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Curcumin Offers Antioxidant Protection to Cryopreserved Bovine Semen

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ABSTRACT

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Evidence shows that oxidative stress associated with sperm cryopreservation may lead to a significant decrease of the structural integrity and functional activity of male gametes. Curcumin (CUR) has become a substance of scientific interest for its free radical-quenching abilities, which could enhance the post-thaw quality of male gametes. This study assessed the effects of CUR on the post-thaw vitality and selected oxidative stress markers of bovine spermatozoa. Thirty ejaculates collected from 10 breeding bulls were divided into two aliquots and cryopreserved in the absence (control) or presence of CUR (50 μmol/l). Immediately before use, the control or experimental straws were thawed at 37°C for 20 s. CUR administration led to a significantly higher preservation of spermatozoa motion ($P < 0.001$) as well as membrane ($P < 0.05$) and acrosomal ($P < 0.01$) integrity in comparison with the control. Moreover, spermatozoa exposed to CUR exhibited a significantly higher mitochondrial activity ($P < 0.001$). Significantly decreased amounts of reactive oxygen species ($P < 0.01$) and superoxide ($P < 0.001$) were detected following CUR supplementation. Finally, a significant decrease of oxidative damage to proteins ($P < 0.01$), lipids ($P < 0.001$), and DNA ($P < 0.05$) was recorded in samples to which CUR was administered in comparison to the control. In this study, CUR proved to act as an efficient antioxidant molecule offering protection to male gametes against oxidative damage during cryopreservation.

Keywords: bulls; *Curcuma longa*; cryopreservation; oxidative stress; spermatozoa

Over the past decades, artificial insemination (AI) has significantly contributed to the advancement of controlled reproduction in cattle. Short- or long-term semen preservation as a crucial pillar of animal AI comes hand in hand with numerous advantages such as genetic improvement, protection of genetic resources, decreased incidence of sexually transmitted diseases, and enhanced transportation of genetic material across countries (Bailey et al. 2003).

Lamentably, the development of more efficient *ex vivo* semen preservation protocols is slow and complicated, as a significant proportion of male gametes loses their structural integrity and/or functional activity during storage at low temperatures, causing the semen sample useless for AI (Bailey et al. 2003; Tatone et al. 2010).

The unique structural architecture and physiological roles render spermatozoa to be particularly sensible to temperature fluctuations. Such

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vulnerability towards freezing and thawing may lead to cellular damage associated with cold shock and oxidative stress (OS). High concentrations of polyunsaturated fatty acids (PUFAs) present in the sperm membrane are the primary target for lipid peroxidation (LPO), while increasing concentrations of reactive oxygen species (ROS) may lead to further sperm cell deterioration during semen storage at low temperatures (Aitken 1995). Male reproductive fluids contain an array of antioxidant molecules which should normally provide protection against the detrimental effects of ROS (Bansal and Bilaspuri 2011). Nevertheless, this system is prone to fail to protect spermatozoa against oxidative insults following cryopreservation (Bucak et al. 2010, 2012).

Extensive research has led to the creation of numerous methods to improve the process of sperm cryopreservation, among which antioxidants could become a potential remedy to counteract alterations to spermatozoa exposed to low temperatures. Currently, an important goal is to define an antioxidant or a combination of different ones exhibiting high ROS scavenging activity, metal chelation properties, and the ability to regulate the activities of inherent antioxidant molecules that could contribute to the alleviation of the undesirable effects of sperm chilling or freezing, hence to prolong the sperm activity and vitality (Bansal and Bilaspuri 2011).

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6- heptadiene-3,5-dione] (CUR) is a major phytochemical commonly found in turmeric (*Curcuma longa*) – a tropical rhizome of the *Zingiberaceae* family. CUR is the biomolecule responsible for the vibrant yellow colour of *Curcuma longa* and is currently recognized to be in charge for the majority of its remedial effects (Aggarwal et al. 2007).

Although CUR has been defined as an effective ROS scavenger as well as a powerful LPO inhibitor (Bucak et al. 2012; Tvrda et al. 2016), inconsistent evidence is available with respect to its exact roles in male fertility. Several *in vivo* (Salahshoor et al. 2012) and *in vitro* studies (Bucak et al. 2012; Soleimanzadeh and Saberivand 2013; Tvrda et al. 2016) emphasize the energy-promoting and protective properties of CUR on male reproductive structures. On the other hand, different reports suggest negative roles of CUR in cellular signaling linked to spermatogenic processes (Naz 2011; Xia et al. 2012), stressing out a more complex and cautious approach towards CUR, due to its possible toxic or contraceptive properties.

