2019).

Despite the advantages and widespread use of IVP, bull effects contribute to considerable variation in embryo rates, even under similar conditions (Otoi *et al.*, 1993; Palma and Sinowatz, 2004; Alomar *et al.*, 2008). Differences in *in vitro* fertility have been associated with the sperm trait profile of samples used for *in vitro* fertilization (IVF) (Tanghe *et al.*, 2002; Tartaglione and Ritta, 2004; Alomar *et al.*, 2006, 2008; Simões *et al.*, 2013; Siqueira *et al.*, 2018) and it has been shown convincingly that sperm oxidative stress impairs fertilization ability, embryo development, fetal growth and offspring health (Silva *et al.*, 2007; Lane *et al.*, 2014; de Castro *et al.*, 2016).

During IVF, sperm are exposed to higher oxygen concentrations (approximately 20%) when compared with *in vivo* conditions (1.5–8%) (Fischer and Bavister, 1993). The effects of high oxygen concentrations in the *in vitro* environment have also been correlated with the generation of reactive oxygen species (ROS) (Goto *et al.*, 1993; Takahashi, 2012). Although ROS are free radicals and highly reactive oxidizing agents with critical roles in physiological functions such as

sperm capacitation, acrosome reaction and oocyte binding (Ford, 2001), any imbalance between ROS production and antioxidant levels has a negative influence on sperm capacity, as a result of oxidative stress (Sikka *et al.*, 1995).

Mammalian sperm are rich in polyunsaturated fatty acids, increasing their susceptibility to ROS-mediated damage. These free radicals react with unsaturated fatty acids, initiating a cascade of lipid peroxidation (Lenzi *et al.*, 2000). Furthermore, by-products of lipid peroxidation are also harmful for sperm, damaging their membranes, DNA and proteins (Agarwal *et al.*, 2014).

The main source of sperm for *in vitro* fertilization is frozen–thawed semen, which has a higher lipid peroxidation index compared with fresh semen (Chatterjee and Gagnon, 2001; Kadirvel *et al.*, 2009). Lipid peroxidation in frozen–thawed semen occurs due to the collapse of antioxidant enzyme defences, coupled with the generation of ROS during cryopreservation (Alvarez and Storey, 1992). Furthermore, dilution of seminal plasma, in addition to temperature reduction (Medeiros *et al.*, 2002), may exacerbate the oxidative environment, due to removal of seminal proteins that prevent sperm membrane changes and inhibit lipid peroxidation (Del Valle *et al.*, 2017).

Selection of a viable and motile sperm population is usually performed before IVF (Tanghe *et al.*, 2002; Suzuki *et al.*, 2003; Samardzija *et al.*, 2006; Machado *et al.*, 2009). Different methods, such as swim up, centrifugation and Percoll® gradient, are used to select viable and motile sperm, as well as to remove seminal plasma and cryoprotectant. Percoll is the most widely used density separation reagent in bovine IVF, and is use is related to sperm quality, increasing the proportion of motile sperm (Henkel and Schill, 2003; Suzuki *et al.*, 2003; Machado *et al.*, 2009). Although selection methods improve sperm traits, it has not been well established whether Percoll gradient selection removes cells damaged by cryopreservation-induced oxidative stress. Trait profiles of frozen–thawed sperm used for IVF and their oxidative potential could strongly affect IVP yields. Therefore, the hypothesis of this study was that the level of native oxidative stress in semen straws could affect sperm traits and the oxidative potential of sperm samples during IVF, despite the use of sperm selection and preparation (Percoll centrifugation). To test this hypothesis, we evaluated the influence of native lipid peroxidation index of commercial cryopreserved semen on sperm traits after Percoll gradient selection and also, the lipid peroxidation index of the sperm samples when kept under IVF conditions.

Materials and methods

Ethics Committee approval for animal use was granted by the School of Veterinary Medicine and Animal Science of University of São Paulo (protocol CEUA 7246170117).

Reagent and solutions

All chemical reagents and solutions used were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated.

Semen preparation

In total, 48 semen batches from 14 Nellore bulls donated from two commercial artificial insemination centres in Brazil were used. The cryopreservation procedure was performed according to standard protocols from each centre and both centres used egg yolk-based extenders. For each one of the 48 batches, five semen straws from the same batch/ejaculate were used. Each straw was thawed, processed and analyzed independently for all variables in an independent replicate. Each straw was considered as a technical replicate of the same biological sample (batch/ejaculate). The mean of five replicates was determined and used for ranking, comparisons between groups and correlations ($n = 48$).

