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# EPICATECHIN IMPROVES FROZEN SPERM VITALITY BY ITS ANTIOXIDANT AND CRYOPROTECTIVE ACTIONS

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**ABSTRACT**

This study served to elucidate the effects of three selected doses of epicatechin (EPI; 25, 50 or 100 µmol/L) on the motion and oxidative profile of bovine spermatozoa subjected to the process of cryopreservation. Furthermore, we focused on the impact of EPI on the expression patterns of heat shock proteins 90 and 70 as well as the BAX and Bcl-2 proteins involved in the process of apoptosis. The experimental samples were compared with a native control as well as specimens cryopreserved in the absence of any supplement. Our dada indicates that particularly 50 and 100 µmol/L EPI assured a higher preservation of the sperm motility ($p<0.001$) in comparison with the cryopreserved control. The best protection of the sperm DNA ($p<0.05$), lipids ($p<0.05$) and proteins ($p<0.01$) were observed in the samples exposed to 100 µmol/L EPI in comparison to untreated frozen samples. The presence of higher EPI concentrations in the cryopreservation medium led to significant stabilization of the heat shock protein 90 ($p<0.01$ in case of 50 µmol/L EPI; $p<0.001$ with respect to 100 µmol/L EPI) as well as a higher maintenance of the BAX:Bcl-2 ratio ($p<0.001$) when compared to the untreated frozen control. In summary, we may assume that EPI exhibits antioxidant properties which enable the molecule to prevent excessive damage to biomolecules essential for the cell survival and offer an additional layer of protection to spermatozoa exposed to low temperatures, all of which may be translated into a higher post-thaw sperm motility and activity.

**Keywords:** epicatechin, cryopreservation, spermatozoa, oxidative damage, protein expression, heat shock proteins, apoptosis

## INTRODUCTION

Sperm cryopreservation, when properly executed, enables long-term storage of male gametes in a state of metabolic arrest that prevents cellular aging while maintaining their viability and fertilizing potential, therefore it allows them to be used when needed (**Hezavehei *et al.*, 2018**). The role of sperm storage at low temperatures as a critical pillar of reproductive technologies enables to manage or accelerate the rate of genetic improvement, while minimizing the risks of horizontal or vertical transmission of venereal diseases among the animals and leading to a significant cost reduction of the breeding process (**Curry, 2000; Peris-Frau *et al.*, 2020**). Semen storage is also a valuable tool for the protection of genetic resources and biodiversity of protected or endangered animal species (**Fickel *et al.*, 2007**).

A key factor in the biology of sperm cryopreservation is that male gametes are small cells with a relatively large surface (**John Morris *et al.*, 2012**) which will affect the viscosity and transition temperature of the intracellular cytosol (**Hezavehei *et al.*, 2018**). In the absence of cryoprotective agents, cold shock, osmotic stress, and ice crystal formation may lead to the deterioration of organelles and molecules crucial to maintain sperm structural integrity and functional activity (**Hezavehei *et al.*, 2018; Ambar *et al.*, 2020**). Sperm freezing procedures are furthermore accompanied by an outburst of reactive oxygen species (ROS), supraphysiological levels of which may promote the loss of membrane fluidity, apoptosis, mitochondrial swelling, and DNA damage (**Peris-Frau *et al.*, 2020**).

One of the most suitable strategies for the prevention of sperm deterioration during the freeze-thaw procedure is to enhance the semen extender by a multitude of supplements. Although a variety of studies have revealed an enhancement of the sperm quality and fertilizing ability following the administration of energetic molecules, antioxidants, proteins or cryoprotective agents, current attention has shifted to natural biomolecules and extracts with a variety of beneficial effects on the male gamete. Indeed, there are various medicinal herbs known to manage a variety of reproductive ailments and are used as remedies in traditional medicine (**Roozbeh *et al.*, 2021; Tvrdá *et al.*, 2021**).

Catechins are polyphenolic flavonols which are ought to be the primary bioactive components of green tea. Strawberries, cocoa, black grapes, and apricots are also rich in catechins (**Dos Santos *et al.*, 2020**). Green tea catechins encompass four biomolecules, namely epicatechin (EPI), epicatechin-3-gallate, epigallocatechin, and epigallocatechin-3-gallate (**Roychoudhury *et al.*, 2017**), all of which have been reported to be suitable in the prevention of osteoarthritis, cardiovascular issues, Parkinson's disease, or cancer (**Isemura *et al.*, 2019; Dos Santos *et al.*, 2020**). Moreover, catechins have been shown to be involved in the metabolism of lipids and carbohydrates, hence these could be effective in the management of diabetes mellitus or liver dysfunction (**Isemura *et al.*, 2019**). Epicatechin has been shown to present with a notable ROS-scavenging activity (**Pirker *et al.*, 2021**) and ability to chelate transition metals such as chromium and cadmium. At the same time, EPI is capable to reduce iron and copper, and thus the molecule prevents the Fenton reaction associated with generation of a highly toxic hydroxyl radical (**Roychoudhury *et al.*, 2017; Pirker *et al.*, 2021**).

Over the past years, several reports have indicated potential benefits of catechin supplementation to extenders for bovine (**Tvrda *et al.*, 2019**), boar (**Boonsorn *et al.*, 2010**), goat (**Silva *et al.*, 2019**) and canine (**Wittayarat *et al.*, 2013**) semen. Moreover, it has been suggested that catechins, and specifically EPI may provide in vitro protection to spermatozoa against oxidative insults (**Moretti *et al.*, 2012; Jamalan *et al.*, 2016; Tvrda *et al.*, 2019**). Most previous studies agree that EPI stabilizes the membranous structures of male gametes, leading to a more solid sperm morphology. Furthermore, it has been suggested that EPI could modulate sperm capacitation and acrosome reaction by interfering with cholesterol efflux from the membranes and intracellular $Na^+/K^+$ ATPase (**De Amicis *et al.*, 2012**).