Specific questions related to the potential protective and antioxidant activity of CUR supplemented to cryopreservation media on the post-thaw sperm viability still need to be answered. As such, this study was designed to assess the impact of curcumin on the motion characteristics, structural, metabolic, and oxidative profile of frozen-thawed bovine spermatozoa.

MATERIAL AND METHODS

Sample collection. Ejaculates for this study were collected from ten adult Simmental-Fleckvieh breeding bulls (Slovak Biological Services, Nitra, Slovakia). The animals were of comparable age and kept under homogeneous feeding and housing conditions. Three semen samples were acquired regularly from each animal using a sterilized artificial vagina. Following collection, sperm concentration and motility were assessed manually with the help of a phase-contrast microscope BX43 (Olympus Optical Co., Ltd., Japan)(200×). A minimum 85% motility and 1×10^9 spermatozoa/ml were the established cut-off values for the sample to be used for the experiments. All collected ejaculates complied with the above-mentioned criteria.

Semen extending, freezing and thawing. All ejaculates were split into two identical aliquots and extended to 11×10^6 spermatozoa/ml with Triladyl (Minitüb GmbH, Germany), comprising 20% (w/v) egg yolk, Tris, citric acid, sucrose, buffering agents, glycerol, antibiotics, and distilled water. For the experimental group, 50 µmol/l CUR pre-dissolved in dimethyl sulfoxide (DMSO) (both Sigma-Aldrich, USA) were added to the extender, while the control group contained an equivalent portion of DMSO. The final DMSO concentration in both groups was set to 0.5%. The selected CUR dose was decided upon our previous standardization reports (Tvrda et al. 2015, 2016). The samples were transferred into 0.25 ml French straws, cooled at 4°C for 2 h, and subsequently frozen according to the following scheme: –3°C/min from +4 to –10°C; –40°C/min from –10 to –100°C; –20°C/min from –100 to –140°C using a digital freezer (Digitcool 5300 ZB 250; IMV, France). Lastly, the straws were transferred into liquid nitrogen (–196°C) and stored for 30 days.

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Prior to the experiments, the straws were allowed to thaw at 37°C in a water bath for 20 s. For the assessment of the mitochondrial function and OS markers, thawed spermatozoa were centrifuged, washed, and resuspended in phosphate-buffered saline (Dulbecco's PBS; Sigma-Aldrich).

Spermatozoa motion assessment. Sperm motility parameters were evaluated with the computer-assisted sperm analysis (CASA) system (Version 14.0 TOX IVOS II.; Hamilton-Thorne Biosciences, USA). Fluorescent illumination and the IDENT stain (Hamilton-Thorne Biosciences) were used for the sample processing. Ten µl of sample were loaded into the Makler counting chamber (depth 10 µm, 37°C; Sefi Medical Instruments, Israel) and at least 300 cells were evaluated for primary and secondary motility characteristics, including motility (MOT, %), progressive motility (PROG, %), straight linear velocity (VSL, µm/s), average path velocity (VAP, µm/s), curvilinear velocity (VCL, µm/s), path straightness (STR, %), amplitude of lateral head displacement (ALH, µm), beat cross frequency (BCF, Hz), and linearity (LIN, %).

Membrane integrity. Eosin-nigrosin staining protocol was used to examine the integrity of the spermatozoa membranes (Moskovtsev and Librach 2013). Ten µl of each sample were mixed first with 20 µl 5% eosin and then with 20 µl 10% nigrosin (both Sigma-Aldrich). The mixture was smeared on a pre-warmed glass slide and air dried at 37°C. All slides were blindly evaluated by one observer using bright filed microscopy (1000×) and oil immersion. At least 200 cells per sample were evaluated as either live (spermatozoa with white heads) or dead (spermatozoa with red heads) and expressed in percent values.

Acrosome integrity. Acrosome integrity was evaluated using the fast green-rose bengal staining protocol developed by Pope et al. (1991). The staining solution consisted of 1% fast green (Sigma-Aldrich), 1% rose bengal (Sigma-Aldrich), and 40% ethyl alcohol (Centralchem, Slovakia) which were dissolved in 0.1 M citric acid–0.2 M disodium phosphate buffer (Sigma-Aldrich). Twenty µl of each sample were mixed with 20 µl of the fast green-rose bengal stain and incubated at laboratory temperature for 70 s. The mixture was smeared on a pre-warmed glass slide and air dried at 37°C. All slides were blindly evaluated by one observer using bright filed microscopy (1000×) and oil immersion. At least 200 cells per slide were assessed for the presence or absence of acrosome, which was expressed in percent values.

