JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

THEOBROMINE AS AN EFFECTIVE PREVENTION AGAINST CRYODAMAGE OF BOVINE SPERMATOZOA

Filip Benko[1], Štefan Baňas[1], Michal Ďuračka[2], Eva Tvrdá[1]*

Address(es): Ing. Filip Benko, PhD.
[1] Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Institute of Biotechnology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia.
[2] Slovak University of Agriculture in Nitra, AgroBioTech Research Centre, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia.

*Corresponding author: filip.benko@uniag.sk

https://doi.org/10.55251/jmbfs.11527

ARTICLE INFO

Received 9. 7. 2024
Revised 21. 7. 2025
Accepted 30. 8. 2025
Published 1. 10. 2025

Regular article

OPEN ACCESS

ABSTRACT

This research examined a possible protective effects of three selected theobromine (TBR) doses (12.5, 25 and 50 µM) on the cryopreserved bovine spermatozoa following sperm motility, oxidative profile and the expression patterns of heat shock proteins (HSPs) 70 and 90 as well as apoptosis-associated BAX (pro-apoptotic Bcl2-associated X) and Bcl-2 (anti-apoptotic B-cell lymphoma 2) proteins. Cryopreserved samples supplemented with TBR were compared with a native control ($Ctrl_N$) and a cryopreserved control ($Ctrl_C$) without TBR treatment. Our results demonstrate that the administration with TBR led to a significant improvement of sperm progressive motility ($p<0.0001$) when compared to $Ctrl_C$. The highest levels of protection for sperm DNA ($p<0.01$), lipids ($p<0.0001$) and proteins ($p<0.001$) were detected in samples treated with 25 and 50 µM of TBR against $Ctrl_C$. Administration of 25 and 50 µM of TBR significantly preserve HSP90 protein expression ($p<0.0001$) while HSP70 protein expression stay without changes when compared with the unsupplemented frozen control. No significant differences were observed in the expression patterns of Bcl-2 protein, however a significant decline of BAX protein was observed in the frozen samples enriched with 25 ($p<0.05$) and 50 ($p<0.001$) µM of TBR after comparison with the $Ctrl_C$. In summary, we may consider that TBR can be effective agent in stabilizing the sperm membranes by preventing reactive oxygen species (ROS) induced lipid peroxidation, protein oxidation and subsequent oxidative damage to molecules critical for a proper sperm function. These protective properties of TBR may lead to higher post-thaw quality and viability of bovine spermatozoa.

Keywords: theobromine, cryopreservation, bovine spermatozoa, oxidation, apoptosis, heat shock proteins

INTRODUCTION

As sperm cryopreservation enables an effective distribution of genetically superior animals across the globe, it is an important factor in the efficiency and success of artificial insemination (AI). Furthermore, ejaculates are stored at low temperatures to enhance *in vitro* methods used in veterinary andrology, and to preserve genetic diversity and potential of protected, endangered, and valuable animals **(Barbas and Mascarenhas, 2009)**. Several factors contribute to the damage of spermatozoa during the cryopreservation process, including cell acidosis, energetic deficiency, reactive oxygen species (ROS) production, ice crystal formation, hyperosmolarity, and protein denaturation **(Baust *et al.*, 2009)**. It is thought that cryocapacitation is responsible for low vitality of frozen spermatozoa, which could lead to an unsuccessful fertilization and consequently a diminished fertility rates if used for AI. Spermatozoa suffering from cryoshock may develop more aggressive interactions with their surrounding environment during their transfer to the female reproductive tract due to altered structural changes **(Cormier *et al.*, 1997)** therefore, the oocyte has a significantly limited chance of being fertilized **(Ugur *et al.*, 2019)**. The exact mechanisms of cryocapacitation are nevertheless not yet fully understood. During cryopreservation, ROS are more likely to be generated, resulting in oxidative stress that impairs the sperm quality **(Lone *et al.*, 2018)**. The reason lies in the presence of higher amounts of polyunsaturated fatty acids (PUFAs) in the sperm plasma membrane **(Sarlos *et al.*, 2002)**, that are highly sensitive to higher ROS levels **(Gadea *et al.*, 2013)**. Furthermore, the seminal antioxidant system cannot deal with higher ROS levels, resulting in an imbalance between their production and detoxification, resulting in oxidative stress **(Dowling and Simmons, 2008)**. There are several enzymatic antioxidants, referred to as natural antioxidants, such as glutathione peroxidase, glutathione reductase, superoxide dismutase, and catalase **(Alvarez *et al.*, 1987; Alvarez *et al.*, 1989)**, all of which contribute to the natural defense system of sperm cell **(Silva *et al.*, 2011; Parttyka *et al.*, 2012)**. As semen extenders develop, alternative cryoprotective supplements are being added that could be of benefit for the sperm health by mitigating potentially toxic effects of cooling and freezing. Due to their biological effects, low toxicity, and high bioavailability, bioactive molecules derived from plants are gaining increasing scientific interest. There are several alternative biomolecules that can significantly ameliorate or reverse cell cryo-induced structural or functional damage, including curcumin, lycopene, epicatechin, kaempferol or theobromine, in addition to having antioxidant and metabolic stimulating properties at the same time. By screening natural sources, new antibiotic replacement substances for animal production have also been identified **(Mazurova *et al.*, 2015)**. In addition to their ability to scavenge ROS and modulate the intrinsic antioxidant enzymes, various *in vitro* studies have demonstrated that natural bioactive molecules exhibit antibacterial activity as well as remarkable antioxidant properties **(Tvrdá *et al.*, 2016; Tvrdá *et al.*, 2019; Kunová *et al.*, 2019)**. Theobromine (3, 7-dimethylxanthine; TBR) is the principal methylxanthine present in cocoa beans, coffee, tea, and chocolate. The molecule is a vasodilator, diuretic, and heart stimulator. It has a role as an adenosine receptor antagonist, and a bronchodilator agent. At the same time, an enhancement of buffalo sperm motility, viability, acrosomal integrity was observed in the presence of TBR when compared to control and other methylxanthines during cryopreservation **(Bishist *et al.*, 2020)**. We focused on the impact of TBR on the central oxidative characteristics of frozen-thawed bovine sperm which is unclear due to the insufficient number of research studies of its properties. Additionally, we looked at any possible effects of TBR on the expression patterns of heat shock proteins (HSPs) 70 and 90 acting as primary indicators of sperm freezability **(Parmar *et al.*, 2021)** as well as the Bcl2-associated X (BAX) protein and the B-cell lymphoma 2 (Bcl-2) protein, which play essential roles in the regulation of sperm apoptosis **(Martin *et al.*, 2007)**.

