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CRYOCAPACITATION AND ITS ASSOCIATION WITH OXIDATIVE FEATURES IN CRYOPRESERVED BOVINE SPERMATOZOA

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ABSTRACT

The goal of our research was to evaluate the impact of cryopreservation on the antioxidant activity, lipid peroxidation and protein carbonylation as well as its connection with the progress of cryocapacitation in bovine spermatozoa. As biological material we used semen obtained from 20 sexually mature Holstein bulls. Each ejaculate was divided into three equal aliquots as follows: the first part or control (CTRL) was incubated in physiological saline solution while the second part (CAP) was incubated in a capacitation medium at specific conditions. The third part of each sample was cryopreserved (CRYO) and stored in liquid nitrogen at -196°C for further analysis. The motility of spermatozoa was assessed with CASA (computer assisted sperm analysis), while the capacitation status was evaluated by the chlortetracycline (CTC) fluorescent staining. Total antioxidant capacity (TAC) and the level of lipid peroxidation (LPO) was measured with a combined spectro-fluoro-luminometer. The presence of protein oxidation was detected by the traditional DNPH (2,4-dinitrophenylhydrazine) method and evaluated spectrophotometrically. Based on our data, the motility was significantly decreased ($P<0.0001$; $P<0.01$) in the CRYO group against CAP and CTRL. There was a significant increase ($P<0.01$; $P<0.05$) of acrosome-reacted spermatozoa (AR-pattern) in the CRYO group when compared to CTRL and CAP, while TAC was statistically decreased ($P<0.05$) between the CRYO and CAP experimental group. In the case of protein oxidation and LPO, both parameters were significantly higher ($P<0.0001$) in the CRYO group when compared to CAP or CTRL. In summary, cryopreservation induces capacitation-like changes and promotes oxidative damage in frozen-thawed bovine spermatozoa.

Keywords: cryocapacitation, bull, spermatozoa, antioxidant activity, lipid peroxidation, protein oxidation

INTRODUCTION

The major development of cryobiotechnologies is dating back in the half of the 20th century when glycerol was used as cryoprotective additive for cryopreservation of mammalian spermatozoa for the first time. In general, low temperatures during cryopreservation process can negatively affect the structure and functionality of spermatozoa, which caused membrane and mitochondrial disruption, loss of sperm motility and viability, destabilization of plasmatic membrane lipid composition or premature capacitation known as cryocapacitation. Capacitation is a maturation process of spermatozoa, which involves a series of complex changes on the level of plasmatic membrane before acrosome reaction (AR) and fusion with oocyte (**Yeste, 2016; Chauhan *et al.*, 2018; Asa *et al.*, 2020**). The term cryocapacitation refers to cryoinduced membrane reorganization and loss of polyunsaturated fatty acids (PUFA's) and degradation of cholesterol associated with other typical capacitation hallmarks like higher concentration of intracellular calcium and protein phosphorylation (**Ledesma *et al.*, 2019**). Cryodamage of spermatozoa is often related to osmotic stress, cold shock, and formation of intracellular or extracellular ice crystals. Nevertheless, a large quantity of free radicals and reactive oxygen species (ROS) is generated during freezing and after thawing, which are able to easily diffuse through the cellular membranes and lead into development of oxidative stress (OS). This condition is a consequence of imbalance between the generation and accumulation of ROS and cell ability to detoxify these highly reactive intermediates. Destabilization of the plasmatic membrane due to cryopreservation arise when membrane undergo thermotropic lipid phase transition from the liquid into gel phase (**Srivastava *et al.*, 2013; Tvrdá *et al.*, 2016a; Gangwar *et al.*, 2018**). The activity of seminal antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) vary between fresh and post-thaw spermatozoa. Naturally, antioxidants protect spermatozoa against free radicals generated by the mitochondrial metabolism. Previous studies reported a dramatic decline in the total antioxidant capacity (TAC) and enzymatic activity of antioxidants due to cryopreservation and associated cryoinjury (**Lone *et al.*, 2016; Castro *et al.*, 2016**). Mammalian spermatozoa are susceptible to oxidative damage because of the high content of PUFA's. As a vital component of sperm membrane PUFA's are responsible for the better membrane fluidity and sensitivity. However, they also became a primary target for ROS, which lead into lipid peroxidation (LPO). LPO is characterized as an oxidative degradation of lipids, after interaction between free radicals and lipid carbon-carbon bounds, specifically in PUFA's. One of the main end products of LPO is malondialdehyde (MDA), which is considered as a biomarker for the cellular oxidative damage and membrane injury, mostly in the enol form. It is highly reactive three-carbon dialdehyde substance, which comes from peroxidation of membrane PUFA's and arachidonic acid metabolism (**Ayala *et al.*, 2014; Ahmed *et al.*, 2017; Alyethodi *et al.*, 2021**). The presence of the protein-bound carbonyls is often used as a marker of global protein oxidation. It is defined as a covalent modification of proteins due to direct reaction with ROS or indirect reaction with the secondary by-products of OS. Protein carbonylation is irreversible modification, which occurs when ROS attack amino acid side chains of lysine, arginine, threonine, or proline in the presence of transition metals and formation of reactive ketones and aldehydes or reactive carbonyl compounds produced during glycoxidation and lipoperoxidation, which had the ability to bind into amino acid side chains (**Celi & Gabai, 2015; Weber *et al.*, 2015**). The aim of the study was to evaluate the impact of cryoinjury on the oxidative properties of cryopreserved bovine spermatozoa including the antioxidant activity, lipid peroxidation and protein oxidation and its association with progress of cryocapacitation.