***Mitochondrial activity* (*MTT test*)**. Sperm mitochondrial activity was assessed with the metabolic activity (MTT) test. This assay reflects on the transformation of a yellow tetrazolium molecule (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) to purple formazan by succinate dehydrogenase of functional mitochondria. The MTT tetrazolium (Sigma-Aldrich) was administered to the sperm suspension and incubated for 2 h (shaker, 37°C, 95% air atmosphere, 5% CO_2). Following incubation, isopropanol (Centralchem) was added to the suspension to stop the reaction. Optical density was assessed at a wavelength of 570 nm against 620 nm as a reference with the Multiskan FC microplate photometer (Thermo Fisher Scientific Inc., USA). The data were acquired from five independent experiments and the results are presented in percentage of the control set to 100 % (Tvrda et al. 2016).

***Assessment of superoxide production* (*NBT test*)**. The nitroblue-tetrazolium (NBT) assay was applied to evaluate the intracellular generation of the superoxide radical. The NBT test is based on the production of purple NBT formazan crystals, which are created as a result of the reduction of yellow nitroblue tetrazolium chloride (2,2'-bis(4-nitrophenyl)-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'diphenylene) to ditetrazolium chloride; Sigma-Aldrich) by superoxide. The NBT tetrazolium was dissolved in Dulbecco's PBS including 1.5 % DMSO and administered to the cell suspension. Following incubation (shaker, 37°C, 95% air atmosphere, 5% CO_2) for 1 h, spermatozoa were washed twice with PBS and centrifuged (300 *g*) for 10 min. Finally, the suspension was dissolved in 2 M potassium hydroxide (KOH; Centralchem) in DMSO. Optical density was assessed at a wavelength of 620 nm against 570 nm as a reference with the Multiskan FC microplate photometer (Thermo Fisher Scientific Inc.). The data were acquired from five independent experiments and the results are exhibited in percentage of the control set to 100% (Tvrda et al. 2015).

Reactive oxygen species quantification. ROS production in the control and experimental samples was evaluated using the chemiluminescence assay with luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione; Sigma-Aldrich) as the probe (Kashou et al. 2013). Tested samples comprised luminol (10 µl,

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5 mM) and 400 µl of the sperm suspension. The negative control consisted of 400 µl PBS and 10 µl luminol, while the positive control was composed of 400 µl of PBS, 50 µl of hydrogen peroxide (30%, 8.8 M; Sigma-Aldrich), and 10 µl luminol. Both controls were prepared in triplicates. The luminescent signal was recorded on a 48-well plate for 15 min with the help of the Glomax Multi$^+$ Combined Spectro-Fluoro Luminometer (Promega Corp., USA). The results are expressed as relative light units (RLU)/s/10^6 sperm.

Evaluation of oxidative DNA damage. DNA isolation from spermatozoa was performed using the GenElute^{TM} Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich) and the obtained DNA was quantified using the Genesys 10 spectrophotometer (Thermo Fisher Scientific Inc.) at a dual wavelength of 260/280 nm. DNA damage was assessed using the colourimetric EpiQuik^{TM} 8-OHdG DNA Damage Quantification Direct Kit (Epigentek Group Inc., USA), enabling a direct quantification of 8-hydroxy-2'-deoxyguanosine (8-OHdG) using a strip-well microplate. The amount of 8-OHdG was quantified at 450 nm using an ELISA reader (Multiskan FC microplate photometer; Thermo Fisher Scientific Inc.). The results are expressed as % 8-OHdG.

Oxidative damage to proteins and lipids. Frozen-thawed sperm samples were centrifuged (800 *g*, 25°C, 10 min) and washed with PBS twice. The cell fraction was lysed using sonication (28 kHz, 30 s, ice) and RIPA buffer (Sigma-Aldrich) with protease inhibitor cocktail (Sigma-Aldrich). Each sample was subsequently centrifuged (11 828 *g*, 4°C, 15 min) to purify the lysates from the residual cell fragments. The resulting cell lysates were stored at –80°C for further assessment.

The extent of LPO proportional to the malondialdehyde (MDA) concentration was evaluated using the thiobarbituric acid reactive substances (TBARS) assay, modified for a 96-well plate. The end product resulting from the reaction of MDA and thiobarbituric acid (Sigma-Aldrich) was measured at a wavelength 530–540 nm using an ELISA reader (Multiskan FC microplate photometer) (Tvrda et al. 2016). The MDA concentration is expressed in µmol/g protein.