MATERIAL AND METHODS

Semen Collection and Cryopreservation

Semen samples were collected from 20 healthy and sexually mature Holstein bulls (Slovak Biological Services, a.s., Nitra, Slovakia) by using artificial vagina **(Tvrda *et al.*, 2019)**. Each sample was divided into five equal aliquots. The first, serving as the native control ($Ctrl_N$), was transported in the laboratory, diluted in phosphate-buffered saline solution (without calcium and magnesium; Sigma-Aldrich, St. Louis, MO, USA) at 1:40, and immediately assessed as specified below (motility, oxidative profile and proteomic analysis). The residual aliquots were diluted to a final concentration of 11×10^6 sperm/mL in a semen extender comprising Triladyl (Minitub GmbH, Tiefenbach, Germany), 20% (*w/v*) egg yolk, sugar, buffers, Tris, citric acid, glycerol, antibiotics, and distilled water. For the experimental groups, the extender was supplemented with 12.5 µM, 25 µM and 50 µM of TBR (Sigma-Aldrich, St. Louis, MO, USA) in DMSO (dimethyl sulfoxide; Sigma-Aldrich, St. Louis, MO, USA), while the cryopreserved control group ($Ctrl_C$) was enriched with an equal amount of DMSO (final concentration of 0.5%).

DMSO was used in $Ctrl_C$ since it served as a medium through which TBR was delivered to the experimental groups. All diluted samples were loaded into 0.25 mL French straws, cooled down to 4 °C for 2 h and subsequently frozen using a digital freezing machine (Digitcool 5300 ZB 250; IMV, Paris, France). Finally, the straws were plunged into liquid nitrogen and stored for one month. Before analysis, the straws were thawed in a 37 °C water bath for 90 s and processed exactly like the native control **(Baňas *et al.*, 2022; Tvrda *et al.*, 2019; Baňas *et al.*, 2023b)**.

Sperm motility assessment

Sperm progressive motility was assessed through the computer assisted sperm analysis (CASA; Version 14.0 TOX IVOS II.; Hamilton-Thorne Biosciences, Beverly, CA, USA) as previously published **(Baňas *et al.*, 2022)**.

Oxidative profile

All control and experimental specimens were centrifuged (300× *g*, 20 °C, 10 min), washed with PBS twice and treated with a lysis solution composed of 150 mmol/L 1,4-dithiothreitol (Sigma-Aldrich, St. Louis, MO, USA), 25 mmol/L tris(2-carboxyethyl) phosphine (Sigma-Aldrich, St. Louis, MO, USA) and 2% β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA). The suspensions were 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 with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), the yield and quality were determined using the combined spectro-flluoro-luminometer GloMax® Multi$^+$ (Promega, Madison, WI, USA) at 260 nm **(Wu *et al.*, 2015)**. The extent of oxidative damage to the sperm DNA was assessed with the EpiQuik™ 8-OHdG DNA Damage Quantification Direct Kit (EpiGentek Inc., Farmingdale, NY, USA) according to the instructions by the manufacturer. The amount of 8-hydroxy-2′-deoxyguanosine (8-OHdG) was proportional to the OD intensity measured and is expressed in % **(Baňas *et al.*, 2022; Vorilhon *et al.*, 2018)**. Proteins from washed-out spermatozoa were extracted with RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA) and protease inhibitor (Sigma-Aldrich, St. Louis, MO, USA). Following an overnight extraction, the samples were centrifuged at 11,828× *g* for 10 min at 4 °C and the supernatants collected for further analyses. Protein concentration was determined using the commercial Total protein kit (DiaSys, Holzheim, Germany) and the RX Monza instrument (Randox, Crumlin, UK) **(Benko *et al.*, 2021)**. Oxidative damage to the proteins expressed through the levels of protein carbonyls was evaluated using the 2,4-dinitrophenylhydrazine (DNPH) method as previously published **(Baňas *et al.*, 2023a)**. Protein carbonyls are expressed in nmol/mg protein. Lipid peroxidation (LPO) expressed through the levels of malondialdehyde (MDA) was determined using the TBARS assay according to **Tvrda *et al.* (2019)** while MDA concentration was expressed as µmol/g protein.

Western blotting

Randomly selected samples from each control and experimental group were treated with 4× Laemli buffer (BioRad, Hercules, CA, USA), β-mercaptoethanol and boiled at 95 °C for 10 min. The pre-treated samples were loaded (25 µg protein, 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, CA, USA). Gel electrophoresis was run for 2 h at 90 V; subsequently, the gel was visualized with the ChemiDoc Imaging System (BioRad, Hercules, CA, USA). 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. The resulting membranes were incubated for 3 × 10 min in tris buffered saline (TBS), and then blocked either with 5% milk (Sigma-Aldrich, St. Louis, MO, USA; for BAX and Bcl-2) or 5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA; for HSP70 and HSP90) in TBS containing 0.1% Tween-20 (Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 2 h. Subsequently, the membranes were exposed to the following primary antibodies against the proteins of interest overnight at 4 °C (Table 1).

Table 1 Used primary antibodies for Western blotting

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
HSP90	HSP90α (D1A7) mAb	Monoclonal/IgG	1:1 000	5 % BSA in TBS/0.1% Tween-20	rabbit	#4872	Cell Signaling Technology; Danvers, MA, USA
HSP70	HSP70 Ab	Polyclonal/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 5 × 10 min in 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. Finally, the membranes were washed 3 × 10 min in TBS/0.2% Tween-20 at room temperature. For protein visualization, the membranes were incubated with the ECL substrate (GE Healthcare, Chicago, IL, USA) for 5 min and processed with the ChemiDoc Imaging System. Relative quantification of the protein expression was performed with BioRad Image Software 6.1 (BioRad, Hercules, CA, USA). For all blots, the expression of a housekeeping protein was assessed. In this case, rabbit β-actin Antibody (Cell Signaling Technology; Danvers, MA, USA), diluted at 1:1000 in 5% BSA/TBS/0.1% Tween-20 was used **(Baňas *et al.*, 2022)**. The results are interpreted as relative quantification of the native control.