In this study, for the sperm preparation protocol, including centrifugation force and mediums, we tested the most commonly used protocol in IVP laboratories in Brazil (Machado *et al.*, 2009; Ramos-Deus *et al.*, 2020). Therefore, after thawing (37°C; 30 s), straws were evaluated for visual motility. Samples were subjected to a Percoll density gradient (400 µl Percoll 45% over 400 µl Percoll 90%) and centrifuged at 6600 *g* for 5 min. After centrifugation, the extender in the supernatant was collected to assess lipid peroxidation, whereas the pellet containing motile sperm was washed at 1100 *g* for 3 min in 1 ml Fert-TALP without inducers of sperm capacitation (Parrish *et al.*, 1988). The selected population was then assessed for motility, and its concentration analyzed using a Neubauer chamber and diluted in an appropriate volume of Fert-TALP to a final concentration of 25×10^6 motile sperm/ml. Samples were then analyzed using fluorescent probes under flow cytometry [FITC-PSA with propidium iodide (PI), tetraethylbenzimidazolycarbocyanine iodide (JC-1) and acridine orange] and incubated under IVF conditions (with Fert-TALP with capacitation agents for 20 h under mineral oil at 38.5°C, 5% CO_2 in air, and high humidity) to evaluate oxidative potential.

Experimental design, based on ranking and selection of bulls by lipid peroxidation

To evaluate the influence of lipid peroxidation on cryopreserved samples, spermatozoa trait profiles were analyzed after Percoll gradient selection and the samples were grouped based on native lipid peroxidation index in the extender. The 48 semen straws were divided retrospectively into four groups, with median and quartiles used to determine the limiting values for each group. Group 1 included semen straws that were between the maximum value of lipid peroxidation and the upper quartile (highest, $n = 13$); Group 2 included semen straws between upper quartile and median (high, $n = 11$); Group 3 included the median and the lower quartile (average, $n = 12$); and Group 4 included lower quartile and the minimum value of lipid peroxidation (low, $n = 12$).

Lipid peroxidation

To analyze lipid peroxidation, the TBARS test was used for two samples: (1) supernatant recovered after Percoll centrifugation containing the remaining semen extender fraction; and (2) sperm selected using Percoll and incubated under the same *in vitro* fertilization conditions. Sperm in this group (25×10^6 motile sperm/ml) were incubated with 475 µl of Fert-TALP with capacitation agents for 20 h under mineral oil at 38.5°C, in 5% CO_2 in air, and high humidity.

The TBARS reaction evaluates malondialdehyde (MDA) concentrations as products of lipid peroxidation. This analysis was performed as described earlier by Hamilton *et al.* (2016). Samples were analyzed to evaluate the spontaneous lipid peroxidation content by the production of TBARS. Initially, ice-cold trichloroacetic acid 10% (ratio 2:1) was added. Samples were then centrifuged at 20,800 *g* for 15 min (5°C) to precipitate proteins and debris. The supernatant was recovered and transferred to cryotubes, 1% thiobarbituric acid was added (ratio 1:1) and the sample incubated at 95°C in a water bath for 15 min. In this assay, thiobarbituric acid reacts with MDA to produce a pink-coloured

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complex. Thiobarbituric acid reactive substances were quantified using a cuvette and a spectrophotometer (Ultrospec 3300 pro®, Amersham Biosciences) at a wavelength of 532 nm and lipid peroxidation index reported as nanograms of TBARS/ml. The values obtained were compared with a standard curve for MDA concentration.

Motility

Total sperm motility was subjectively estimated by examination using phase contrast microscopy at ×100 magnification by placing 5 µl of semen sample on a warmed slide (37ºC) overlaid with a coverslip. Sperm motility was estimated at two time points: immediately after thawing (Motility post thaw) and after selection using Percoll (Motility post Percoll).