The presence of carbonyl groups was quantified using the 2,4-dinitrophenylhydrazine (DNPH) method. One ml of the sample previously pretreated with trichloroacetic acid (TCA; 20% w/v; Sigma-Aldrich) was mixed with 1 ml DNPH (10 mM in 2 N HCl; Sigma-Aldrich) and incubated at 37°C for 1 h. Following a second administration of 1 ml trichloroacetic acid, the suspension was incubated at 4°C for 10 min before centrifugation at 11 828 *g* for 15 min. The resulting pellet was washed three times with 1 ml of ethanol/ethyl acetate (1/1; v/v) and subsequently resuspended in 1 ml 6 M guanidine HCl (Sigma-Aldrich). The absorbance was measured at 360 nm, using 6 M guanidine HCl as a blank. The molar absorption coefficient of 22 000/M/cm was applied to calculate the amount of protein carbonyls in each sample. Protein carbonyls are expressed in nmol/mg protein (Weber et al. 2015).

In order to normalize all obtained data, the total amount of proteins was quantified with the DiaSys Total Protein (DiaSys, Germany) commercially available kit and the Randox RX Monza clinical chemistry analyzer (Randox Laboratories, UK). The assay follows the Biuret method, where copper sulfate interacts with proteins to create a violet-blue colour complex in an alkaline environment. The resulting colour intensity measured at 540 nm is proportional to the final amount of proteins.

Statistical analysis. All data are expressed as mean ± SEM. GraphPad Prism program (Version 5.0 for MS Windows; GraphPad Software, www.graphpad.com) was used for the statistical analysis. Descriptive statistics was performed at first. Paired *t*-test was applied for specific statistical analysis, following the presumption that values in each row stand for paired observations. The level of significance was set at $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$.

RESULTS

The effects of CUR on the motion parameters and structural integrity of frozen-thawed bovine spermatozoa are presented in Table 1. CUR administration to the semen extender led to a significantly higher preservation of spermatozoa motility ($P < 0.001$) and progressive motility ($P < 0.001$) in comparison with the control. Significantly higher values were additionally observed in the experimental group for all selected secondary sperm motility parameters ($P < 0.001$ in relation to VAP, VCL, VSL, BCL, STR, and LIN; $P < 0.01$ in the case of ALH) (Table 1). Moreover, CUR supplementation to the cryopreservation medium led to a signifi-

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Table 1. Effect of curcumin supplementation on the motility characteristics, membrane and acrosome integrity of frozen-thawed bovine spermatozoa

Parameter	Control (n = 30)	Curcumin (n = 30)
MOT (%)	49.51 ± 0.53	63.62 ± 0.85***
PROG (%)	31.79 ± 0.54	44.23 ± 0.71***
VAP (μm/s)	70.87 ± 0.51	80.08 ± 0.48***
VSL (μm/s)	60.41 ± 0.51	69.67 ± 0.45***
VCL (μm/s)	102.10 ± 0.66	110.90 ± 0.52***
ALH (μm)	3.99 ± 0.13	4.54 ± 0.16**
BCF (Hz)	19.24 ± 0.50	24.61 ± 0.85***
STR (%)	64.46 ± 1.07	70.26 ± 0.51***
LIN (%)	56.74 ± 0.41	62.31 ± 0.60***
Membrane integrity (%)	72.85 ± 0.68	81.60 ± 0.30*
Acrosomal integrity (%)	70.80 ± 0.47	82.70 ± 0.52**

MOT = motility, PROG = progressive motility, VAP = average path velocity, VSL = straight linear velocity, VCL = curvilinear velocity, ALH = amplitude of lateral head displacement, BCF = beat cross frequency, STR = path straightness, LIN = linearity
*P < 0.05, **P < 0.01, ***P < 0.001

cantly higher membrane integrity (P < 0.05) and acrosome stability (P < 0.01) in comparison with the control (Table 1).

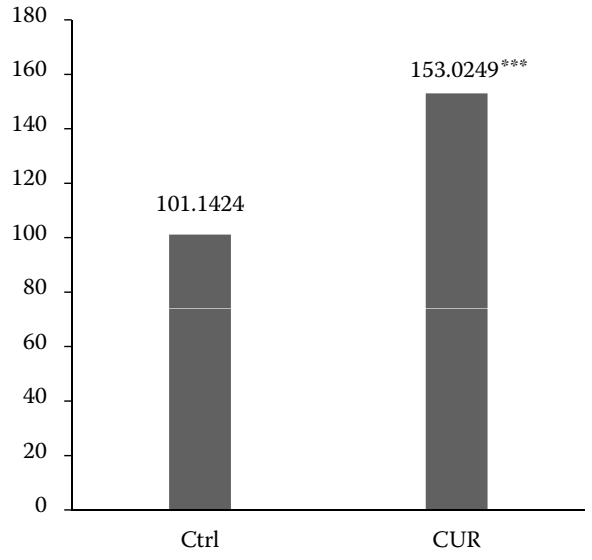

Figure 1. Effect of curcumin administration on the mitochondrial activity of frozen-thawed bovine spermatozoa
each bar represents mean (± SEM) optical density as the percentage of the control, set to 100%; data are expressed as a percentage of the control value and were obtained from five independent experiments; level of significance was set at *P < 0.05, **P < 0.01, ***P < 0.001