Statistics

Statistical analysis was performed with the GraphPad Prism program (version 9.2.0 for Mac; GraphPad Software, La Jolla, CA, USA). One-way ANOVA and Dunnett's test were selected for the analysis of obtained data. The level of significance was set at following levels (****p<0.0001; ***p<0.001; **p<0.01 and *p<0.05).

RESULTS AND DISCUSSION

Sperm progressive motility

Obtained data from CASA analysis (Figure 1) showed a significant improvement (p<0.0001) of post-thaw sperm progressive motility in all cryopreserved groups treated with different concentrations of TBR (12.5, 25 and 50 µM) compared to non-treated cryopreserved control ($Ctrl_C$).

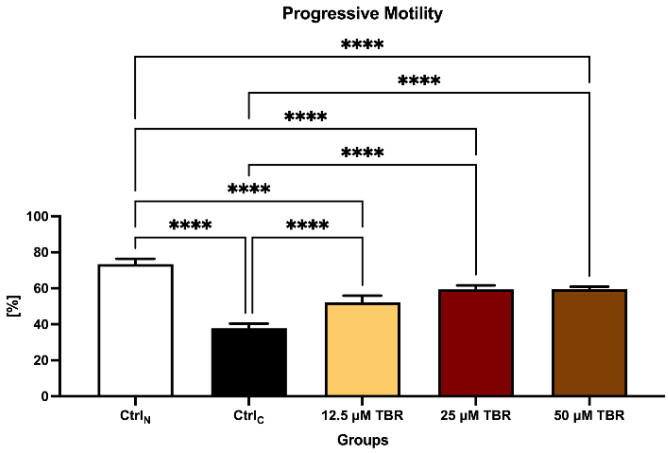

Figure 1 Progressive motility of bovine spermatozoa.
Legend: $Ctrl_N$ – native control; $Ctrl_C$ – cryopreserved control without TBR; 12.5, 25 and 50 µM TBR – cryopreserved experimental groups treated with TBR; ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05

Our results observing an improved sperm quality agree with **Bishist *et al.* (2020)** who compared untreated semen of Murrah buffalo with pentoxiphylline, theophylline, and TBR supplemented semen. Similarly, **Kumar *et al.* (2023)** also confirmed a beneficial potential of 1,3-dimethylxanhtine known as theophylline for the quality parameters of bull semen including increase of sperm motility, concentration, viability and membrane integrity compared to non-treated groups. Based on the previous findings, methylxanthines such as caffeine or theophylline exhibit stimulatory effects for post-thaw motility of cryopreserved goat ejaculate,

but its use is dose-dependent **(Sinha *et al.*, 1995)**. In contrast, it seems that methylxanthines treatment had no effect on the motility of pooled turkey semen following different storage regimes as **Parkhurst *et al.* (2000)** reported.

Oxidation damage

As indicated in Figure 2, sperm DNA was more susceptible to oxidative damage when exposed to cryogenic temperatures. A significant increase of DNA oxidation by the presence of 8-hydroxy-2′-deoxyguanosine (8-OHdG) was detected in the $Ctrl_C$ vs. $Ctrl_N$ ($p<0.001$) as well as TBR treated groups ($p<0.01$). However, a significant decrease ($p<0.01$) in 8-OHdG levels was observed in the groups supplemented with 25 and 50 µM of TBR compared to non-treated $Ctrl_C$ group. An experimental group supplemented with 12.5 µM of TBR stay without changes.

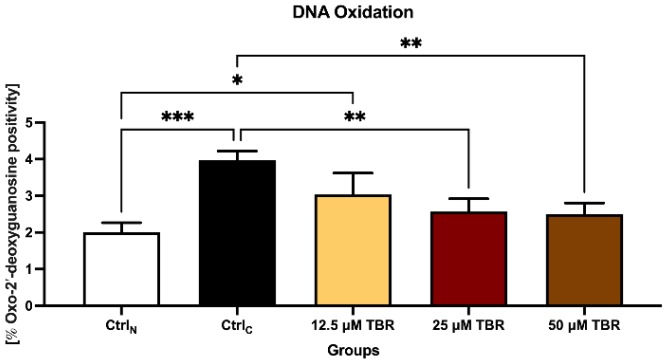

Figure 2 The level of DNA oxidation through to presence of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in bovine spermatozoa.
Legend: $Ctrl_N$ – native control; $Ctrl_C$ – cryopreserved control without TBR; 12.5, 25 and 50 µM TBR – cryopreserved experimental groups treated with TBR; ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05

In the case of protein oxidation (Figure 3), a significant increase ($p<0.0001$; $p<0.001$) in the concentration of protein carbonyls was observed in all cryopreserved groups with or without TBR compared with the native control ($Ctrl_N$), but the concentration of protein carbonyls decreased significantly in all groups supplemented with TBR against $Ctrl_C$ group (25 and 50 µM of TBR - $p < 0.001$; 12.5 µM of TBR – $p<0.01$).

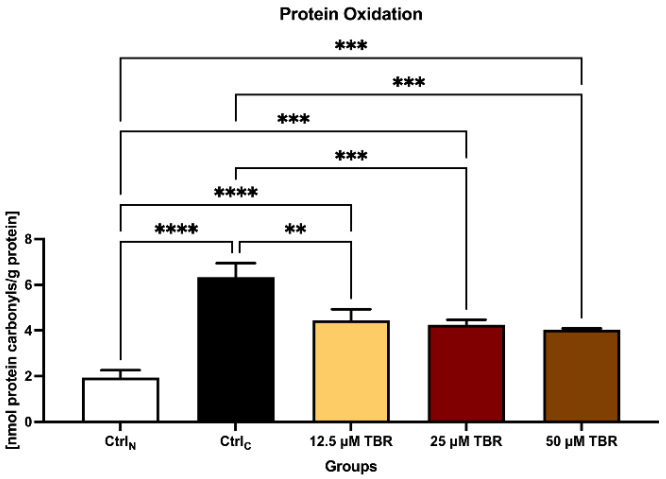

Figure 3 Protein oxidation of bovine spermatozoa.
Legend: $Ctrl_N$ – native control; $Ctrl_C$ – cryopreserved control without TBR; 12.5, 25 and 50 µM TBR – cryopreserved experimental groups treated with TBR; ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05

According to previous analysis, a similar trend was observed for the level of sperm lipid peroxidation (Figure 4). A significant increase of the lipid peroxidation represented by the presence of malondialdehyde (MDA) was detected in all cryopreserved groups against native $Ctrl_N$ but a continual significant decrease of MDA concentration in the TBR treated groups (50 µM of TBR – $p<0.0001$; 12.5 and 25 µM of TBR – $p<0.001$) was obvious after comparison with non-treated $Ctrl_C$ group.