MATERIAL AND METHODS

Sample collection and cultivation

Sexually mature and healthy adult Holstein bulls (n=20) were used for this experiment. Semen collection was performed by using artificial vagina at local breeding facility (Slovenské biologické služby, a.s., Nitra). Primary motility of all samples was evaluated by the CASA (computer-assisted sperm analysis) system with minimum of 80% of motile spermatozoa. After that, samples were transported to the laboratory for further analysis. All samples were divided into three equal parts, the first part (control) was incubated in the physiological saline solution (IMUNA PHARM, a.s., Šarišské Michaľany) and the second part (capacitated) was incubated with capacitation medium prepared according to **Heydari *et al.* (2020**). The incubation conditions were set as follows: 30 min. at 39°C and 5% concentration of CO_2. Immediately after the incubation, sperm motility in both groups (control and capacitated) was assessed with CASA system. Successful *in vitro* capacitation was associated with hyperactivated motility, which was observable under microscope.

Cryopreservation, thawing and washing procedure

Third (cryopreserved) part of each sample was diluted with Triladyl, which contained 20% (w/v) of egg yolk, then transferred by using automatic straw filler (Minitüb GmbH, Germany) into 0.25 ml French straws, cooled (4°C/2 hours), cryopreserved (-3°C/min. from 4°C to -10°C; -40°C/min. from -10°C to -100°C; -20°C/min. from -100°C to -140°C) with digital freezer and stored in liquid nitrogen at -196°C at least for one month. Then, samples in containers filled with liquid nitrogen were transported into laboratory for the further investigation. All cryopreserved samples in straws were thawed at 37°C/1-2 min. with heating pad, transferred into labelled 0.5 ml tubes and after-thawing motility was observed using CASA system. After the evaluation of motility, 500 µl of PBS (phosphate buffer solution; Sigma-Aldrich, St. Louis, MO, USA) were added into the samples, which were centrifuged for 10 min./5000 RPM. This washing procedure was repeated twice for the extraction of cryoprotectant residues and samples were subjected to another analysis.

Evaluation of sperm motility

The evaluation of the quantity of motile spermatozoa (%, MOT) was performed by CASA system (version 14.0 TOX IVOS II.; Hamilton-Thorne Biosciences, Beverly, MA, USA) when ten µl of every sample was pipetted into Makler's counting chamber (depth 10 µm, 37°C; Sefi Medical Instruments, Haifa, Israel) for microscopic observation. The final data were assessed with the Animal Motility program (Hamilton-Thorne, biosciences, Beverly, MA, USA).

Capacitation status

The assessment of capacitation status was performed by chlortetracycline (CTC) assay for fluorescent microscopy. The course of capacitation was identified by specific patterns, which depends on the absorption of fluorescent dye by the cell. Three specific fluorescent patterns were recognized: F-pattern for non-capacitated spermatozoa (fluorescence over the whole region of sperm head), B-pattern for capacitated spermatozoa (fluorescence of the sperm head except in the post-acrosomal region) and AR-pattern for acrosome-reacted spermatozoa (no fluorescence of the sperm head, only bright band in the equatorial segment) (**Zanganeh *et al.*, 2013**).

