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Antioxidant effects of lycopene on bovine sperm survival and oxidative profile following cryopreservation

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ABSTRACT: Reactive oxygen species overgeneration as a side effect of semen cryopreservation may lead to lipid peroxidation, protein degradation, DNA fragmentation and cell death, resulting in a decrease of sperm survival and fertilisation ability. Lycopene has been proposed as a potential supplement to semen extenders because of its antioxidant properties. The aim of this study was to evaluate the effects of lycopene on the structural integrity, functional activity and selected oxidative stress parameters of cryopreserved bovine sperm. Thirty bovine ejaculates were split into two aliquots and diluted with a commercial semen extender supplemented with 1.5 mmol/l lycopene or containing no supplement (control), cooled down to 4 °C, frozen and kept in liquid nitrogen. Prior to experiments, frozen straws were thawed at 37 °C for 20 s. Lycopene addition resulted in a higher sperm motility ($P < 0.001$), progressive motility ($P < 0.001$) and all secondary motion characteristics ($P < 0.001$ with respect to the average path velocity, linear velocity, velocity of curvilinear motion, beat cross frequency, path straightness and linearity; $P < 0.01$ in the case of the amplitude of lateral head displacement). Furthermore, lycopene exhibited protective effects on the sperm membrane ($P < 0.05$) and acrosomal ($P < 0.01$) integrity in comparison to control. An assay for metabolic function revealed that lycopene supplementation to the cryopreservation medium resulted in a higher preservation of the sperm mitochondrial activity ($P < 0.001$). Reactive oxygen species production as well as intracellular superoxide generation were decreased following lycopene addition ($P < 0.01$ in the case of reactive oxygen species; $P < 0.001$ with respect to superoxide production). Finally, the presence of lycopene led to a decrease in protein carbonyl production ($P < 0.01$), lipid peroxidation ($P < 0.001$) as well as oxidative DNA damage ($P < 0.05$) when compared to control. In conclusion, lycopene exhibited significant reactive oxygen species-trapping and antioxidant properties which may prevent oxidative damage to frozen-thawed sperm, and, thus, enhance the post-thaw vitality of male reproductive cells in cattle breeding.

Keywords: oxidative stress; reactive oxygen species; bulls; supplements

Although semen cryopreservation is an essential pillar of animal reproductive biotechnologies, its full potential remains to be realised, as a considerable proportion of mammalian sperm lose their fertility during the freezing and thawing process (Bailey et al. 2003). Even in case of relatively cryoresistant bovine sperm, eight-to tenfold more frozen-thawed cells are necessary to achieve equivalent fertility rates when compared to fresh semen (Shannon and Vishwanath 1995). Sperm

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cryopreservation often leads to the overgeneration of reactive oxygen species (ROS) primarily due to heat shock, exposure to atmospheric oxygen and removal of seminal plasma (Bailey et al. 2003). Subsequent oxidative insults may lead to profound alterations in sperm motion behaviour, membrane integrity, lipid peroxidation (LPO), DNA damage, cell death and, ultimately, to reduced fertilisation capacity (Aitken et al. 1989; Agarwal 2003; Ball 2008; Tvrda et al. 2011).

Research in recent years has led to the design of new semen extenders supplemented with protective and antioxidant substances which could reduce the undesirable effects of cryopreservation on male reproductive cells, and thus protect and prolong sperm activity and vitality (Andreea and Stela 2010).

Lycopene (ψ,ψ-carotene; LYC) is a natural carotenoid and a powerful antioxidant found in tomatoes, watermelons, papayas and pink grapefruits (Atasoy 2012). Previous *in vivo* as well as *in vitro* reports have demonstrated that LYC may improve sperm motility, viability, morphology and testicular oxidative balance following exposure to medication (Atessahin et al. 2006a; Atessahin et al. 2006b), organic pollutants (Raj et al. 2012; Tamiselvan et al. 2013), oxidative stress promoters (Zini et al. 2010; Tvrda et al. 2016a) or mycotoxins (Tas et al. 2010). At the same time, pivotal studies have emphasised the potential of LYC as a suitable antioxidant supplement to extenders for mammalian or avian semen (Mangiagalli et al. 2007; Uysal and Bucak 2007; Rosato et al. 2012a; Rosato et al. 2012b; Bucak et al. 2015).

This study was therefore designed to evaluate the effects of lycopene on the motility characteristics, and on the structural, metabolic and oxidative profile of cryopreserved bovine sperm, in order to explore the potential of lycopene to contribute to a higher post-thaw semen quality.

MATERIAL AND METHODS

Semen collection. Ejaculates from ten adult Simmental-Fleckvieh breeding bulls (Slovak Biological Services, Nitra, Slovakia) were used in the study. The animals were of similar age and were kept under uniform feeding and housing conditions.

Three semen samples were collected from each bull on a regular collection schedule using an artificial vagina. Immediately after collection, sperm concentration and motility were estimated using phase-contrast microscopy (× 200). Only fresh semen with a minimum of 1×10^9 sperm/ml and 70% progressive motility was used for the subsequent experiments. All samples met the criteria set by the common standards for the production of insemination doses.