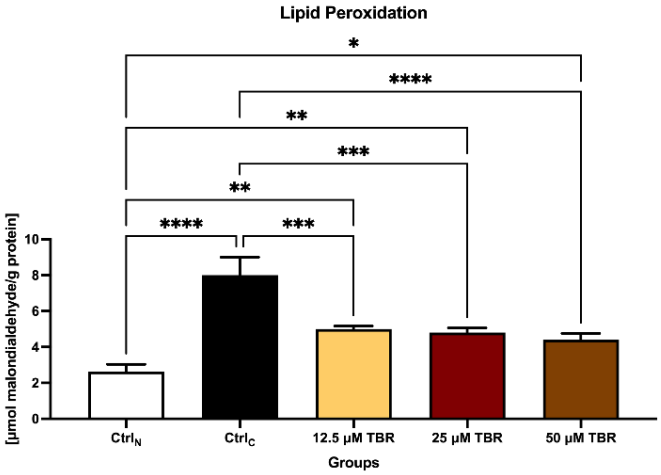

Figure 4 Lipid peroxidation of bovine spermatozoa.
Legend: $Ctrl_N$ – native control; $Ctrl_C$ – cryopreserved control without TBR; 12.5, 25 and 50 µM TBR – cryopreserved experimental groups treated with TBR; ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05

Researchers have been studying TBR differences and similarities with caffeine in recent years. However, studies on its effects on the qualitative properties of spermatozoa are just at the beginning. According to animal and *in vitro* studies, the nutraceutical potential benefits include cognitive enhancement, nerve cell protection, and anti-inflammatory properties. A number of recent studies have demonstrated TBR potential as an antitumoral, anti-inflammatory, and cardiovascular protector biomolecule without the unwanted side effects usually associated with caffeine. While TBR exhibits significant adenosine receptor-independent behaviour, including the reduction of oxidative stress in cells and the regulation of gene expression, it also inhibits the enzymatic activity of phosphodiesterases and blocks adenosine receptors as its main mechanisms of action. ATP can form non-covalent stacking complexes with TBR and caffeine, which are both methylxanthines **(Gattuso *et al.*, 2011)**. Based on the previous findings, after-thawing treatment with methylxanthines like caffeine and its stimulating potential depends on the right dose. **Alipour *et al.* (2018)** reported that lower doses of caffeine from 0.5 – 4 mM/L had no effect on membrane integrity or MDA content. On the other hand, caffeine supplementation promotes sperm viability as well as kinetic parameters of sperm motility such as velocity, linearity and beat cross frequency. In addition, methylxanthines may alter the cell metabolism. In millimolar concentrations, both TBR and caffeine have been shown to bind to DNA as well as to RNA. These findings, however, have yet to be fully understood in terms of their physiological implications. Following consumption of cocoa, methylxanthines may interact with DNA or RNA, resulting in induced or repressed gene expression **(Johnson *et al.*, 2012)**.

Protein expression

Heat shock proteins (HSP90/HSP70) as well as apoptosis-associated proteins (BAX/Bcl-2) were analyzed and semi-quantified using the Western blot technique; their expression patterns are displayed in Figure 5. Beta actin was used as an internal control for data normalization.

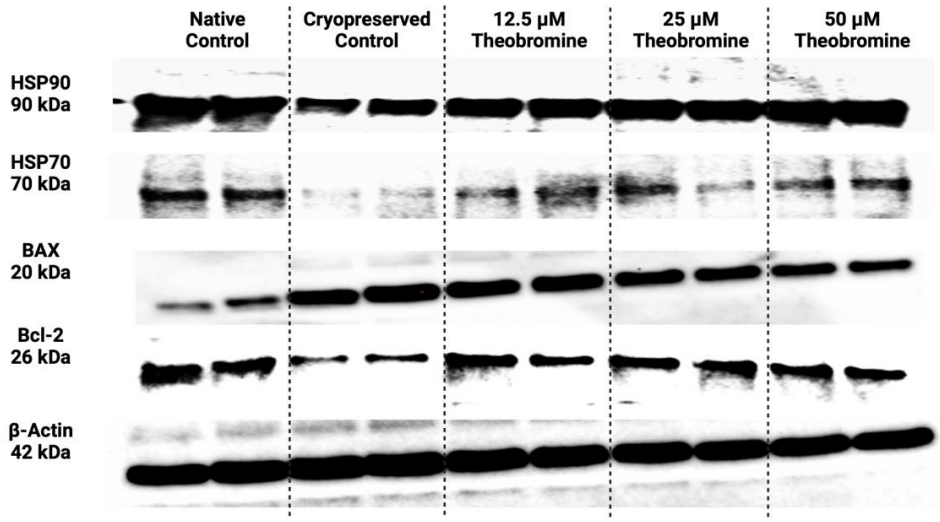

Figure 5 Heat shock (HSP90/HSP70) and apoptosis-associated (BAX/Bcl-2) proteins expression patterns in native control and cryopreserved bovine spermatozoa with or without theobromine treatment against β-actin as a control.

Our data from Western blotting revealed a significant decline ($p<0.001$) of HSP90 (Figure 6) amount in the cryopreserved control ($Ctrl_C$) against the native one ($Ctrl_N$) as well as TBR treated groups. In comparison with the non-treated cryopreserved control ($Ctrl_C$), a significant improvement in the HSP90 protein expression was recorded in all groups supplemeted with TBR (12.5 µM of TBR – $p<0.001$; 25 and 50 µM of TBR - $p<0.0001$).