In this study, we strived to assess the impact of epicatechin supplementation on prime oxidative characteristics of cryopreserved bovine spermatozoa. Furthermore, we studied any possible effects of epicatechin on expression patterns of heat shock proteins (HSP) 70 and 90 considered to be act as primary indicators of sperm freezability (**Parmar *et al.*, 2019**) as well as Bax and Bcl-2 proteins known to play essential roles in the regulation of sperm apoptosis (**Martin et al., 2007**).

## MATERIAL AND METHODS

### Semen collection and processing

Semen samples were collected from 20 healthy and sexually mature bulls of the Holstein breed (Slovak Biological Services, a.s., Nitra, Slovakia) using specific procedural steps (**Tvrda *et al.*, 2018**).

Each ejaculate was divided into five equal fractions. The first fraction considered as the native control ($Ctrl_N$) was diluted in phosphate buffered saline (without calcium and magnesium; Sigma-Aldrich, St. Louis, MO, USA) using a dilution ratio of 1:40, and immediately evaluated by the techniques mentioned below. The residual fractions were diluted to a final concentration of $11\times10^6$ sperm/mL in an extender containing Triladyl (Minitub GmbH, Tiefenbach, Germany), 20% (w/v) egg yolk, sugar, buffers, Tris, citric acid, glycerol, and antibiotics (spectinomycin, tylosin, gentamicin and lincomycin) and diluted with distilled water. For the experimental groups, the extender was supplemented with 25 µmol/L, 50 µmol/L or 100 µmol/L EPI (Sigma-Aldrich, St. Louis, MO, USA) in DMSO (dimethyl sulfoxide; Sigma-Aldrich, St. Louis, MO, USA) while the cryopreserved control group ($Ctrl_C$) was administered an equal amount of DMSO (final concentration of 0.5%). Diluted semen samples were loaded into 0.25 mL straws, cooled down to 4°C for 2 h and subsequently frozen using a digital freezing machine (Digitcool 5300 ZB 250; IMV, France). Finally, the straws were plunged into liquid nitrogen (–196°C). After storage for one month, the straws were thawed in a 37°C water bath for 20 s immediately before use. Following thawing, the specimens were subjected to specific analyzes of qualitative parameters (**Tvrda *et al.*, 2019**).

**CASA analysis**

Sperm motion was assessed using computer aided sperm analysis (CASA; Version 14.0 TOX IVOS II.; Hamilton-Thorne Biosciences, Beverly, CA, USA). The analysis was set up as follows: frame rate – 60 Hz; minimum contrast – 20; static head size – 0.25–5.00; static head intensity – 0.40–2.00; static elongation – 20–100; default cell size – 4 pixels; default cell intensity – 40. Ten microliters of each sample were pipetted into a Makler counting chamber (depth 10 µm, 37°C; Sefi Medical Instruments, Haifa, Israel) and immediately assessed. Ten microscopic fields were subjected to each analysis to ensure capture of at least 300 cells.

**Oxidative damage to sperm DNA**

All control and experimental samples were centrifuged (300×g, 20°C, 10 min) and washed with PBS twice. For DNA extraction, a lysis solution consisting of 25 mmol/L tris(2-carboxyethyl)phosphine (Sigma-Aldrich, St. Louis, MO, USA), 150 mmol/L 1,4-dithiothreitol (Sigma-Aldrich, St. Louis, MO, USA) and 2% β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA) was added to the washed-out cells, which were subsequently vortexed for 5 min, diluted 1:1 in nuclease free water (Qiagen, Hilden, Germany), and then incubated with 200 µg/mL proteinase K (Sigma-Aldrich, St. Louis, MO, USA) at 56°C for 2 h. DNA was extracted from the resulting lysates with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA yields and quality were determined using the the GloMax®-combined spectro-flluoro-luminometer (Promega, Madison, WI, USA) at 260 nm (**Wu *et al.*, 2015**).
The EpiQuik™ 8-OHdG DNA Damage Quantification Direct Kit (EpiGentek Inc., Farmingdale, NY, USA) was used to quantify the extent of oxidative damage to the sperm DNA by measuring the quantity of 8-hydroxydeoxyguanosine (8-OHdG), an oxidized nucleoside of DNA. Three hundred ng of extracted DNA stored in TE buffer (Thermo Fisher Scientific, Waltham, MA, USA) were added and bound to each strip pretreated to increase its DNA affinity. 8-OHdG was detected using capture and detection antibodies following the instructions of the manufacturer. The detected signal is enhanced with an enhancer solution and then quantified colorimetrically by reading the absorbance at 450 nm with the GloMax®-Multi Microplate Multimode Reader (Promega, Madison, WI, USA). The amount of 8-OHdG was proportional to the OD intensity measured and is expressed in % (**Vorilhon *et al.*, 2018**).

**Protein extraction**

Proteins from intact washed-out spermatozoa were used for further assays. To achieve this, the cells were treated with 1 mL RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA) with protease inhibitor (Sigma-Aldrich, St. Louis, MO, USA) to prevent protein degradation. The samples were subsequently left in the refrigerator overnight. The next day, the samples were mixed again and centrifuged at 11 828×g for 10 min at 4°C and the supernatants collected for further analyses (**Benko *et al.*, 2021**).
Protein determination was performed using a photometric test, based on the Biuret reaction. In this assay, a reaction occurs between proteins and copper ions, forming complexes in an alkaline solution with resulting a blue-violet color. We used a commercial Total protein kit (DiaSys, Holzheim, Germany) for protein determination and the assay was performed on the RX Monza instrument (Randox, Crumlin, UK).