Total antioxidant capacity (TAC)

Quantification of total antioxidant capacity was performed with antioxidant assay using luminol and horseradish peroxidase conjugate. Trolox was used as a standard (concentration range 5-100 mmol/L) and the induction of chemiluminescence reaction was assessed with signal reagent consisted of Tris-HCl, hydrogen peroxide, 4-iodophenol and luminol. Chemiluminescence was quantified with Glomax Multi$^+$ combined spectro-fluoro-luminometer on 96-well plate in 10 cycles/1 min. and final data were expressed as µmol Trolox Eq./ mg protein (**Muller *et al.*, 2013; Tvrdá *et al.*, 2020**).

Protein oxidation

Determination of protein oxidation and the presence of protein-bound carbonyls was performed by the traditional DNPH (2,4-dinitrophenylhydrazine) method. First, 1 mL of each sample was treated with 1 mL of 20 % trichloroacetic acid (TCA) and incubated at 4°C/10 min. and centrifuged at 3000 RPM/5 min. Then supernatant was extracted and 1 mL of DNPH was added to the samples, mixed, and incubated 1 hour in the dark at laboratory temperature. Following incubation, 1 mL of TCA was added, and samples were incubated at 4°C/10 min. before centrifugation (3000 RPM/5 min.). After that, supernatant was discarded, and pellet was washed and centrifuged (3000 RPM/5 min.) three times by adding 1 mL of ethanol/ethyl acetate for the removal of DNPH residues. Washed sample pellets were resuspended in 1 mL of 6 M guanidine-HCl solution, and the absorbance was measured spectrophotometrically at 360 nm. The amount of the protein carbonyls was expressed as nmol/mg protein (**Weber *et al.*, 2015; Ďuračka *et al.*, 2016**).

Lipid peroxidation (LPO)

The level of lipid peroxidation was expressed through the concentration of malondialdehyde by using TBARS assay. All samples were subjected to the sodium dodecyl sulfate (SDS) and thiobarbituric acid (TBA), which was dissolved in 20% acetic acid. Immediately, treated samples were boiled for one hour at 90-100°C and placed on ice for approximately 10 min. Following centrifugation (3800 RPM/10 min.), supernatant was collected and used for quantification of MDA (µmol/g protein) as the end-product of LPO, which was measured with combined spectro-fluoro-luminometer Glomax Multi$^+$ on 96-well plate at 530-540 nm (**Tvrdá *et al.*, 2016b; Benko *et al.*, 2019**).

Statistical analysis

All data were statistically processed with the GraphPad Prism program (version 6.0 for Windows, GraphPad Software incorporated, San Diego, California, USA, https://www.graphpad.com/). Significant differences between groups were evaluated by One-way ANOVA and Tukey's test. The data collected from experimental groups are expressed as percentage of the control (±SD). The levels of significance were set at P<0.05; P<0.01; P<0.001; P<0.0001.

RESULTS AND DISCUSSION

Sperm motility

Figure 1 represented the percentage of motile spermatozoa, when significantly highest (P<0.0001) sperm motility was observed in the CAP group compared to the CRYO group. On the other hand, a statistical decline (P<0.0001; P<0.01) of motile spermatozoa was recorded in the CRYO experimental group versus CAP as well as CTRL group. There were no significant changes between CAP and CTRL group. Before fertilization, spermatozoa undergo a series of membrane modifications like the influx of calcium ions and phosphorylation of tyrosine, which resulted in hyperactivated motility. Hyperactivation is essential part of capacitation and it is characterized as a high-amplitude asymmetrical beating of sperm flagellum (**Marquez & Suarez, 2004**). The main key of successful hyperactivation lays in tyrosine phosphorylation of the flagellar proteins of spermatozoa. It is not unusual that cryopreserved spermatozoa after thawing are hyperactivated because of the poor calcium efflux, which lead into the accumulation of cytosolic calcium ions in the cell. When the levels of intracellular calcium reach threshold values, spontaneous capacitation and acrosome reaction is induced without the activation of specific sperm/oocyte plasma membrane receptors or associated signal transduction pathway (**Bailey *et al.*, 2000; Talukdar *et al.*, 2015**).