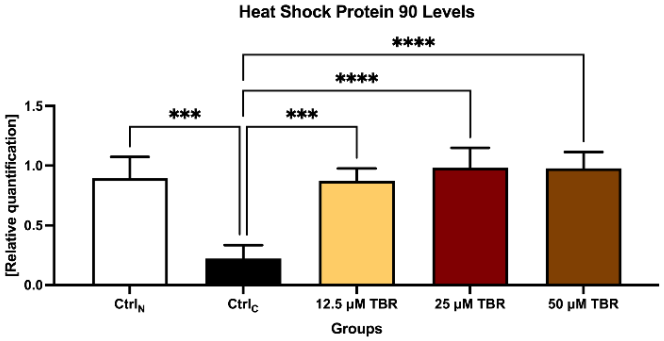

Figure 6 Heat shock protein 90 (HSP90) expression pattern in bovine spermatozoa.
Legend: $Ctrl_N$ – native control; $Ctrl_C$ – cryopreserved control without TBR; 12.5, 25 and 50 µM TBR – cryopreserved experimental groups treated with TBR; ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05

The amount of HSP70 protein (Figure 7) in the untreated cryopreserved control was significantly lower ($p<0.05$) after the exposure to low temperature when compared with the native control $Ctrl_N$. Among all experimental groups treated with TBR no significant differences were recorded in the comparison with untreated $Ctrl_C$.

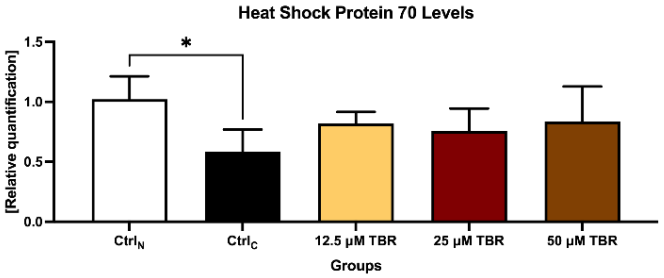

Figure 7 Heat shock protein 70 (HSP70) expression pattern in bovine spermatozoa
Legend: $Ctrl_N$ – native control; $Ctrl_C$ – cryopreserved control without TBR; 12.5, 25 and 50 µM TBR – cryopreserved experimental groups treated with TBR; ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05

As revealed in (Figure 8) , level of the pro-apoptotic BAX protein was significantly increased ($p<0.0001$) following the cryopreservation process ($Ctrl_C$) when compared to the native control ($Ctrl_N$). The supplementation of 25 and 50 µM of TBR significantly decreased ($p<0.05$; $p<0.001$) the level of BAX against the cryopreserved control.

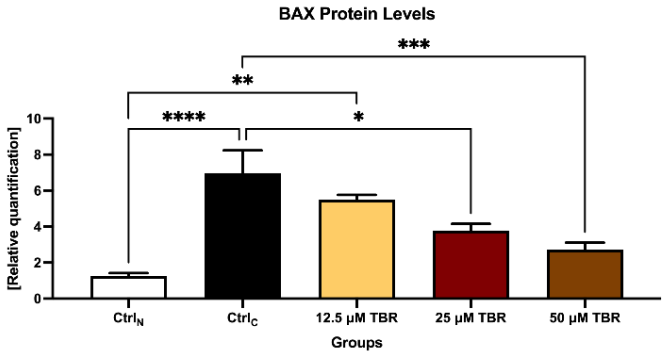

Figure 8 Pro-apoptotic BAX protein expression pattern in bovine spermatozoa
Legend: $Ctrl_N$ – native control; $Ctrl_C$ – cryopreserved control without TBR; 12.5, 25 and 50 µM TBR – cryopreserved experimental groups treated with TBR; ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05

On the contrary, the level of anti-apoptotic Bcl-2 protein (Figure 9) showed a significant decline ($p<0.05$) in all cryopreserved groups with or without TBR supplementation against native control ($p<0.0001$; $p<0.05$). However, there is a visible improvement in groups treated with different concentrations of TBR, but without significant changes compared to non-treated cryopreserved group ($Ctrl_C$).

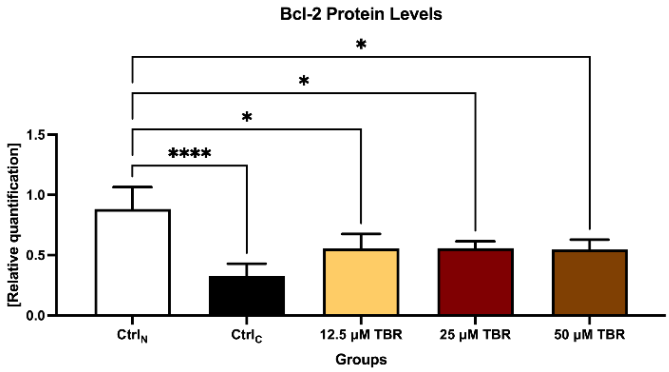

Figure 9 Anti-apoptotic Bcl-2 protein expression pattern in bovine spermatozoa
Legend: $Ctrl_N$ – native control; $Ctrl_C$ – cryopreserved control without TBR; 12.5, 25 and 50 µM TBR – cryopreserved experimental groups treated with TBR; ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05

Susceptibility of the sperm to apoptosis was determined by a ratio of proapoptotic (BAX) and anti-apoptotic (Bcl-2) proteins (Figure 10). A significant increase ($p<0.0001$) of the apoptosis-associated proteins ratio was detected in all cryopreserved groups with or without presence of TBR but there is a significant continuous decline ($p<0.0001$) of BAX/Bcl-2 ratio in TBR treated groups from 12.5 to 50 µM of TBR in comparison with cryopreserved control without TBR ($Ctrl_C$).

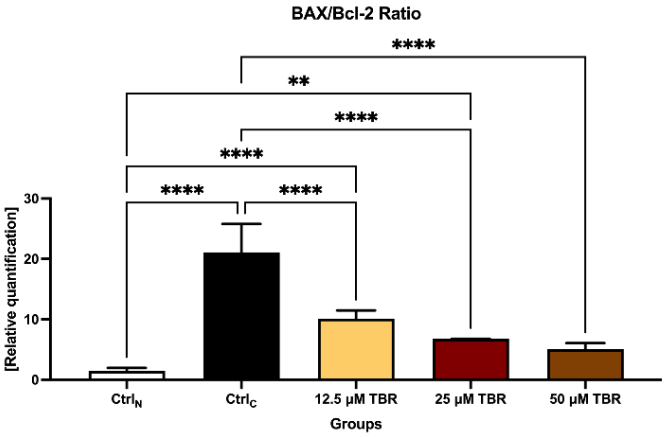

Figure 10 Apoptotic-associated proteins (BAX/Bcl-2 ratio) expression patterns
Legend: Ctrl$_N$ – native control; Ctrl$_C$ – cryopreserved control without TBR; 12.5, 25 and 50 µM TBR – cryopreserved experimental groups treated with TBR; ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05