**Protein oxidation**

Oxidative damage to the proteins expressed through the levels of protein carbonyls was evaluated using the 2,4-dinitrophenylhydrazine (DNPH) method. One mL of the lysate pretreated with trichloroacetic acid (TCA; 20% w/v; Sigma-Aldrich, St. Louis, MO, USA) was mixed with 1 mL DNPH (10 mmol/L in 2 N HCl; Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37°C for 1 h. Following a second treatment with 1 mL TCA, the suspension was incubated at 4°C for 10 min and centrifuged (11 828×g, 4°C, 15 min). The resulting pellet was washed three times with 1 mL ethanol/ethyl acetate (1/1; v/v; Sigma-Aldrich, St. Louis, MO, USA) and subsequently resuspended in 1 mL 6 mol/L guanidine HCl (Sigma-Aldrich, St. Louis, MO, USA). The absorbance was measured at 360 nm, using 6 mol/L guanidine HCl as a blank. The molar absorption coefficient of 22 000/M/cm was used to quantify protein carbonyls in each sample. Protein carbonyls are expressed in nmol/mg protein (**Tvrda *et al.*, 2019**).

**Lipid peroxidation**

Lipid peroxidation (LPO) expressed through the levels of malondialdehyde (MDA) was determined using the TBARS assay. Each sample was treated with 5% sodium dodecyl sulphate (Sigma-Aldrich, St. Louis, MO, USA) and subjected to 0.53% thiobarbituric acid (TBA; Sigma-Aldrich, St. Louis, MO, USA) dissolved in 20% acetic acid (pH 3.5; Centralchem, Bratislava, Slovakia) and subsequently boiled at 90–100 °C for 1 h. Afterwards, the samples were placed on ice for 10 min and centrifuged (1750×g, 4°C10 min). The supernatant was pipetted to a clear 96-well plate and absorbance was measured with the GloMax®-Multi Microplate Multimode Reader. MDA concentration is expressed as µmol/g protein (**Tvrda *et al.*, 2019**).

**Western blotting**

Prior to the assay, all lysates were normalized, i.e., protein concentration was adjusted using ultrapure (UHQ) water to reach the final concentration of 25 µg protein. The samples were treated with 4x Laemli buffer (BioRad, Hercules, CA, USA) and β-mercaptoethanol. The samples were then boiled at 95°C for 10 minutes.
The pre-treated samples were loaded (20 µL) into Mini-PROTEAN TGX Stain-free polyacrylamide gels (BioRad, Hercules, CA, USA), along with 7 µL of Precision Plus Protein marker (BioRad, Hercules, USA). Gel electrophoresis was run at 90 V for 2 hours at 90 V, subsequently the gel was visualized with the ChemiDoc Imaging System (BioRad, Hercules, CA, USA). For the blotting procedure, the gels were transferred to PVDF membranes (Trans-Blot Turbo Pack; BioRad, Hercules, CA, USA) using the Trans-Blot Turbo Transfer System (BioRad, Hercules, CA, USA), at 7 min, 25 V and 2.5 A. After completion of the blot, the sandwich was disassembled, and the membrane was incubated for 3x10 min in tris buffered saline (TBS), composed of Tris base (Sigma-Aldrich, St. Louis, MO, USA), sodium chloride (Centralchem, Bratislava, Slovakia) and UHQ water. This step was followed by membrane blocking either with 5% milk (Sigma-Aldrich, St. Louis, MO, USA; in case of BAX and Bcl-2) or 5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA; in case of HSP70 and HSP90) in TBS containing 0.1% Tween-20 (Sigma-Aldrich, St. Louis, MO, USA). Membrane blocking was performed on a stirrer at room temperature for 2h. Subsequently, the membranes were treated with primary antibodies against the proteins of interest overnight at 4°C. Antibodies used for the detection of target proteins are specified in Table 1.

**Table 1** Antibodies used in the Western blot analysis

| Target protein | Antibody | Clonality/Isotype | Dilution | Blocking solution | Source | ID | Manufacturer |
|---|---|---|---|---|---|---|---|
| BAX | anti-BAX antibody (BCL2-Associated X Protein) N-Term | Polyclonal/IgG | 1:1 000 | 5% milk in TBS/0.1% Tween-20 | rabbit | #ABIN6990475 | Antibodies Online; Dunwoody, GA, USA |
| Bcl-2 | anti-Bcl-2 antibody (B-Cell CLL/lymphoma 2) N-Term | Polyclonal/IgG | 1:1 000 | 5% milk in TBS/0.1% Tween-20 | rabbit | #ABIN2857047 | Antibodies Online; Dunwoody, GA, USA |
| HSP70 | HSP70 Antibody | Polyclonal/IgG | 1:1 000 | 5% BSA in TBS/0.1% Tween-20 | rabbit | #4872 | Cell Signaling Technology; Danvers, MA, USA |
| HSP90 | HSP90α (D1A7) mAb | Monoclonal/IgG | 1:1 000 | 5% BSA in TBS/0.1% Tween-20 | rabbit | #8165 | Cell Signaling Technology; Danvers, MA, USA |

The next day, the membranes were washed 5x10 min in wash buffer composed of 1% milk or 1% BSA respectively, in TBS/0.2% Tween-20, and subsequently incubated with a secondary anti-rabbit antibody (GE Healthcare, Chicago, IL, USA) diluted 1: 15 000 in 1% milk or 1% BSA respectively, in TBS/0.2% Tween-20 for 1 h. Following incubation, the membranes were washed 3x10 minutes in TBS/0.2% Tween-20 at room temperature and using a stirrer.
To visualize the target proteins, membranes were incubated with the ECL substrate (GE Healthcare, Chicago, IL, USA) in the dark for 5 minutes. After incubation the

membranes were placed to the ChemiDoc Imaging System, which automatically calculated the protein visualization time based on the light signal emitted by the membranes.