Flow cytometry

Flow cytometry analyses were performed with a Guava EasyCyte™ Mini System (Guava® Technologies, Hayward, CA, USA) with a blue laser (488 nm) that emitted 20 mW visible laser radiation and three channels for fluorescence detection (525, 583 and 680 nm). We assessed 20×10^3 gated cells per sample, with data from each assay analyzed using FlowJo software v.10.2 (Flow Cytometry Analysis Software, Tree Star Inc., Ashland, OR, USA). Cells were identified and selected excluding debris, probe particles and non-single sperm events by applying a gate on forward scatter (FSC) versus a green fluorescence dot plot. The data corresponding to each fluorescence signal were given as arbitrary fluorescence units, recorded after logarithmic amplification, and the selected population was represented by percentage (%). Control samples, in addition to negative and positive samples, were used to elaborate thresholds and gates for each analysis to achieve the highest determination coefficient. This set-up for gain and voltage of channels used was applied to all samples. More details for control samples and fluorescent probes are available in a previous report (Siqueira *et al.*, 2018).

In total, 187.5×10^3 events were used for fluorescein-conjugated *Pisum sativum* agglutinin (FITC-PSA) with PI and JC-1 stains, and 375×10^3 sperm were used for chromatin resistance to acid denaturation. To assess the acrosome integrity and plasma membrane integrity, FITC-PSA was used in conjunction with PI to produce a simultaneous evaluation. Sperm samples were incubated for 5 min with 7 µg/ml PI and 24 µg/ml FITC while protected from light. The FITC fluorescence emission was detected at 515–520 nm and PI fluorescence emission was detected at 630–650 nm. Emission of green fluorescence using FITC-PSA indicates a damaged acrosome, whereas emission of red fluorescence using PI indicates a damaged plasma membrane.

JC-1 was used to assess sperm mitochondrial membrane potential, and cells were incubated for 5 min with 1 µM JC-1 while protected from light. Fluorescence detection was performed at 590 nm. Sperm with a high mitochondrial membrane potential emit greater intensities of yellow fluorescence compared with sperm with low mitochondrial membrane potential (Uribe *et al.*, 2017).

Chromatin resistance analysis was based on a sperm chromatin structure assay (SCSA) (Evenson *et al.*, 2002), modified as described previously (Castro *et al.*, 2018). The assay evaluates the susceptibility of chromatin to fragmentation and uses an acid detergent challenge to denature double-stranded DNA. This method allows acridine orange (AO) dye to bind to DNA, emitting either red fluorescence for single-stranded denatured chromatin or green fluorescence for double-stranded DNA, indicating resistant chromatin.

Samples with high motility were selected as a negative control for acrosome and membrane damage and high mitochondrial membrane potential. For positive controls (damaged acrosome and membrane and reduced mitochondrial membrane potential), a subset of the negative control sample was subjected to five cycles of freezing (liquid nitrogen) and thawing (water bath at 60ºC).

For the AO positive control, sperm were incubated with an acid solution (hydrochloric acid and acid detergent 1.2 M; pH 0.1) for 5 min to induce total sperm DNA denaturation (Castro *et al.*, 2018).

Statistical analyses

Statistical analysis was performed using Statistical Analysis System 9.3 software (SAS Institute, Cary, NC, USA) for Windows. All data were tested for normality of residues and homogeneity of variance. Group and bull effects were included in the statistical model. Comparisons of means among experimental groups were performed using a Tukey test. The GLM procedure was used to evaluate the effects of lipid peroxidation groups on dependent variables. A correlation test (Spearman) was performed between sperm traits and lipid peroxidation on sperm samples. Results are presented as mean ± standard error (SEM). A 5% significance level was used to reject the null hypothesis.

Results and Discussion

Ranking by lipid peroxidation

Lipid peroxidation is an important marker of oxygen-induced damage (Aitken *et al.*, 1989). In aerobic conditions, as during cryopreservation, sperm undergo spontaneous lipid peroxidation that may lead to complete loss of motility (Alvarez *et al.*, 1978). In this study, we verified whether the level of lipid peroxidation in the extender of cryopreserved semen influenced sperm traits of Percoll-selected populations or the oxidative potential of samples subjected to IVF conditions.

Ranking of samples according to lipid peroxidation index (TBARS) in semen extender generated four treatment groups, based on quartiles (233.23 ± 21.46; 144.81 ± 2.69; 108.50 ± 1.84; 58.75 ± 4.69 ng/ml for Groups 1, 2, 3, and 4, respectively; Fig. 1).