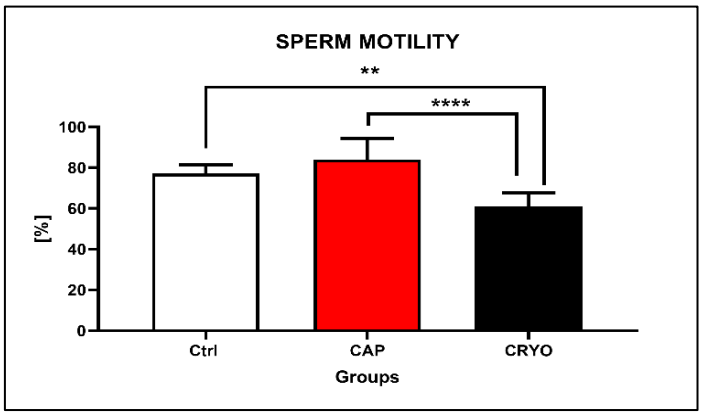

Figure 1 Sperm motility of *in vitro* capacitated and cryopreserved bovine spermatozoa. The graph showed the percentage (%, MOT) of motile spermatozoa in the groups (±SD). (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001)

Capacitation patterns

Evaluation of the capacitation status indicates that the CRTL had significantly higher (P<0.0001) proportion of non-capacitated spermatozoa "F-pattern" (Figure 2a) against the CAP and CRYO group. Also a significant decrease (P<0.0001) of non-capacitated spermatozoa was observed in the CAP group compared to the CRYO as well as CTRL. In the case of capacitated cells "B-pattern" (Figure 2b), there was an increase in the CAP group with a significance rate (P<0.0001) against both CRYO and CTRL group. The differences were also detected between the CTRL and both experimental groups (CAP and CRYO) with statistically decreased (P<0.0001) proportion of capacitated spermatozoa. In contrast, the data point out to a significant increase (P<0.001; P<0.05) of acrosome-reacted spermatozoa (Figure 2c) "AR-pattern" in the CRYO group in comparison to the CAP as well as CTRL. There were no significant differences between the CAP and CTRL group in the percentage of cells, which underwent AR. Our results were supported by the **Yoon *et al.* (2015)**, in their research capacitation status was evaluated during every step of cryopreservation including dilution/cooling, addition of cryoprotectant and freezing/thawing. The percentage of acrosome-reacted spermatozoa (AR-pattern) increased significantly (P<0.05), while the amount of non-capacitated (F-pattern) decreased after thawing. In the study of **Longobardi *et al.* (2017)** the percentage of AR-pattern was statistically higher (P<0.05) in the frozen spermatozoa compared to the native samples. Previous findings confirmed that cryopreserved bovine spermatozoa are able to imitate capacitation-like changes by membrane destabilization, redistribution of phospholipids, removal of cholesterol and calcium influx. As mentioned earlier, induced premature capacitation or cryocapacitation is disadvantageous process because spermatozoa spontaneously undergo acrosome reaction, which decrease fertilizing ability and quality of cryopreserved semen (**Mostek *et al.*, 2017**). **Srivastava *et al.* (2013)** finds a direct connection between

cholesterol content of frozen-thawed spermatozoa and the acrosome membrane integrity. Cells with higher membrane cholesterol concentration are less sensitive to undergo a spontaneous capacitation as well as AR, while spermatozoa with lower cholesterol content capacitate faster. The intact acrosome, which contain hydrolytic enzymes such as hyaluronidase and acrosin is essential for the progress of capacitation. Acrosomal enzymes are released during AR right after conjunction of outer acrosomal with the plasma membrane. Frozen-thawed spermatozoa exhibit lost or partially damaged acrosome, which was supported by several studies (**Jeong *et al.*, 2009; Nur *et al.*, 2010**).