Relative quantification of the protein expression was assessed using BioRad Image Software 6.1 (BioRad, Hercules, USA). For all the blots, the expression of a housekeeping protein was assessed. In this case, we used rabbit β-actin Antibody (#4967; Cell Signaling Technology; Danvers, MA, USA), diluted in a ratio of 1: 1000 in 5% BSA/TBS/0.1% Tween-20 (**Benko *et al.*, 2021**). The results are interpreted as relative quantification of the native control.

### Statistical analysis

Statistical analysis was carried out using the GraphPad Prism program (version 9.2.0 for Mac; GraphPad Software, La Jolla, CA, USA). One-way ANOVA was used for statistical evaluations. Dunnett's test was selected as a follow-up test to ANOVA, based on a comparison of every mean to a control mean, and creating a confidence interval for the difference between the two means. The comparative analysis was performed in the following sequence:

- Native control ($Ctrl_N$) was compared to the cryopreserved control ($Ctrl_C$),
- Experimental groups were compared to both controls.

For the cryopreservation experiments, Dunnett's test was used to compare the experimental groups to the untreated control. The level of significance was set at $^{****}p<0.0001$, $^{***}p<0.001$, $^{**}p<0.01$ and $^{*}p<0.05$.

## RESULTS AND DISCUSSION

### Sperm motility

The CASA analysis revealed that the cryopreservation procedure had a significantly negative impact on the sperm motion behavior (Figure 1). Nevertheless, we observed that while the lowest motility was present in the cryopreserved control ($Ctrl_C$; $p<0.0001$ in comparison with $Ctrl_N$), a significant improvement in the percentage of motile spermatozoa was observed in the presence of all three EPI doses ($p<0.01$ in case of 25 µmol/L EPI; $p<0.001$ with respect to 50 and 100 µmol/L EPI in comparison with $Ctrl_C$).

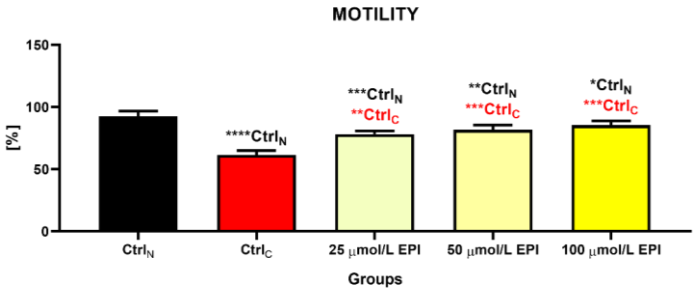


**Figure 1** Motility of bovine spermatozoa in a native state and cryopreserved in the absence or presence of different epicatechin (EPI) concentrations
**Legend:** $^{****}p<0.0001$, $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$. $Ctrl_N$ – versus native control, $Ctrl_C$ – versus cryopreserved control.

Our data on the sperm motility agree with **Greifova *et al.* (2018)** indicating beneficial effects of EPI on the motion activity of bovine spermatozoa incubated in vitro for 24 h. Similarly, our previous study (**Tvrdá *et al.*, 2019**) suggests that a concentration range of 12.5–100 µmol/L EPI provided protection to the loss of sperm motility as a consequence of their exposure to a prooxidant (ferrous ascorbate) over a period of 6 h. According to **Purdy *et al.* (2004)** various concentrations (25–100 µmol/L) of catechin led to a significant improvement of cooled goat sperm motility following 96 h. Furthermore, it was reported that the motion of boar spermatozoa was higher following exposure to catechin (25 µmol/L and 50 µmol/L) after 24 h and 48 h (**Boonsorn *et al.*, 2010**). On the other hand, **Jamalan *et al.* (2016)** observed that not even a wide concentration range of catechins (at 25–1000 µmol/L) did not exhibit any significant impact on human spermatozoa motility exposed to lead, aluminium, or cadmium. What is more, **Moretti *et al.* (2012)** concluded that while spermatozoa supplemented with lower EPI concentrations presented with a significantly improved progressive motility of human gametes, high doses of EPI (200 and 400 µmol/L) led to a significant decline of the motion characteristics. These conflicting reports may therefore indicate that the exact effects of EPI on the sperm motility may depend on the dosage, sperm processing technique, species, or time of analysis.

### Oxidative changes to sperm DNA, proteins, and lipids

As observed in Figure 2, exposure to low temperatures had a negative impact on the susceptibility of sperm DNA towards oxidative damage since the highest amount of 8-OHdG was detected in the $Ctrl_C$ group ($p<0.001$ against $Ctrl_N$). While none of the EPI concentrations selected for the experiments was able to completely protect sperm DNA against oxidative insults, a dose-dependent decrease of 8-OHdG was observed proportionately to the increasing EPI concentration supplemented to the extender. Significantly decreased levels of 8-OHdG in comparison to $Ctrl_C$ were observed particularly in the case of 100 µmol/L EPI ($p<0.05$).