The aim of this study was to evaluate the native lipid peroxidation of commercial semen straws, without any induction, to better represent commercial IVF laboratory conditions. The determination of native lipid peroxidation index of each straw was possible only after thawing, precluding a prospective grouping. Therefore we used retrospective grouping in this study. Retrospective grouping of samples based on lipid peroxidation index has previously been used successfully to evaluate the effects of oxidative stress on sperm quality and fertility (Simões *et al.*, 2013; Hamilton *et al.*, 2016). However, the retrospective division of groups had some limitations, such as not controlling the distribution of samples, bulls and centres among groups. We also performed correlation analysis as a post-test to analyze the behaviour of these variables in datasets independently of group division.

Sperm evaluations

Plasma membrane integrity and mitochondrial membrane potential

Percoll selection provides a highly motile sperm population with resistant chromatin and intact acrosomes and plasma membranes

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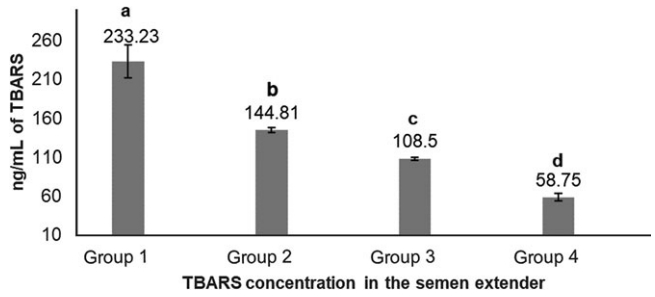

Figure 1. Means (+SEM) TBARS concentration (ng/ml) in extender from Percoll® supernatant, ranked from highest to lowest concentrations and allocated into four lipid peroxidation groups. $^{a-d}$Groups without a common superscript differed ($P < 0.05$).

(Tanghe *et al.*, 2002; Suzuki *et al.*, 2003; Samardzija *et al.*, 2006; Machado *et al.*, 2009), making it a useful tool for IVF. In our study, Percoll selection did not select solely the population with an intact plasma membrane. Group 1 (highest level of lipid peroxidation in semen extender after Percoll) had a lower plasma membrane integrity sperm population and lowest percentage of sperm with high mitochondrial membrane potential (Table 1 and Figs 2 and 3) when compared with the other groups based on the content of lipid peroxidation in the semen extender. Additionally, Group 4 (lowest lipid peroxidation content in extender) had a higher percentage of sperm with intact plasma membranes and a higher percentage of sperm with high mitochondrial membrane potential. Furthermore, there was a negative linear correlation in semen extender between percentage of sperm with plasma membrane integrity, high mitochondrial membrane potential and TBARS index (Table 2: Rho = −0.3; $P = 0.01$; Rho = −0.5; $P = 0.0005$, respectively).

Similarly, there was increased oxidation in plasma membranes and mitochondria in sperm exposed to pro-oxidative conditions (Silva *et al.*, 2007). It is well known that sperm are very susceptible to oxidative stress, as a result of their relative lack of cytoplasm that limits enzymatic antioxidant protection (Aitken *et al.*, 1998; Chatterjee and Gagnon 2001). This situation would allow free radicals to attack lipids in the sperm membrane and mitochondria (Aitken *et al.*, 2012). Disruption to the electron transport chain could lead to a decrease in proton pumping with consequent impairment to membrane potential (Ricci *et al.*, 2004).

Despite some studies not reporting changes to the sperm plasma membrane (Silva *et al.*, 2007), and mitochondrial membrane potential (de Castro *et al.*, 2016), or DNA (Silva *et al.*, 2007) as a consequence of induced oxidative stress, all these studies reported decreased fertility as a result of lipid peroxidation or oxidative stress. Therefore, lipid peroxidation could decrease sperm fertility, even without some detectable changes in these sperm traits.

In our study, Percoll did not completely remove sperm with oxidative damage. Corroborating our results, although Percoll selected sperm with greater motility and viability, this selection did not increase fertility rates (Suzuki *et al.*, 2003; Machado *et al.*, 2009). Also, the level of lipid peroxidation within the sperm plasma membrane negatively influenced blastocyst rates (Silva *et al.*, 2007). Taken together, these findings could explain why Percoll selection described in previously studies failed to improve fertility.