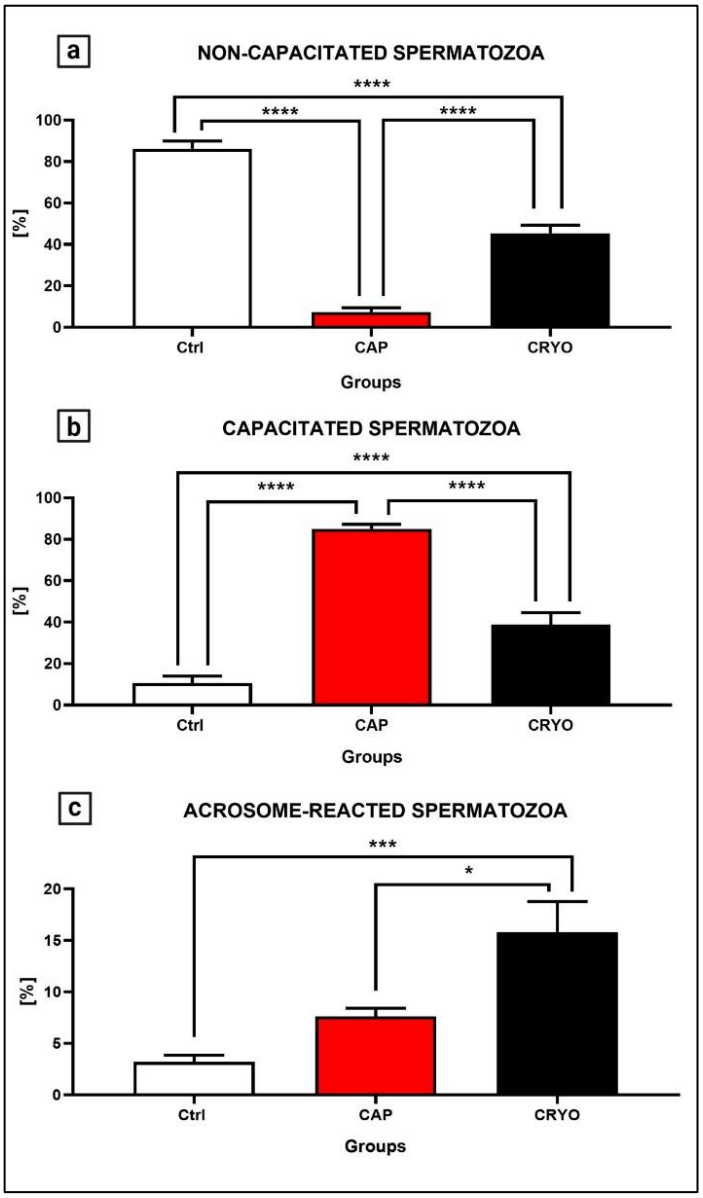

Figure 2 Capacitation status of *in vitro* capacitated and cryopreserved bovine spermatozoa with CTC patterns evaluation. Each column represented the percentage proportion of CTC patterns between groups (±SD). (**a**) F-pattern, non-capacitated spermatozoa; (**b**) B-pattern, capacitated spermatozoa; (**c**) AR-pattern, acrosome-reacted spermatozoa. (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001)

Total antioxidant status

Analysis of the total antioxidant capacity (Figure 3) revealed a statistical decline (P<0.05) only between the CRYO and CAP group. However, no significant changes in the TAC were visible between the CAP against CTRL. It is well known that cryopreservation and thawing procedure are responsible for the development of oxidative damage due to increased ROS generation and decreased antioxidant protection (**Luno *et al.*, 2014**). Naturally, antioxidants in the seminal plasma (SP) play a major role in the protection of spermatozoa against OS and protect cell against ROS produced by the mitochondrial metabolism. Unfortunately, SP of semen samples used for cryopreservation is often removed or extremely diluted and lose protective abilities. Then, antioxidant protection of cryopreserved spermatozoa mainly depends on the intracellular antioxidants, which can be limited due to oxidation of PUFA's and reduced cytoplasmic content. It can be assumed that these antioxidants can cover only basal physiological ROS production but their low concentrations inside the cell cannot handle major oxidative damage (**Castro *et al.*, 2016**). SP contains both enzymatic antioxidants, including superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) catalase (CAT) and other non-enzymatic low molecular weight scavengers (retinol, α-tocopherol, ascorbic acid, selenium, pyruvate, urate, taurine and hypotaurine) which together determine TAC (**Barranco *et al.*, 2015**). **Aleythodi *et al.* (2021**) suggested that cryopreservation is responsible for the higher ROS-generation and lower TAC in bovine semen, but it depends on the individual quality of samples obtained from different bulls. **Gürler *et al.* (2015**) noticed negative correlation between TAC and LPO in frozen bull semen but LPO also positively correlated with the activity of SOD, while GPx was not related. **Nichi *et al.* (2006**) observed higher activity of GPx in *Bos taurus* compared to *Bos indicus* bulls as a response of spermatozoa to heat stress. It brings the theory that enzymatic activity of seminal plasma antioxidants could be used as an indicator of heat or cold shock caused by temperature changes. Cryopreservation is also responsible for a significant redistribution or abundance of the proteins with ROS scavenging activity. Following cryopreservation, antioxidant enzymes such as GPx, GR and SOD were redistributed on the surface of ram spermatozoa (**Marti *et al.*, 2008**). These facts, together with decreased antioxidant activity of SOD and GR could be the reason of increased cryo-susceptibility of frozen-thawed spermatozoa to LPO and OS (**Bilodeau *et al.*, 2000; Peris-Frau *et al.*, 2020**).