Table 2. Effect of curcumin supplementation on the oxidative stress markers of frozen-thawed bovine spermatozoa

Parameter	Control (n = 30)	Curcumin (n = 30)
ROS (RLU/s/10^6 sperm)	40.19 ± 7.62	23.80 ± 4.51**
Protein carbonyls (nmol/mg protein)	3.92 ± 0.51	1.96 ± 0.30**
Oxidative DNA damage (%)	15.33 ± 0.15	5.55 ± 0.07*
LPO (μmol/g protein)	5.13 ± 0.71	2.55 ± 0.46***

ROS = reactive oxygen species, RLU = relative light units, LPO = lipid peroxidation
*P < 0.05, **P < 0.01, ***P < 0.001

The MTT test revealed that the CUR presence in the semen extender led to a significantly higher sperm mitochondrial activity (P < 0.001, Figure 1).

As revealed in Figure 2, the superoxide generation was significantly decreased in the presence of CUR when compared to the control (P < 0.001, Figure 2). Moreover, CUR showed to be effective in the prevention of ROS overgeneration (P < 0.01; Table 2).

Evaluation of the oxidative damage to proteins, lipids, and DNA indicated that CUR administration to the semen cryopreservation medium led to a significant decrease of the levels of protein

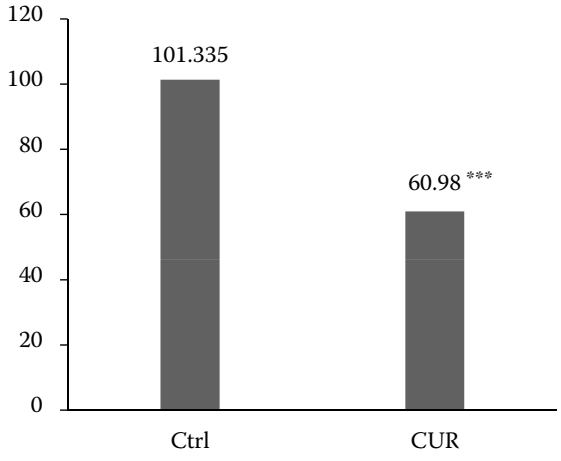

Figure 2. Effect of curcumin administration on the intracellular superoxide production of frozen-thawed bovine spermatozoa
each bar represents mean (± SEM) optical density as the percentage of the control, set to 100%; data are expressed as a percentage of the control value and were obtained from five independent experiments; level of significance was set at *P < 0.05, **P < 0.01, ***P < 0.001

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carbonyls ($P < 0.01$), MDA ($P < 0.001$) as well as 8-OHdG ($P < 0.05$) in comparison with the control (Table 2).

DISCUSSION

Based on previous evidence about the possible beneficial *in vitro* effects of CUR on male gametes (Bucak et al. 2010, 2012; Tvrda et al. 2016), our study investigated the impact of CUR on (1) structural and functional characteristics, and (2) selected oxidative stress markers of bovine spermatozoa subjected to cryopreservation.

Physiologically relevant concentrations of ROS have been shown to participate in maintaining optimal membrane stability, acrosome integrity, and fertilizing potential of mammalian spermatozoa (Aitken and Fischer 1994). On the other hand, their overproduction resulting from repeated freeze-thaw cycles may result in a rapid decrease of sperm motion behaviour, activity, and membrane integrity (Ball 2008; Bucak et al. 2010). Exposure of spermatozoa to low temperatures has been repeatedly associated with adenosine triphosphate depletion, premature capacitation, and acrosome reaction as well as increased morphological alterations (Ball 2008). At the same time, it must be emphasized that a proper sperm motility, intact membranes and mitochondria are essential to enable the sperm transit through the cervical structures followed by their penetration through the cumular cells and *zona pellucida* of the oocyte. In this study, CUR behaved as an efficient promoter of sperm motility and a membrane-protecting agent enhancing the sperm structural integrity following freezing and thawing, leading to a significant maintenance of sperm motility characteristics.