Another consideration is that all straws used in this study were extended in egg yolk-based extender, this type of extender remains the one most often used in Brazil (Leite *et al.*, 2010), and it is an enriched source of aromatic amino acids, substrates for production of H_2O_2 by membrane-bound aromatic amino acid oxidase (AAAO) released from dead sperm (Vishwanath and Shannon, 2000). In this context, in Group 1, high levels of cells with damaged plasma membranes could be considered a cause of higher levels of lipid peroxidation. Therefore, we inferred that changes recorded in this study were likely to be a consequence of oxidative stress rather than a cause.

Lipid peroxidation under IVF conditions

For the TBARS index under IVF conditions, Groups 1 and 2 (higher index of TBARS in semen extender) also had higher values for lipid peroxidation under IVF conditions (Table 1 and Fig. 4). Moreover, there was also a significant positive linear correlation in semen extender between TBARS under IVF conditions and TBARS index (Rho = 0.4, $P = 0.0038$, Table 2).

Similar to our study, sperm previously exposed to oxidative conditions had an increased capacity for generating ROS after induction of capacitation (Aitken *et al.*, 1989). Following this reasoning, the level of oxidative stress present in the cryopreserved samples would influence ROS production and consequently lipid peroxidation and oxidative stress of sperm after Percoll selection for IVF. Current results have highlighted the importance of semen handling during cryopreservation with implications pertaining to the oxidative potential of samples during IVF, despite the higher quality of cells recovered after Percoll selection. Several studies have been undertaken to demonstrate that colloid centrifugation improves the quality and fertilizing ability of cryopreserved sperm, as well as reduce the ROS amount (Martinez-Alborcia *et al.*, 2013; Morrell *et al.*, 2017; Nongbua *et al.*, 2017). Our results do not contradict these earlier studies, as we did not compare lipid peroxidation in the sperm samples before and after colloid centrifugation. This centrifugation is effective for removing extender, and we can assume that it is effective in removing ROS contained in the extender. However, the higher oxidative potential was observed in sperm selected from straws with a higher lipid peroxidation index, and suggested that oxidative stress markers, such as intracellular ROS and those linked to the plasma membrane, remain on the selected sperm samples, affecting later IVP conditions and rates.

Motility, acrosome membrane integrity and chromatin resistance

Sperm motility, acrosome integrity and chromatin resistance were not significantly different between groups of lipid peroxidation on the basis of TBARS levels in the semen extender obtained from supernatants after Percoll centrifugation (Table 1).

Although we did not observe any significant difference among groups regarding motility, we observed a significantly negative correlation between lipid peroxidation index and motility after Percoll selection. Lipid peroxidation levels correlated with loss of motility have been observed previously (Alvarez *et al.*, 1978). Probably, we were not able to detect significant differences among groups because a subjective method is less accurate than objectives methods, making the comparison less sensitive to differences. Unfortunately, in this study, it was not feasible to use a more objective motility assessment.

There was no significant effect of lipid peroxidation index of extender on acrosome integrity or chromatin resistance. It is possible that these two sperm traits are less susceptible to oxidative damage compared with plasma membrane integrity and mitochondrial membrane potential. Consistent with these results, oxidative stress induced by hydrogen peroxide strongly impaired sperm motility but, even at higher doses, this resulted in only 2% of sperm with damaged chromatin, indicating that bull sperm

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Table 1. Means (± SEM) sperm traits separated into groups on the basis of TBARS levels in semen extender (ranked from Groups 1 to 4, highest to lowest lipid peroxidation levels, respectively)

Sperm trait	Group 1	Group 2	Group 3	Group 4	*P*-value
Motility post thaw (%)	51.67 ± 2.39	48.33 ± 1.97	52.52 ± 1.37	51.54 ± 1.84	0.48
Motility post Percoll (%)	52.92 ± 1.89	56.20 ± 1.99	58.16 ± 1.87	56.75 ± 2.50	0.31
Acrosome integrity (%)	87.00 ± 0.90	89.27 ± 0.92	85.62 ± 1.58	88.35 ± 1.45	0.20
Plasma membrane integrity (%)	74.36 ± 1.33[a]	78.63 ± 1.56[b]	74.33 ± 2.03[a]	81.34 ± 1.22[c]	0.004
High mitochondrial membrane potential (%)	73.00 ± 1.03[a]	78.63 ± 1.55[b]	77.66 ± 1.80[b]	81.12 ± 1.25[c]	0.0009
Chromatin resistance (%)	97.97 ± 0.31	98.59 ± 0.27	97.62 ± 0.46	98.04 ± 0.36	0.33
TBARS under IVF conditions (ng/ml)	213.16 ± 18.74[a]	231.63 ± 29.81[a]	185.75 ± 30.31[b]	135.36 ± 12.03[c]	0.026

[a-c]Within a row, means without a common superscript differed ($P < 0.05$).