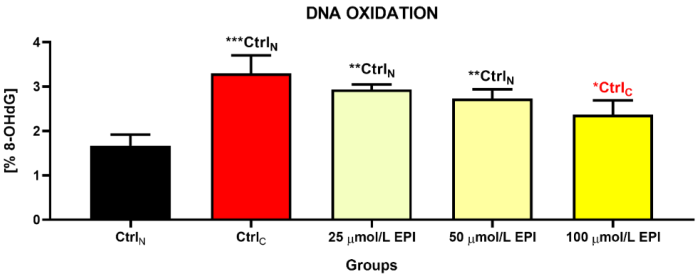


**Figure 2** Oxidative DNA damage of bovine spermatozoa in a native state and cryopreserved in the absence or presence of different epicatechin (EPI) concentrations
**Legend:** $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$. $Ctrl_N$ – versus native control, $Ctrl_C$ – versus cryopreserved control.

Correspondingly to DNA oxidation, the highest concentrations of protein carbonyls were found in the cryopreserved control ($Ctrl_C$) which were significantly different when compared to the native control ($Ctrl_N$; $p<0.001$; Figure 3). A lower degree of oxidative damage to the proteins was observed in all experimental groups supplemented with EPI, however a significantly lower amount of protein carbonyls in comparison to $Ctrl_C$ were recorded following sperm exposure to 100 µmol/L EPI ($p<0.01$).

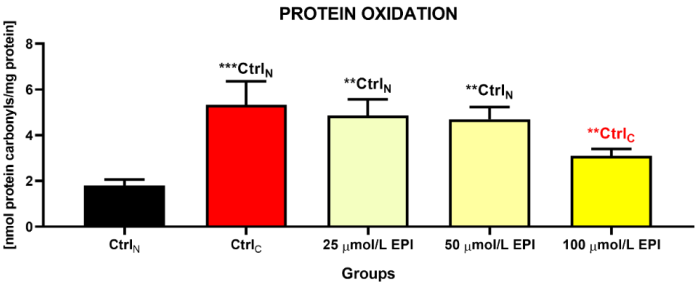


**Figure 3** Oxidative damage to the proteins of bovine spermatozoa in a native state and cryopreserved in the absence or presence of different epicatechin (EPI) concentrations
**Legend:** $^{***}p<0.001$, $^{**}p<0.01$. $Ctr_N$ – versus native control, $Ctrl_C$ – versus cryopreserved control.

The extent of peroxidative damage to the sperm lipids mirrored the same trend as observed in previous analyses of the oxidative profile (Figure 4). The highest degree of oxidative insults to the lipid molecules reflected in the highest MDA concentration were observed in the cryopreserved control ($Ctrl_C$) when compared to the native sperm suspension ($Ctrl_N$; $p<0.01$). A gradual dose-dependent decrease of MDA levels was noted in the experimental groups. While significant differences were observed in the comparative analysis among $Ctrl_N$, 25 µmol/L EPI ($p<0.01$) and 50 µmol/L EPI ($p<0.05$), a significant decline of MDA levels were detected in the experimental group administered with 100 µmol/L EPI in comparison to $Ctrl_C$ ($p<0.05$).

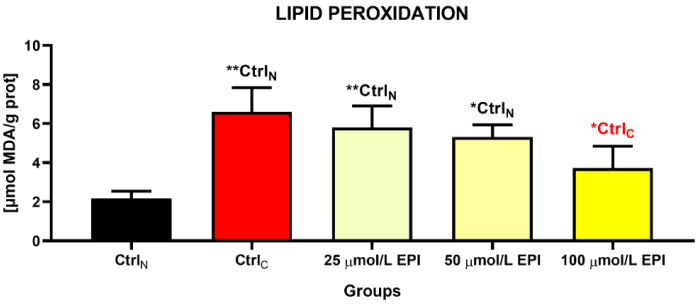


**Figure 4** Oxidative damage to the lipids of bovine spermatozoa in a native state and cryopreserved in the absence or presence of different epicatechin (EPI) concentrations
**Legend:** $^{**}p<0.01$, $^{*}p<0.05$. $Ctrl_N$ – versus native control, $Ctrl_C$ – versus cryopreserved control.

Data from this study indicate that EPI presented with the ability to at least partially prevent excessive oxidative damage to vital biomolecules which may lie in its potential to interact with ROS before these can reach and interact with the sperm proteins, lipids and/or DNA. A similar hypothesis was postulated by **Awoniyi *et al.* (2012)** according to who green tea supplements improved the motion activity, antioxidant properties and diminished lipid peroxidation of epididymal spermatozoa collected from rats exposed to t-butyl hydroperoxide, most likely because of its capability to inhibit ROS production and subsequently prevent excessive oxidative damage. Moreover **Ding *et al.* (2015)** reported that supplementation of catechins to irradiated mice prevented any potential germ cell loss and ameliorated radiation-induced testicular OS, leading to a higher sperm concentration, motility, and morphology. Similar beneficial effects were recorded by **Zanchi *et al.* (2015)** suggesting that in accordance with our results, green tea catechins significantly reduced LPO, protein oxidation and DNA fragmentation in male reproductive cells of mice exposed to cyclophosphamide. Accordingly, lower levels of MDA and DNA fragmentation were noted in previous studies on cooled dog spermatozoa (**Wittayarat *et al.*, 2013**) and cryopreserved boar semen from boars (**Gale *et al.*, 2015**) and bovine spermatozoa challenged by oxidative pressure (**Tvrda *et al.*, 2019**). Moreover, **Sapanidou *et al.* (2014)** revealed that exposure of frozen-thawed bovine spermatozoa to 2 µg/mL and 5 µg/mL of green tea extract for 2 h led to a significant increase of sperm vitality with a concomitant lower MDA production in comparison to the untreated control.