Using a polyphenolic extract of cocoa, **Oleaga *et al*. (2012)** have shown that the expression of genes occurs differently in human breast cancer cells. Our study supports the theory, that gene expression of the key proteins in frozen-thawed spermatozoa of bulls positively reacts to the administration of TBR in the samples. TBR analog pentoxifylline reduces inflammation and stimulates macrophages that are anti-inflammatory during wound repair **(Sunil *et al.*, 2014)**. Following our data, we may hypothesize that TBR has a positive effect on the protection and soothing the detrimental effects of the sperm cryodamage. A study by **Macedo *et al*. (2023)** shows that male sheep reproduce better with a diet supplemented with 10% cocoa meal, and present with a greater percentage of Sertoli cells. In addition, cocoa meal supplementation improves total sperm count as well as daily sperm production. From our data we may indicate that the carefully chosen concentrations of TBR do not have a negative impact on the vulnerable structure of spermatozoa altered by the cryopreservation in the laboratory conditions. This study found that cryopreserved controls had significantly lower levels of HSP70 and HSP90 than native control groups, suggesting that bovine spermatozoa may reduce HSPs in their plasma membranes during the freeze–thaw process **(Cao *et al.*, 2003)**. TBR was proven to be an effective asset for stabilization of the balance amongst the pro-apoptotic and anti-apoptotic proteins particularly at concentrations of 25 and 50 µM TBR. It has been reported that neither HSP90 nor HSP70 has been detected in seminal plasma before cryopreservation, nor in cryopreservation medium after thawing **(Shan *et al.*, 2020; Zhang *et al.*, 2015a;b)**. In our previous study with kaempferol **(Baňas *et al.*, 2023)**, a significant reduction in the sperm motility in the frozen-thawed samples was directly connected with the downregulation of both HSPs. The number of dead semen cells in frozen-thawed samples was also increased, since dead cells cannot synthesize proteins any longer **(Peris-Frau *et al.*, 2020)**. Since it has been hypothesized that HSPs may also play a role in the protection of antioxidant enzymes, we may speculate that reduced HSP levels in cryopreserved samples may reduce the resistance of sperm proteins, lipids, and DNA to oxidative damage caused by ROS overgeneration during the freeze–thaw process. Our Western blot data confirmed previous findings that cryopreservation supports the activation of the apoptotic machinery **(Paasch *et al.*, 2004)**. Limited information is available with respect to the effects of natural biomolecules on the HSP expression patterns during sperm cryopreservation. We can speculate that lower levels of HSPs in cryopreserved samples may decrease sperm protein, lipid, and DNA's resistance to oxidative damage caused by ROS overproduction during the cryopreservation process due to the hypothesis that HSPs may also protect antioxidant enzymes. There is very limited information available about how natural biomolecules may affect HSP expression patterns along the freeze-thaw process. In assisted reproduction procedures, early apoptotic spermatozoa can be difficult to identify and/or remove because of the unclear mechanisms involved in sperm cryodamage, leading to a higher probability of failed fertilization and embryogenesis **(Karabulut *et al.*, 2018)**. The ratio of the pro-apoptotic BAX and the anti-apoptotic Bcl-2 protein plays a key role in determining the fate of cells. While Bcl-2 prevents the activity of pro-apoptotic proteins that form mitochondrial pores **(Llambi *et al.*, 2011)**, BAX activates apoptosis **(Dalal *et al.*, 2016)**. Our results agree with our previous study with epicatechin **(Baňas *et al.*, 2022)** which disclosed a significant enhancement of the BAX/Bcl-2 ratio which was in a direct association with a higher vitality of frozen-thawed spermatozoa. Cryopreserved spermatozoa have been evaluated in a small number of studies for their ability to inhibit or mitigate the overactivation of apoptotic machinery. TBR may serve as a lead compound for the development of new pharmaceuticals in the treatment of some human diseases and may provide a safe and natural alternative to some medications we currently use. This opens a new challenge for the researchers to explore the properties of TBR more deeply.

CONCLUSION

We may conclude that supplementation with higher concentrations of TBR, especially with 25 and 50 µM leads to an improved post-thaw vitality of the cryopreserved bovine spermatozoa. In summary, TBR exhibits antioxidant properties that can protect proteins, lipids, and DNA from excessive damage, essential for the structure and function of sperm cells. TBR is able stabilize the ratio between pro- and anti-apoptotic proteins in the cell by preventing the loss of proteins protecting sperm from cryoshock. Our focus on the qualitative parameters of the spermatozoa, positively or negatively altered by TBR, opens a new view on the impact of natural antioxidants and their use in the cryopreservation of the breeding bulls.

Acknowledgments: This research was supported by the Slovak Research and Development Agency grant no. APVV-21-0095 and by the project VEGA 1/0067/24.

REFERENCES

Alipour, P., Daghigh Kia, H. & Moghaddam, G. (2018). Evaluating caffeine antioxidant properties on Ghezel ram sperm quality after freeze-thawing. *Revue de Medicine Veterineire*, *169*(10-12), 233-240.

Alvarez, J.G. & Storey B.T. (1989). Role of glutathione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. *Gamete Research*, *23*(1), 77-90. https://doi.org/10.1002/mrd.1120230108

Alvarez, J. G., Touchstone J.C., Blasco L. & Storey B.T. (1987). Spontaneous Lipid Peroxidation and Production of Hydrogen Peroxide and Superoxide in Human Spermatozoa Superoxide Dismutase as Major Enzyme Protectant Against Oxygen Toxicity. *Journal of Andrology, 8*(5), 338-348. https://doi.org/10.1002/j.1939-4640.1987.tb00973.x

Baňas, Š., Benko, F., Ďuračka, M., Lukáč, N. & Tvrdá, E. (2023a). Kaempferol Enhances Sperm Post-Thaw Survival by Its Cryoprotective and Antioxidant Behaviour. *Stresses, 3*(4), 687-700. https://doi.org/10.3390/stresses3040047

Baňas, Š., Benko, F., Ďuračka, M., Lukáč, N. & Tvrdá, E. (2023b). Epicatechin Prevents Cryocapacitation of Bovine Spermatozoa through Antioxidant Activity and Stabilization of Transmembrane Ion Channels. *International Journal of Molecular Sciences*, *24*(3), 2510. https://doi.org/10.3390/ijms24032510