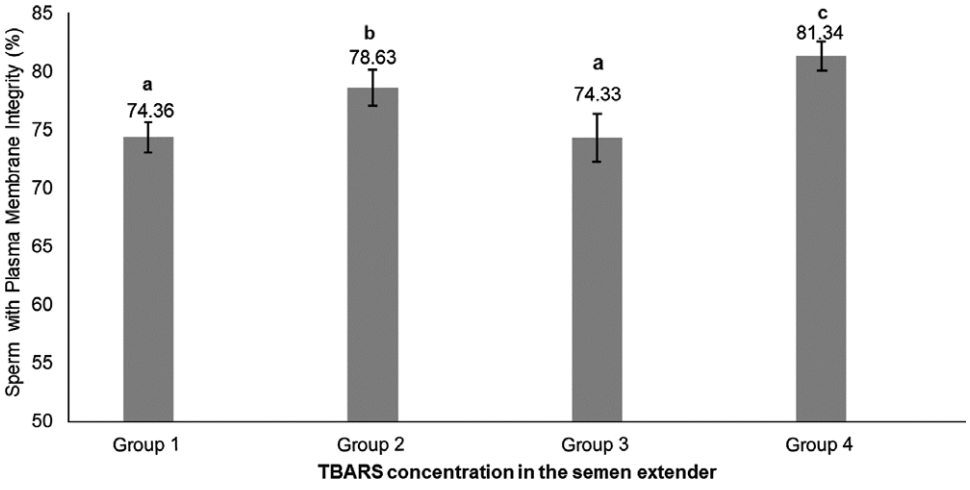

Figure 2. Means (+ SEM) percentage of sperm with plasma membrane integrity in four lipid peroxidation groups, ranked from highest to lowest (Group 1 to Group 4, respectively). [a–c]Groups without a common superscript differed ($P = 0.004$).

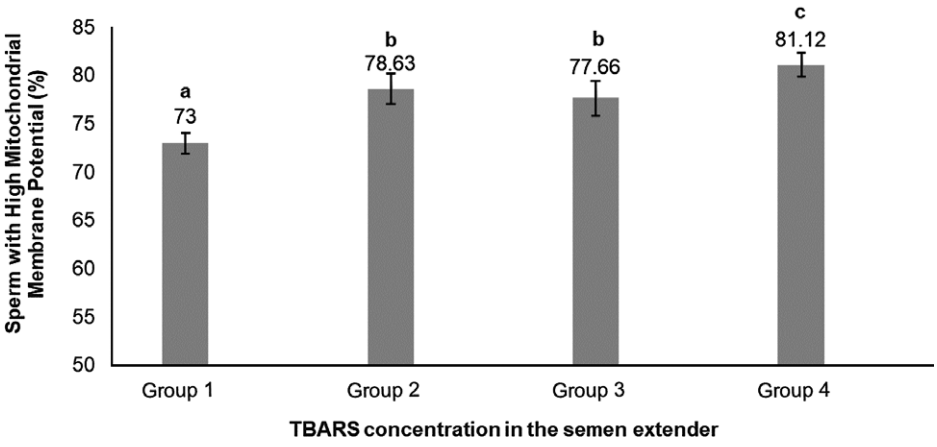

Figure 3. Means (+ SEM) percentage of sperm with high mitochondrial membrane potential in four lipid peroxidation groups, ranked from highest to lowest (Group 1 to Group 4, respectively). [a–c]Groups without a common superscript differed ($P = 0.0009$).

chromatin is relatively resistant to damage and that changes in this sperm trait were relatively rare in this species (Simões *et al.*, 2013; de Castro *et al.*, 2016).

Chromatin resistance was positively correlated with lipid peroxidation under IVF conditions. Relationship analysis also detected positive correlations among acrosome integrity, membrane integrity, mitochondrial potential, and chromatin resistance (Table 2).