Previous reports on the impact of CUR on the sperm mitochondria have speculated about its possible roles in the metabolic signaling of male gametes. Reddy and Aggarwal (1994) suggest that CUR has the ability to inhibit protein kinase C, which plays important roles in modulating the flagellar movement of spermatozoa. Such inhibition could be associated with possible detrimental effects of CUR on the spermatozoa motion (Rithaporn et al. 2003). This hypothesis is however in disagreement with our findings suggesting that CUR exhibits protecting and enhancing effects on the sperm mitochondria against the detrimental impact of low temperatures on the metabolism of male gametes. In addition, our MTT outcomes are consistent with a number of toxicological studies according to which the sperm metabolic activity was protected against stress induced by aflatoxins (Mathuria and Verma 2008), sodium nitrate (El-Wakf et al. 2011), cyclophosphamide (Dev et al. 2013) or ferrous ascorbate (Tvrda et al. 2016).

Numerous scientific reports indicate that one of the possible protective mechanisms of CUR on the sperm structure or function is its ability to scavenge ROS. A different reason for the beneficial effects of CUR on the sperm vitality may lie in its ability to stimulate the internal ROS-quenching and detoxification system of male gametes, consistent with previous studies revealing changes in the antioxidant capacity of male reproductive structures in the presence of CUR (Karbalay-Doust and Noorafshan 2011; Soleimanzadeh and Saberivand 2013; Tvrda et al. 2016). A proper oxidative balance is intricately related to male fertility and as a complex antioxidant network assures beneficial conditions for spermatozoa activity (Aitken 1995). *In vitro* storage and cryoconservation of mammalian semen may lead to severe alterations to the oxidative milieu, either through uncontrolled ROS generation or through aberrations in the antioxidant mechanisms of spermatozoa. As such, we may hypothesize that CUR administration to the semen extender provides a greater protection to the natural oxidative balance of bovine gametes.

Sperm cryopreservation has been consistently determined to be responsible for LPO, sperm DNA damage, alterations to the intracellular enzymatic activity and ultimately, apoptosis and a reduced fertilization capacity (Tatone et al. 2010). As such, we evaluated the effect of CUR supplementation on the most notable OS markers of cryopreserved bovine spermatozoa.

Sperm DNA integrity is a crucial factor for successful fertilization and subsequent embryo development. Numerous studies have revealed significant associations between sperm exposure to low temperatures and oxidative DNA damage (Tatone et al. 2010; Gonzalez-Marin et al. 2012). In this study, a significant preservation of post-thaw DNA integrity was recorded following CUR administration, most likely because of its antioxidant properties, complementing our luminometric assessment of ROS generation. A significant decrease of DNA fragmentation following CUR treatment could be also related to the

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protection of the antioxidant balance as concluded by Soleimanzadeh and Saberivand (2013).

OS may lead to protein aggregation, fragmentation, susceptibility to proteolysis, leading to a disruption of their normal functions (Rahal et al. 2014). Semen cryopreservation may contribute to sperm damage through an excessive generation of protein carbonyls. In this case, CUR was able to diminish the quantity of oxidized proteins in bovine spermatozoa probably due to its direct ROS-trapping properties and ability to prevent the residual ROS to alter the structure or function of the protein molecules found in the spermatozoon. Piper et al. (1998) reported a decreased protein utilization by spermatozoa as a key result of the antioxidant properties of CUR or because of an increased activity of glutamyl cysteine synthase, which may be modulated by CUR.

Lipids have been repeatedly determined as the sperm macromolecules most susceptible to oxidation. Sperm LPO is a self-propagating chain reaction resulting in the deprivation of membrane fluidity and the loss of membrane enzymes and ion channels (Aitken 1995). Our experimental data indicate that CUR was able to decrease peroxidative alterations to sperm membranes, preventing structural or functional damage to male gametes. Rahman (2007) hypothesizes that CUR might be able to counteract oxidative damage, most likely because of its ability to scavenge lipid peroxyls before these can attack the membrane lipids. Such conclusions are consistent with Salama and El-Bahr (2007) emphasizing the protective effects of CUR against damage to lipid molecules present in male reproductive cells and tissues. The outcomes from our TBARS assay are, however, contradicting other reports on frozen-thawed mammalian sperm cells (Bucak et al. 2010, 2012) according to which no significant decrease of MDA was detected following CUR administration. As such, it may be arguable that CUR could protect vital sperm structures against oxidative damage possibly even without interacting with LPO.