On the other hand, our data disagree with **Moretti *et al.* (2012)** who observed no significant positive or negative effect of EPI on the extent of LPO of human spermatozoa subjected to t-butyl hydroperoxide. As such, we may hypothesize on the exact mechanism of action of EPI on the sperm membranous structures that are highly vulnerable to lipid disintegration caused by ROS. Another aspect to be taken into consideration, are the chemical properties of the molecule. Being amphipathic in nature, EPI has been shown to be capable to interact with both plasma proteins and phospholipids. Furthermore, its permeability and its lipid affinity directly depend on the extent of hydroxylation, molecular configuration, and side chain length. Moreover, the stereochemistry of the hydroxyl group located on the C ring and the absence of the carbonyl group may have a direct impact on the antioxidant behavior of the flavanol (**Lambert *et al.*, 2010**).

**Expression patterns of heat shock proteins**

Data collected from the Western blot analysis revealed that HSP90 expression in spermatozoa was significantly negatively affected by low temperatures in comparison to their native state ($p<0.001$; Figure 5). In the meantime, all EPI doses supplemented to the cryopreservation medium were able to provide a certain degree of protection to the protein following the freeze-thaw procedure. In particular, HSP90 protein expression was significantly enhanced in the experimental groups containing 50 µmol/L ($p<0.01$) and 100 µmol/L EPI ($p<0.001$) when compared to the cryopreserved control.

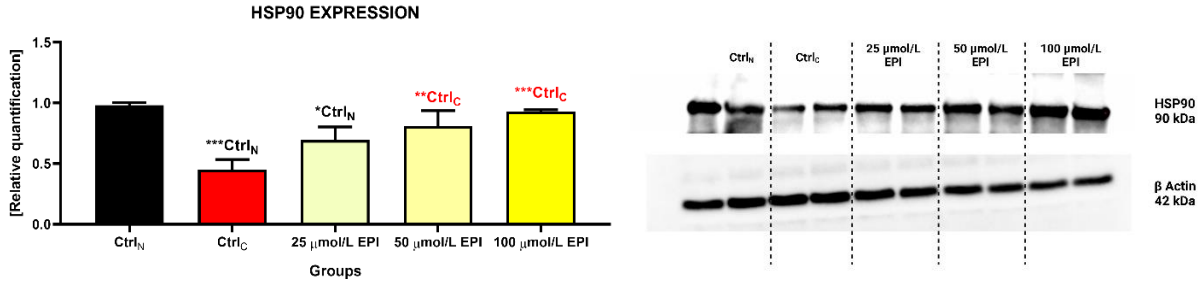


**Figure 5** Heat shock protein 90 (HSP90) expression patterns of bovine spermatozoa in a native state and cryopreserved in the absence or presence of different epicatechin (EPI) concentrations. **Legend:** **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$. Ctrl$_N$ – versus native control, Ctrl$_C$ – versus cryopreserved control.

A similar dose-dependent pattern of behavior was recorded in case of the HSP70 protein expression (Figure 6). Although to a lower degree, cryopreserved spermatozoa exposed to 50 µmol/L and 100 µmol/L EPI presented with a higher expression of HSP70 in comparison to their counterparts frozen in the absence of EPI ($p<0.01$ with respect to 50 µmol/L EPI; $p<0.0001$ in case of 100 µmol/L EPI; respectively, versus Ctrl$_C$).

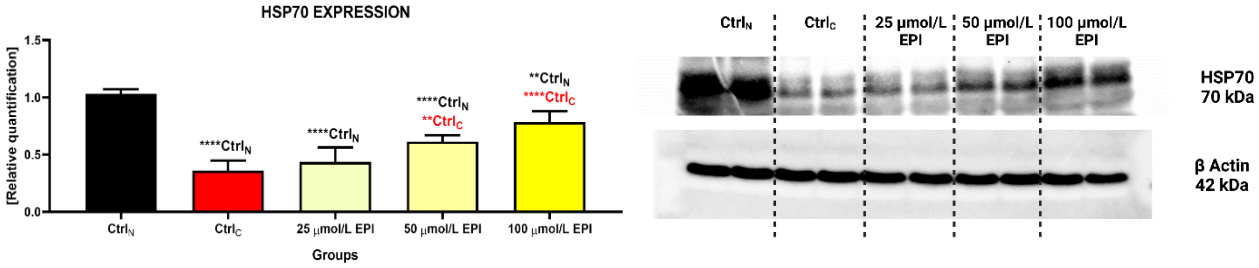


**Figure 6** Heat shock protein 70 (HSP70) expression patterns of bovine spermatozoa in a native state and cryopreserved in the absence or presence of different epicatechin (EPI) concentrations. **Legend:** **** $p<0.0001$, ** $p<0.01$. Ctrl$_N$ – versus native control, Ctrl$_C$ – versus cryopreserved control.

It has been previously elucidated that HSPs play important roles in the amelioration of stress and maintenance of a normal cellular activity since their fluctuations have a direct effect on the acquisition of tolerance (**Shan *et al.*, 2020**). Numerous reports suggest that the HSPs expression levels in the eucaryotic cell may change under thermal or cold stress (**Cao *et al.*, 2003; Wang *et al.*, 2014; Zhang *et al.*, 2015a,b; Karabulut *et al.*, 2018**).