This study evaluated and found that the oxidative index of the extender of commercial semen straws was associated with the sperm trait profile after sperm selection and oxidative potential under IVF conditions. However, this study focused on evaluating this effect in sperm cells. Further studies are necessary to evaluate the effect of lipid peroxidation in bull semen straws and the oxidative potential of these samples on IVP rates.

Conclusion

Oxidative status of cryopreserved semen is associated with plasma membrane integrity and mitochondrial membrane potential of

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Table 2. Correlation coefficients (Rho) and probability (*P*) values between sperm traits and lipid peroxidation

	Motility post thaw	Motility post Percoll	Acrosome integrity	Plasma membrane integrity	High mitochondrial membrane potential	Chromatin resistance	TBARS under IVF conditions	TBARS in semen extender
Motility post thaw		0.12067	0.14092	-0.0091	0.09261	−0.0744	−0.2254	−0.1057
		P 0.4191	*P* 0.3447	*P* 0.9517	*P* 0.5499	*P* 0.6151	*P* 0.1320	*P* 0.4746
Motility post Percoll			−0.2124	0.13659	0.27543	0.04476	−0.0473	−0.31601
			P 0.1565	*P* 0.3654	*P* 0.0738	*P* 0.7651	*P* 0.7576	*P* 0.0305
Acrosome integrity				0.64778	0.46806	0.42116	−0.0144	−0.08453
				P < 0.0001	*P* 0.0014	*P* 0.0032	*P* 0.9251	*P* 0.5721
Plasma membrane integrity					0.66231	0.53157	0.04322	−0.34889
					P < 0.0001	*P* 0.0001	*P* 0.7780	*P* 0.0162
High mitochondrial membrane potential						0.43126	−0.1145	−0.50027
						P 0.0035	*P* 0.4703	*P* 0.0005
Chromatin resistance							0.40979	−0.01245
							P 0.0047	*P* 0.9331
TBARS under IVF conditions								0.41808
								P 0.0038
TBARS in semen extender								

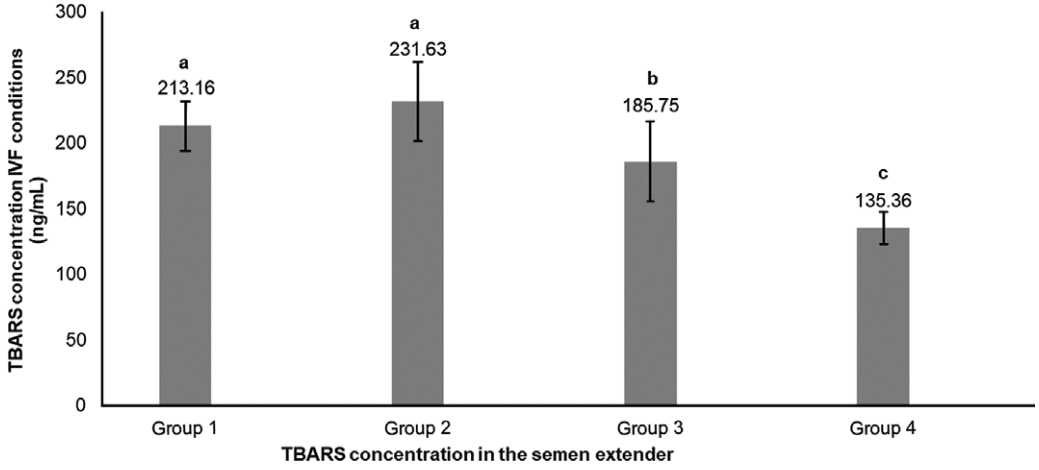

Figure 4. Means (+SEM) lipid peroxidation produced under *in vitro* fertilization conditions in four lipid peroxidation groups, ranked from highest to lowest (Group 1 to Group 4, respectively). [a–c]Groups without a common superscript differed ($P = 0.0026$).

sperm selected for IVF and could also be associated with their oxidative potential under IVF conditions. Percoll selection did not completely remove sperm with oxidative markers.

Ethical approval

Ethics Committee approval for animal use was granted by the School of Veterinary Medicine and Animal Science of University of São Paulo (protocol CEUA 7246170117).

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Competing interests. The authors have declared that no competing interests exist.

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