Baňas, Š., Ďuračka M., Benko F., Žiarovská J., Lukáč N. & Tvrdá E. (2022). Epicatechin improves frozen sperm vitality by its antioxidant and cryoprotective actions. *Journal of Microbiology, Biotechnology and Food Sciences*, *12*, e9490. https://doi.org/10.55251/jmbfs.9490

Barbas, J.P. & Mascarenhas, R.D. (2009). Cryopreservation of domestic animal sperm cells. *Cell and Tissue Bank*ing, *10*(1), 49-62. https://doi.org/10.1007/s10561-008-9081-4

Baust, J.G., Gao D. & Baust J.M. (2009). Cryopreservation: An emerging paradigm change. *Organogenesis*, *5*(3), 90-96. https://doi.org/10.4161/org.5.3.10021

Benko, F., Geschwandtnerová, A., Žiarovská, J., Lukáč, N. & Tvrdá, E. (2021). Expression of HSP90 Gene in the Cryopreserved Bovine Spermatozoa. *Scientific Papers Animal Science Biotechnologies*, *54*(1), 73–77.

Bishist, R., Raina, V.S., Bhakat, M., Mohanty, T.K., Slone, S.A. & Sinha, R. (2020). Effect of Antioxidant Additives on Freezability of buffalo Spermatozoa. *Buffalo Bulletin*, *39*(3), 337-344. https://www.cabidigitallibrary.org/doi/pdf/10.5555/20203451773

Cao, W.L., Wang, Y.X., Xiang, Z.Q. & Li, Z. (2003). Cryopreservation-induced decrease in heat-shock protein 90 in human spermatozoa and its mechanism. *Asian Journal of Andrology*, *5*(1), 43–46.

Cormier, N., Sirard M.A. & Bailey J.L. (1997). Premature capacitation of bovine spermatozoa is initiated by cryopreservation. *Journal of Andrology*, *18*(4), 461-468. https://www.ncbi.nlm.nih.gov/pubmed/9283961

Dalal, J., Kumar, A., Honparkhe, M. & Deka, D. (2016). Singh, N. Minimization of apoptosis-like changes in cryopreserved buffalo bull sperm by supplementing extender with Bcl-2 protein. *Veterinary World*, *9*(5), 432–436. https://doi.org/10.14202/vetworld.2016.432-436

Dowling, D.K. & Simmons, L.W. (2008). Reactive oxygen species as universal constraints in life-history evolution. *Proceedings of the Royal Society B: Biological Sciences*, *276*(1663), 1737-1745. https://doi.org/10.1098/rspb.2008.1791

Gadea, J., Gumbao D., Gomez-Gimenez B. & Gardon J.C. (2013). Supplementation of the thawing medium with reduced glutathione improves function of frozen thawed goat spermatozoa. *Reproductive Biology*, *13*(1), 24-33. https://doi.org/10.1016/j.repbio.2013.01.174

Gattuso G., Manfredi G. & Sammartano S. (2011). Quantitative study on the non-covalent interactions between ATP and caffeine, theophylline and theobromine in aqueous solution. *Fluid Phase Equilibria*, *308*(1-2), 47–54. https://doi.org/10.1016/j.fluid.2011.06.006

Johnson I. M., Prakash H., Prathiba J., Raghunathan R. & Malathi R. (2012). Spectral analysis of naturally occurring methylxanthines (theophylline,