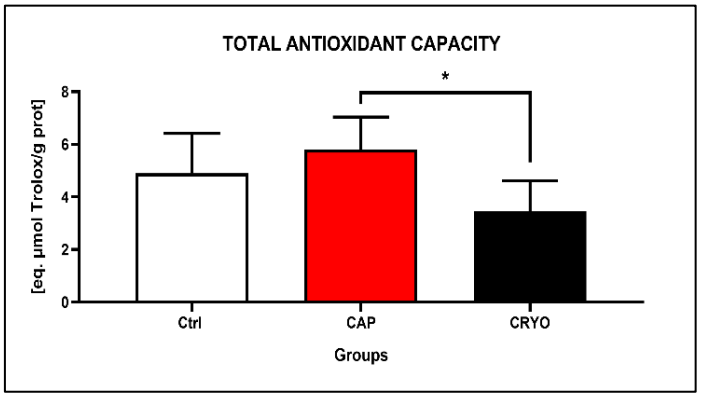

Figure 3 Total antioxidant status of *in vitro* capacitated and cryopreserved bovine spermatozoa. The values in bars showed the level of total antioxidant capacity in each group (±SD). (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001)

Protein oxidation

In the case of the level of protein oxidation (Figure 4), CRYO group showed a dramatic increase in the concentration of oxidized proteins with a significance rate (P<0.0001) in comparison with the CAP group and CTRL, but no differences were observed between the CAP and CTRL. Similarly, **Mostek *et al.* (2017**) confirmed that cryopreservation procedure significantly increased (P<0.05) ROS generation and the concentration of protein carbonyl groups in cryopreserved bovine semen (1.243 nmol/mg) compared to fresh-diluted semen (0.989 nmol/mg) by 20.4%. Freezing-thawing process induces carbonylation of proteins responsible for the energetic metabolism and flagellum organization as well as structural and functional modifications of membrane PUFA's content, which reduce the ability of spermatozoa to move straight forward (**Kogan *et al.*, 2021**). In bull semen, carbonylated proteins are necessary for many physiological functions like the organization of sperm cytoskeleton and flagellum, energetic metabolism, or detoxification. What is more, a lot of these proteins are essential for capacitation process because of generation of superoxide radical (isocitrate dehydrogenase, NADH dehydrogenase), actin polymerization (actin-related proteins M1 and T2) or hyperactivation of sperm motility (ropporin) (**Fujita *et al.*, 2000; O'Flaherty, 2015; Romarowski *et al.*, 2016**). Cryoinduced damage of the protein structure due to carbonylation may cause multiple disturbances of capacitation as a consequence of lack antioxidant defense and oxidative stress development. ROS modify proteins by the oxidation of amino acid residue side chains, cleavage of peptide bonds and formation of new protein-protein covalent bonds. The most vulnerable to oxidation by ROS are thiol groups, which can be found in the enzyme tyrosine phosphatase. The activity of tyrosine phosphatase is directly inhibited due to oxidation of the sulfhydryl group, which initiate capacitation process and AR in spermatozoa (**Bollwein & Bittner, 2018**).