Results collected from the Western blot analysis are consistent with previous studies on frozen-thawed spermatozoa form a variety of mammalian species (**Huang *et al.*, 1999; Cao *et al.*, 2003; Zhang *et al.*, 2015a;b; Varghese *et* al., 2016; Parmar *et al.*, 2021**), indicating that both HSP90 and HSP70 might play an important role as regulators of sperm motility, which may be substantially affected by the cooling process. With the help of Western blotting, we were able to observe this phenomenon in bovine spermatozoa collected from Holstein bulls.

A significant under expression of HSPs in the cryopreserved control in comparison to native semen indicates that bovine spermatozoa may react to the freezing-thawing procedure by degrading HSPs present in the cell (**Cao *et al.*, 2003**). A different hypothesis postulated by **Harrison *et al.* (1972)** suggests that similarly to a variety of proteins, HSPs could be leaking to the extracellular surroundings or the semen extender as a response to cold shock. However, none of previous reports has detected the presence of HSP90 or HSP70 in the cryopreservation medium or seminal plasma prior to the cryopreservation procedure (**Cao *et al.*, 2003; Zhang *et al.*, 2015a;b)**. According to **Zhang *et al.* (2015a;b)**, another reason for the loss of HSPs could be connected to the inability of frozen cells to properly execute HSPs synthesis or the proteins might have been consumed to maintain the assembly and/or conformation of other proteins during the freezing process. Furthermore, frozen-thawed sperm samples often present with an increased number of already dead cells unable to produce any protein anymore (**Peris-Frau *et al.*, 2020**).

In this study, the down-expression of HSP90 and HSP70 was an accompanying phenomenon of a significant decline in the post-thaw sperm motility. This observation is consistent with the postulation by **Wang *et al.* (2005)** according to who a decrease of HSPs precedes the decline of semen quality. At the same time, these data indicate that post-thaw sperm activity, hence the survival time of cryopreserved spermatozoa could be enhanced by an increased HSPs synthesis prior to the freezing procedure (**Zhang *et al.*, 2015b**).

Exposure of cells to low temperatures leads to an increased production of reactive oxygen species (ROS) that impair the sperm vitality and fertilizing potential (**Tvrdá *et al.*, 2018; Tvrda *et al.*, 2018;2019**). It has been hypothesized that HSPs present with the ability to affect the folding of membrane proteins and translocation of polypeptides across the plasma membrane (**Arispe *et al.*, 2002**). It was previously suggested that HSPs underexpression leads to abnormal folding of membrane proteins, with subsequent alterations to the membrane fluidity (**Aboagla and Terada; 2003**). Furthermore, it has been suggested that HSPs could protect vital biomolecules ROS by regulating superoxide dismutase (SOD)

activity. As such, it may be plausible to assume that the decrease of HSPs levels in cryopreserved samples may lead to a, lower resilience of the sperm lipids, proteins and DNA towards oxidative insults catalyzed by ROS generated during the freeze-thaw process, presenting itself as a significant increase of the levels of 8-OHdG, protein carbonyls and MDA.

Very little is known on the effects of natural biomolecules on the fluctuations of protein markers during sperm cryopreservation. In our case it was revealed that higher EPI concentration (50 µmol/L and 100 µmol/L) supplemented to the semen extender led to a significant preservation of both HSPs in cryopreserved spermatozoa. Previous studies have shown that the anthocyanin cyanidin-3-O-β glucopyranoside and its aglycon cyanidin chloride, acted as antioxidants partially via induced expression of HSP70 in the Caco2 cell line (**Hosokawa *et al.*, 1992**). The same effect was also reported in the case of 10-100 µmol/L naringenin (**Li *et al.*, 2014**). Further studies on polyphenols have reported that quercetin, kaempferol and genistein could modulate HSPs (**Burkitt *et al.*, 2000; Putics *et al.*, 2008**). Moreover, different polyphenols such as resveratrol, curcumin, lycopene or even EPI bring glutathione in a reduced form to suppress ROS-mediated cellular damage (**Sarma *et al.*, 2016; Tvrdá *et al.*, 2018; Tvrda *et al.*, 2018;2019**) and ameliorate LPO via decreasing MDA levels and enhancing the activity of enzymatic antioxidants (**van Eden *et al.*, 2015**).

**Expression patterns of proteins involved in cell death**

As revealed by Figure 7, the expression of the pro-apoptotic BAX protein was significantly enhanced following the cryopreservation process as revealed by a significant increase of the protein levels in the cryopreserved control (CtrlC) as opposed to the native sperm sample (CtrlN; $p<0.0001$). Supplementation of particularly 50 and 100 µmol/L EPI led to a significant decrease of the BAX expression behavior in comparison to the frozen-thawed control ($p<0.001$ in case of 50 µmol/L EPI; $p<0.0001$ with regard to µmol/L EPI).