theobromine, and caffeine) binding with DNA. *PLoS ONE*, *7*, e50019. https://doi.org/10.1371/journal.pone.0050019
Karabulut, S., Demiroğlu-Zergeroğlu, A., Yılmaz, E., Kutlu, P. & Keskin, İ. (2018). Effects of human sperm cryopreservation on apoptotic markers in normozoospermic and non-normozoospermic patients. *Zygote*, *26*(4), 308–313. https://doi.org/10.1017/s0967199418000254
Kumar, A., Virmani, M., Kumar, R., Kumar, S., Sarthak, Kanwar, A., Kumar, S. & Patil, C.S. (2023). Inclusion of 1,3-dimethylxanthine improved quality parameters of bull semen. *The Animal Biology,* *25*(4), 11-16. https://doi.org/10.15407/animbiol25.04.011
Kunová, S., Felsociová S., Tvrdá E., Ivanišová E., Kántor A., Žiarovská J., Terentjeva M. & Kačániová M. (2019). Antimicrobial activity of resveratrol and grape pomace extract. *Potravinarstvo*, *13*(1), 363-368. https://doi.org/10.5219/1054
Llambi, F., Moldoveanu, T., Tait, S.W., Bouchier-Hayes, L., Temirov, J., McCormick, L.L., Dillon, C.P. & Green, D.R. (2011). A unified model of mammalian BCL-2 protein family interactions at the mitochondria. *Molecular Cell*, *44*(4), 517–531. https://doi.org/10.1016/j.molcel.2011.10.001
Lone, S.A., Prasad J.K., Ghosh S.K., Das G.K., Balamurugan B. & Verma M.R. (2018). Study on correlation of sperm quality parameters with antioxidant and oxidant status of buffalo bull semen during various stages of cryopreservation. *Andrologia*, *50*, e12970. https://doi.org/10.1111/and.12970
Macedo, D.S., Rocha L.F., Pinheiro E.E.G., Santana A.L., Souza R.S., Bagaldo A.R., Bezerra P.P.A. & Barbosa L.P. (2023). Sheep spermatogenesis is affected by the nutritional levels of cocoa meal, a byproduct of cocoa butter manufacturing rich in theobromine and caffeine. *Revista Brasileira de Zootecnia*, *52*, e20220011. https://doi.org/10.37496/rbz5220220011
Martin, G., Cagnon, N., Sabido, O., Sion, B., Grizard, G., Durand, P. & Levy, R. (2007). Kinetics of occurrence of some features of apoptosis during the cryopreservation process of bovine spermatozoa. *Human Reproduction*, *22*(2), 380–388. https://doi.org/10.1093/humrep/del399
Mazurova, J., Kukla, R., Rozkot, M., Lustykova, A., Slehova, E., Sleha, R., Lipensky, J. & Opletal, L. (2015). Use of natural substances for boar semen decontamination. *Veterinární Medicína*, *60*(5), 235-247. https://doi.org/10.17221/8175-vetmed
Oleaga C., Garcia M., Sole A., Ciudad C.J. Izquierdo-Pulido M. & Noe V. (2012). CYP1A1 is overexpressed upon incubation of breast cancer cells with a polyphenolic cocoa extract. *European Journal of Nutrition*, *51*, 465–476. https://doi.org/10.1007/s00394-011-0231-2
Paasch, U., Sharma, R.K., Gupta, A.K., Grunewald, S., Mascha, E.J., Thomas, A.J., Jr., Glander, H.J. & Agarwal, A. (2004). Cryopreservation and thawing is associated with varying extent of activation of apoptotic machinery in subsets of ejaculated human spermatozoa. *Biology of Reproduction*, *71*(6), 1828–1837. https://doi.org/10.1095/biolreprod.103.025627
Parmar, K.H., Singh, V., Savsani, H.H., Kavani, F.S., Rajoriya, J.S. & Lone, S.A. (2021). Seasonal variation in heat shock proteins (hsp70 and hsp90) and their association with frozen semen quality and fertility in buffaloes. *Cryoletters*, *42*(5), 261–266. https://doi.org/10.1111/j.1365-3040.2006.01596.x
Parkhurst, A.M., Korn, N. & Thurston, R.J. (2000). The effects of methylxanthines on the mobility of stored turkey sperm. *Poultry Science*, *79*(12), 1803-1809. https://doi.org/10.1093/ps/79.12.1803
Parttyka, A., Lukaszewicz E. & Nizanski W. (2012). Effect of cryopreservation on sperm parameters, lipid peroxidation and antioxidant enzymes activity in fowl semen. *Theriogenology*, *77*(8), 1497-1504. https://doi.org/10.1016/j.theriogenology.2011.11.006
Peris-Frau, P., Soler, A.J., Iniesta-Cuerda, M., Martín-Maestro, A., Sánchez-Ajofrín, I., Medina-Chávez, D.A., Fernández-Santos, M.R., García-Álvarez, O., Maroto-Morales, A. & Montoro, V. (2020). Sperm Cryodamage in Ruminants: Understanding the Molecular Changes Induced by the Cryopreservation Process to Optimize Sperm Quality. *International Journal of Molecular Sciences*, *21*(8), 2781. https://doi.org/10.3390/ijms21082781
Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., … & Erlich, H. A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, *239*(4839), 487-491.http://dx.doi.org/10.1088/1367-2630/1/1/006
Sarlos, P., Molnar A. & Kokai M. (2002). Comparative evaluation of the effect of antioxidants in the conservation of ram semen. *Acta Veterinaria Hungarica*, *50*(2), 235-245. https://doi.org/10.1556/AVet.50.2002.2.13
Shan, Q., Ma, F., Wei, J., Li, H., Ma, H. & Sun, P. (2020). Physiological Functions of Heat Shock Proteins. *Current protein & peptide science*, *21*(8), 751–760. https://doi.org/10.2174/13892037mtaywmjenz
Silva, S.V., Soares A.T., Barista A.M., Almeida F.C., Nunes J.F., Peixoto C.A. & Guerra M.M.P. (2011). In vitro and in vivo evaluation of ram sperm frozen in tris egg-yolk and supplemented with superoxide dismutase and reduced glutathione. *Reproduction in Domestic Animals*, *46*(5), 874-881. https://doi.org/10.1111/j.1439-0531.2011.01758.x
Sinha, M.P., Sinha, A.K. & Singh, B.K. Effect of methylxanthines on motility and fertility of frozen-thawed goat semen. (1995). *Theriogenology*, *44*(6), 907-914. https://doi.org/10.1016/0093-691X(95)00274-C
Sunil, V.R., Vayas K.N., Cervelli J.A., Malaviya R., Hall L., Massa C.B., Gow A.J., Laskin J.D. & Laskin D. L. (2014). Pentoxifylline attenuates nitrogen mustard-induced acute lung injury, oxidative stress and inflammation. *Experimental and Molelular Pathology*, *97*(1), 89–98. https://doi.org/10.1016/j.yexmp.2014.05.009
Tvrdá, E., Straka P., Galbavý D. & Ivanič P. (2019). Epicatechin Provides Antioxidant Protection to Bovine Spermatozoa Subjected to Induced Oxidative Stress. *Molecules*, *24*(18), 3226. https://doi.org/10.3390/molecules24183226
Tvrdá, E., Tušimová E., Kováčik A., Paál D., Greifová H., Abdramanov A. & Lukáč N. (2016). Curcumin has protective and antioxidant properties on bull spermatozoa subjected to induced oxidative stress. *Animal Reproduction Science*, *172*, 10-20. https://doi.org/10.1016/j.anireprosci.2016.06.008
Ugur, M.R., Abdelrahman A.S., Evans H.C., Gilmore A.A., Hitit M., Arifiantini R.I., Purwantara B., Kaya A. & Memili E. (2019). Advances in Cryopreservation of Bull Sperm. *Frontiers in Veterinary Science*, *6*, 268. https://doi.org/10.3389/fvets.2019.00268
Vorilhon, S., Brugnon, F., Kocer, A., Dollet, S., Bourgne, C., Berger, M., Janny, L., Pereira, B., Aitken, R.J. & Moazamian, A. (2018). Accuracy of human sperm DNA oxidation quantification and threshold determination using an 8-OHdG immuno-detection assay. *Human Reproduction*, *33*(4), 553–562. https://doi.org/10.1093/humrep/dey038
Wu, H., de Gannes, M.K., Luchetti, G. & Pilsner, J.R. (2015). Rapid method for the isolation of mammalian sperm DNA. *BioTechniques*, *58*(6), 293–300. https://doi.org/10.2144/000114280
Zhang, X.G., Hong, J.Y., Yan, G.J., Wang, Y.F., Li, Q.W. & Hu, J.H. (2015a). Association of heat shock protein 70 with motility of frozen-thawed sperm in bulls. *Czech Journal of Animal Science*, *60*(6), 256–262. https://doi.org/10.17221/8239-cjas
Zhang, X.G., Hu, S., Han, C., Zhu, Q.C., Yan, G.J. & Hu, J.H. (2015b). Association of heat shock protein 90 with motility of post-thawed sperm in bulls. *Cryobiology*, *70*(2), 164–169. https://doi.org/10.1016/j.cryobiol.2014.12.010