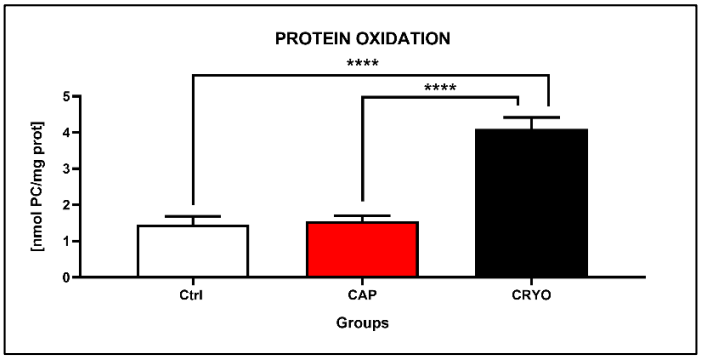

Figure 4 The level of protein oxidation in the *in vitro* capacitated and cryopreserved bovine spermatozoa. The graph represented the concentration of oxidized proteins between the groups (±SD). (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001)

Lipid peroxidation

Data in Figure 5 showed a continuous increase in the MDA concentration between the groups. A significantly higher (P<0.0001) concentration of MDA was detected in the CRYO group compared to CAP and CTRL as well as between CAP and CTRL group with a significance rate (P<0.01). As mentioned before, plasmatic membrane of spermatozoa contains a high concentration of PUFA's, which are vulnerable to oxidative damage. A variety of lipid metabolites are generated due to LPO including MDA, 4-hydroxynonenal, acrolein and peroxyl or alkoxyl radicals. The oxidative damage of PUFA's is initiated by the abstraction of hydrogen or an addition of oxygen radical. The lipid radicals react with oxygen and produce hydroxides and lipid hydroperoxide, which escalate LPO chain reaction. Then membrane phospholipase 2 transported lipid hydroperoxides from out of the membrane and glutathione-peroxidase reduced them into truncated phospholipids, which stop the chain reaction but destabilize the cell membrane (**Repetto *et al.*, 2012; Bollwein & Bittner, 2018**). Destabilization of the membrane also affects functionality of integral proteins and ion channels, which decrease the integrity and fluidity of membrane. Moreover, LPO is responsible for the reduction of sperm motility through to modulation of ion channels, formation of lipid metabolites with axonemal proteins of sperm flagellum and mitochondrial electron transport proteins. As a result of mitochondrial proteins modulation, the electron transport chain is disrupted, which led to efflux of electrons. These electrons are paired with oxygen, which generated additional ROS and increased the level of oxidative damage of spermatozoa (**Aitken *et al.*, 2012; Baker *et al.*, 2015; Moazamian *et al.*, 2015**). According to previous studies, cryopreservation induces LPO and increase the ROS generation after freezing-thawing procedure. Induction of LPO after cryopreservation was reported in boar, bull, buffalo as well as stallion spermatozoa (**Ferrusola *et al.*, 2009; Kadirvel *et al.*, 2009; Yeste *et al.*, 2013**).

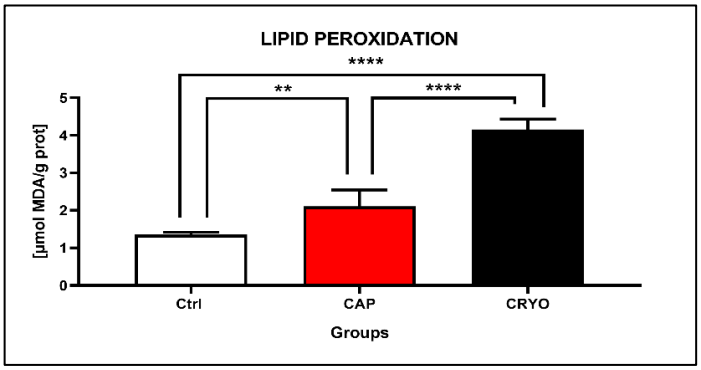

Figure 5 The level of lipid peroxidation in the *in vitro* capacitated and cryopreserved bovine spermatozoa. The values in columns showed the concentration of MDA as a final product of LPO between the groups (±SD). (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001)

CONCLUSION

In summary, we can conclude that cryopreservation induced capacitation-like changes also known as cryocapacitation in cryopreserved bovine spermatozoa. Post-thawed quality of cryopreserved samples was negatively affected on different levels such as the antioxidant activity, lipid peroxidation, protein oxidation or acrosome integrity. These novel findings could help to better understand the process of cryocapacitation at the molecular point of view as well as its association with oxidative features of cryopreserved bovine spermatozoa.

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