The effects of CUR on ejaculated spermatozoa have been investigated before on different mammalian models, however there are still conflicting results to be taken into consideration, particularly with respect to the exact CUR dosage to be supplemented to semen extenders. We used 50 µmol/l CUR based on our previous reports focused on the behaviour of a wider range of CUR concentrations on the bovine sperm survival during extended periods of *in vitro* storage (Tvrda et al. 2015) as well as under induced OS using ferrous ascorbate (Tvrda et al. 2016). A number of reports have studied higher (Bucak et al. 2010, 2012; Soleimanzadeh and Saberivand 2013; Omur and Coyan 2016) as well as lower CUR doses (Naz 2011) with different outcomes. Bucak et al. (2012) reported that the extender supplemented with 0.5 mmol/l CUR led to a lower percentage of alterations to the membrane integrity and total abnormalities in bull sperm, while no significant changes were detected with respect to morphological alterations to the acrosome among the groups. Similarly, cryopreservation diluents containing 2.5, 5 or 10 mmol/l CUR led to a lower percentage of abnormalities to the acrosome or sperm structures in Angora goats (Bucak et al. 2010). Moreover, 1 and 2 mmol/l CUR provided a significantly higher protection to the post-thaw rat and ram sperm motion characteristics, mitochondrial and membrane stability (Soleimanzadeh and Saberivand 2013; Omur and Coyan 2016). Inversely, Bucak et al. (2010, 2012) reported that CUR administration to extenders for Holstein bull (2.5 mmol/l) or Angora goat semen (0.5 mmol/l) led to nonsignificant differences in the sperm motility parameters, except for linearity. Finally, Naz (2011) stressed out that co-incubation of human or mouse spermatozoa in the presence of 125 µmol/l CUR resulted in a decline of the sperm forward motility as well as processes related to the capacitation and acrosome reaction. Administration of concentrations higher than 250 µmol/l CUR led to a complete inhibition of the spermatozoa motility. We may therefore speculate that CUR has the ability to exhibit a dual biological activity: while lower CUR concentrations may protect and stimulate the activity of male reproductive cells, higher CUR doses may exhibit toxic effects on the sperm vitality. As such, the exact critical dosage of CUR may be still an important issue to be studied in detail, as it may be affected by the semen processing protocol, time of exposure or animal species.

CONCLUSION

In conclusion, our data point out the beneficial and protective effects of curcumin against the cryodamage to bovine spermatozoa. The experimental outcomes of this study suggest that curcumin administration to semen cryopreservation media could offer protection to the sperm

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structure, function, and oxidative profile. As such, curcumin administration could be recommended to facilitate the development and enhancement of semen processing and storage protocols in the cattle industry. At the same time, further research should be conducted in order to address the precise molecular pathways underlying the protective and antioxidant roles of curcumin in male reproduction.

REFERENCES

Aggarwal B.B., Sundaram C., Malani N., Ichikawa H. (2007): Curcumin: the Indian solid gold. Advances in Experimental Medicine and Biology, 595, 1–75.

Aitken R.J. (1995): Free radicals, lipid peroxidation, sperm function. Reproduction, Fertility and Development, 7, 659–668.

Aitken J., Fisher H. (1994): Reactive oxygen species generation and human spermatozoa: the balance of benefit and risk. BioEssays, 16, 259–267.

Bailey J.L., Morrier A., Cormier N. (2003): Semen cryopreservation: successes and persistent problems in farm species. Canadian Journal of Animal Science, 83, 393–401.

Ball B.A. (2008): Oxidative stress, osmotic stress and apoptosis: impacts on sperm function and preservation in the horse. Animal Reproduction Science, 107, 257–267.

Bansal A.K., Bilaspuri G.S. (2011): Impacts of oxidative stress and antioxidants on semen functions. Veterinary Medicine International, 2011, 23–32.

Bucak M.N., Sariozkan S., Tuncer P.B., Sakin F., Atessahin A., Kulaksiz R., Cevik M. (2010): The effect of antioxidants on post-thawed Angora goat (Capra hircus ancryrensis) sperm parameters, lipid peroxidation and antioxidant activities. Small Ruminant Research, 89, 24–30.

Bucak M.N., Basspinar N., Tuncer B.P., Coyan K., Sariozkan S., Akalin P.P., Buyukleblebici S., Kucukgunay S. (2012): Effects of curcumin and dithioerythritol on frozen-thawed bovine semen. Andrologia, 44, 102–109.

Dev K.R., Yadamma K., Reddy K.D. (2013): Protective effects of curcumin in cyclophosphamide induced sperm head abnormalities in male mice. International Journal of Pharmacy and Biological Sciences, 4, 1131–1137.