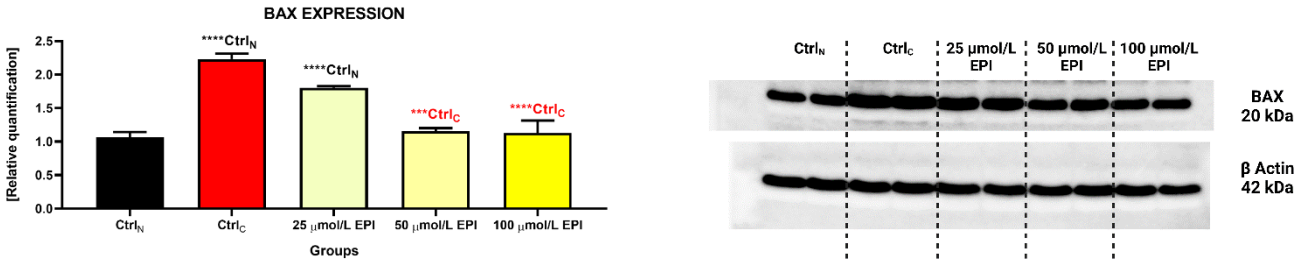


**Figure 7** BAX expression patterns of bovine spermatozoa in a native state and cryopreserved in the absence or presence of different epicatechin (EPI) concentrations.
**Legend:** **** $p<0.0001$, *** $p<0.001$. $Ctrl_N$ – versus native control, $Ctrl_C$ – versus cryopreserved control.

Inversely, the anti-apoptotic Bcl-2 protein was significantly underexpressed in the untreated frozen-thawed sample in comparison with the native control ($p<0.0001$; Figure 8). While a slight improvement in the expression patterns of Bcl-2 were observed following the administration of 25 µmol/L EPI, a significant improvement in Bcl-2 protein levels were observed in the experimental groups containing 50 and 100 µmol/L EPI when compared to the cryopreserved control ($p<0.0001$).

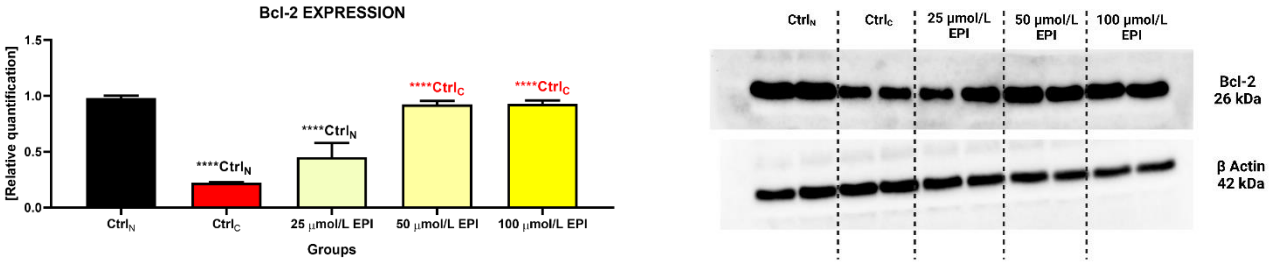


**Figure 8** Bcl-2 expression patterns of bovine spermatozoa in a native state and cryopreserved in the absence or presence of different epicatechin (EPI) concentrations.
**Legend:** **** $p<0.0001$. $Ctrl_N$ – versus native control, $Ctrl_C$ – versus cryopreserved control.

Apoptosis defined as "programmed cell death" has been suggested to play an intricate role in the cryodamage to the sperm cell, as the freezing and thawing process has been shown to increase the activation of specific caspases in a variety of mammalian gametes (**Anzar *et al.*, 2002; Said *et al.*, 2010; Karabulut *et al.*, 2018; Kaur and Atreja, 2018, Topraggaleh *et al.*, 2021).** The exact apoptotic machinery involved in the etiology of sperm cryopreservation, including those resulting in cryoinjury of sperm proteins, lipids or DNA, have not been fully elucidated. Nevertheless, currently it is not possible to distinguish apoptotic cells during artificial reproduction techniques, and thus eliminate damaged spermatozoa which may cause an impaired fertilization and embryogenesis, with subsequent implications on the pregnancy, implantation and/or abortion rates (**Karabulut *et al.*, 2018**).

The primary role of the anti-apoptotic protein Bcl-2 is to prevent the action of pro-apoptotic proteins responsible for the formation of pores in the mitochondrial system (**Llambi *et al.*, 2011**). Bcl-2 and BAX ratio in the sperm cell decides on the fate of the male gamete where Bcl-2 inhibits and BAX promotes cell death (**Dalal *et al.*, 2016**). While being in agreement with our data, the pro-apoptotic BAX protein has been detected in bovine spermatozoa, whereas the anti-apoptotic factor Bcl-2 has not (**Dogan *et al.*, 2013**). This discrepancy may have been caused by the selected protocol for protein extraction, Western blot procedure or antibody used for the protein detection.

To our knowledge, only one study has addressed the potential of natural biomolecules on the expression patterns of pro- and/or apoptotic proteins in cryopreserved spermatozoa. According to **Rezaei *et al.* (2020)** 5 mmol/L of lycopene significantly decreased BAX and increased Bcl-2 mRNA levels. We may agree that similarly to EPI, lycopene is a potent ROS-quencher and inhibits these before they can reach mitochondria leading to membrane hyperpolarization, collapse of the mitochondrial membrane potential, translocation of BAX and BAD, and finally cytochrome c release (**Redza-Dutordoir and Averill-Bates, 2016**).

**CONCLUSION**

Summarizing the data collected in this study, we may conclude that a concentration range of 50 and 100 µmol/L epicatechin is suitable for the improvement of bovine sperm vitality following the freeze-thaw process. Epicatechin has shown to possess antioxidant properties that may prevent excessive damage to biomolecules critical to the sperm structural integrity and functional activity. Furthermore, appropriately selected epicatechin concentrations may prevent the loss of proteins involved in the protection of male gametes against thermal and cold shock and prevent the loss of viable spermatozoa to apoptosis by stabilizing the ratio of pro- and anti-apoptotic proteins in the cell. Nevertheless, specific mechanism of the biomolecule at the cellular and molecular level needs to be addressed in more detail in future studies.

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