El-Wakf A., Elhabiby E.S.M., El-Kholy W.M., El-Ghany E.A.E. (2011): Use of tumeric and curcumin to alleviate adverse reproductive outcomes of water nitrate pollution in male rats. Natural Science, 9, 229–239.

Gonzalez-Marin C., Gosalvez J., Roy R. (2012): Types, causes, detection and repair of DNA fragmentation in animal and human sperm cells. International Journal of Molecular Sciences, 13, 14026–14052.

Karbalay-Doust S., Noorafshan A. (2011): Ameliorative effects of curcumin on the spermatozoon tail length, count, motility and testosterone serum level in metronidazole-treated mice. Prague Medical Report, 112, 288–297.

Kashou A.H., Sharma R., Agarwal A. (2013): Assessment of oxidative stress in sperm and semen. Methods in Molecular Biology, 927, 351–361.

Mathuria N., Verma R.J. (2008): Curcumin ameliorates aflatoxin-induced toxicity in mice spermatozoa. Fertility and Sterility, 90, 775–780.

Moskovtsev S.I., Librach C.L. (2013): Methods of sperm vitality assessment. In: Carrell D.T., Aston K.I. (eds): Spermatogenesis: Methods and Protocols. Springer Science and Business Media, LLC, New York, USA, 13–19.

Naz R.J. (2011): Can curcumin provide an ideal contraceptive? Molecular Reproduction and Development, 78, 116–123.

Omur A.D., Coyan K. (2016): Protective effects of the antioxidants curcumin, ellagic acid and methionine on motility, mitochondrial transmembrane potential, plasma membrane and acrosome integrity in freeze-thawed Merino ram sperm. Veterinarni Medicina, 61, 10–16.

Piper J.T., Singhal S.S., Salameh M.S., Torman R.T., Awasthi Y.C., Awasthi S. (1998): Mechanisms of anticarcinogenic properties of curcumin: the effect of curcumin on glutathione linked detoxification enzymes in rat liver. The International Journal of Biochemistry and Cell Biology, 30, 445–456.

Pope C.E., Zhang Y.Z., Dresser B.L. (1991): A simple staining method for quantifying the acrosomal status of cat spermatozoa. Journal of Zoo and Wildlife Medicine, 2, 87–95.

Rahal A., Kumar A., Singh V., Yadav B., Tiwari R., Chakraborty S., Dhama K. (2014): Oxidative stress, prooxidants, and antioxidants: the interplay. BioMed Research International, 2014, Article ID 761264.

Rahman K. (2007): Studies on free radicals, antioxidants, and co-factors. Journal of Clinical Interventions in Aging, 2, 219–236.

Reddy S., Aggarwal B.B. (1994): Curcumin is a non-competitive and selective inhibitor of phosphorylase kinase. FEBS Letters, 341, 19–22.

Rithaporn T., Monga M., Rajasekaran M. (2003): Curcumin: a potential vaginal contraceptive. Contraception, 68, 219–223.

Salahshoor M.R., Jalili C., Khazaei M., Khani F. (2012): Effects of curcumin on reproductive parameters in male mice. Journal of Clinical Research in Paramedical Sciences, 1, 1–3.

Salama A.F., El-Bahr S.M. (2007): Effect of curcumin on cadmium induced oxidative testicular damage in rats. Journal of Medical Research Institute, 28, 167–173.

https://doi.org/10.17221/33/2017-CJAS

Soleimanzadeh A., Saberivand A. (2013): Effect of curcumin on rat sperm morphology after the freeze-thawing process. Veterinary Research Forum, 4, 185–189.

Tatone C., Di Emidio G., Vento M., Ciriminna R., Artini P.G. (2010): Cryopreservation and oxidative stress in reproductive cells. Gynecological Endocrinology, 26, 563–567.

Tvrda E., Lukac N., Jambor T., Lukacova J., Massanyi P. (2015): Curcumin in male fertility: effects on spermatozoa vitality and oxidative balance. Journal of Microbiology, Biotechnology and Food Sciences, 4, 120–124.

Tvrda E., Tusimova E., Kovacik A., Paal D., Greifova H., Abdramanov A., Lukac N. (2016): Curcumin has protective and antioxidant properties on bull spermatozoa subjected to induced oxidative stress. Animal Reproduction Science, 172, 10–20.

Weber D., Davies M.J., Grune T. (2015): Determination of protein carbonyls in plasma, cell extracts, tissue homogenates, isolated proteins: focus on sample preparation and derivatization conditions. Redox Biology, 5, 367–380.

Xia X., Cai H., Qin S., Xu C. (2012): Histone acetylase inhibitor curcumin impairs mouse spermiogenesis – an in vitro study. PLoS ONE, 7, e48673.

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