Semen cryopreservation. Each ejaculate was divided into two equal fractions and diluted to a final concentration of 11×10^6 sperm/ml in an extender consisting of Triladyl (Minitub GmbH, Tiefenbach, Germany), 20% (w/v) fresh egg yolk, Tris, citric acid, sugar, buffers, glycerol and antibiotics (tylosin, gentamicin, spectinomycin and lincomycin) and diluted with distilled water. For the experimental group, the extender was supplemented with 1.5 mmol/l LYC (Sigma-Aldrich, St Louis, USA) previously dissolved in butylated hydroxytoluene (BHT; Sigma-Aldrich) while the control group were administered an equal amount of BHT. The selected LYC concentration was chosen on the basis of previous studies carried out in our laboratory (Tvrda et al. 2016a; Tvrda et al. 2016b).

Diluted semen samples were loaded into 0.25 ml French straws, cooled down to 4 °C over 2 h and subsequently frozen at a programmed rate of –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 freezing machine (Digitcool 5300 ZB 250; IMV, France). Finally, the straws were plunged into liquid nitrogen (–196 °C). After storage for one month, frozen straws were thawed in a 37 °C water bath for 20 s immediately before use. Mitochondrial tests and oxidative stress assays were performed on thawed sperm samples following centrifugation (300 × *g*, 25 °C, 5 min), washing and resuspension in phosphate-buffered saline (Dulbecco's PBS; Sigma-Aldrich).

Sperm motility assessment. Sperm motion characteristics were assessed using computer-aided sperm analysis (CASA; Version 14.0 TOX IVOS II.; Hamilton-Thorne Biosciences, Beverly, USA). In order to avoid false positive results, the samples were stained with the IDENT Stain, a DNA-specific dye based on Hoechst bisbenzimide (Hamilton-Thorne Biosciences) and analysed under fluorescent illumination.

The system 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

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elongation – 20–100; default cell size – 4 pixels; default cell intensity – 40. Ten microliters of each sample were placed 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 in order to ensure capture of at least 300 cells. The following motility features were recorded: motility (%), progressive motility (%), average path velocity (VAP, µm/s), linear velocity (VSL, µm/s), velocity of curvilinear motion (VCL, µm/s), amplitude of lateral head displacement (ALH, µm), path straightness (STR, %), beat cross frequency (BCF, Hz) and linearity (LIN, %).

Eosin-nigrosin staining. The eosin-nigrosin staining method was used to evaluate the functional integrity of the sperm membrane. This technique is based on the ability of eosin to penetrate into non-viable cells (Moskovtsev and Librach 2013). Five microliters of each sample were placed on a tempered glass slide, mixed with 10 µl 5% eosin (Sigma-Aldrich), followed by 10 µl 10% nigrosin (Sigma-Aldrich). The mixture was smeared on a glass slide and left to air-dry at 37 °C. The slides were observed using bright field microscopy at × 1000 using oil immersion. At least 200 sperm per slide were identified as either dead (red heads) or live (white heads), and expressed as a percentage rate. All slides were assessed blindly by one observer.

Acrosomal integrity. The acrosomal status was assessed following the fast green-rose bengal staining protocol designed by Pope et al. (1991). This single-step staining method applies a mixture consisting of 1% fast green (Sigma-Aldrich), 1% rose bengal (Sigma-Aldrich) and 40% ethyl alcohol (Centralchem, Bratislava, Slovak Republic) in 0.1 M citric acid – 0.2 M disodium phosphate buffer (Sigma-Aldrich). Twenty microliters of the thawed sample were mixed with 20 µl of the staining solution and incubated for 70 s at room temperature. Ten microliters of the mixture were smeared on a tempered glass slide and air-dried at 37 °C. Acrosomal integrity was evaluated using bright field microscopy at × 1000 using oil immersion. At least 200 cells per slide were evaluated for the presence or absence of acrosome, and expressed as a percentage rate. All slides were assessed blindly by one observer.

Mitochondrial activity (MTT assay). Sperm viability was evaluated by the MTT assay, which measures metabolic activity. This colorimetric assay measures the conversion of a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to blue formazan particles by mitochondrial succinate dehydrogenase of intact mitochondria within living cells.

The MTT salt (Sigma-Aldrich) was dissolved in Dulbecco's PBS (Sigma-Aldrich) and added to the cell suspension. After a 2 h incubation (shaker, 37 °C, 95% air atmosphere, 5% CO_2), the cells and formazan crystals were dissolved in isopropanol (Centralchem). The optical density was determined at a wavelength of 570 nm against 620 nm as reference using the Multiskan FC microplate photometer (Thermo Fisher Scientific Inc., Waltham, USA). Data were obtained from five independent experiments and expressed in percentage of the control, which was set to 100% (Tvrda et al. 2016b).

Quantification of superoxide production (NBT test). The nitroblue-tetrazolium (NBT) test was used to assess the intracellular formation of superoxide radicals. This assay is conducted by counting the cells containing blue NBT formazan deposits, which are formed by reduction of the membrane-permeable, water-soluble, yellow-coloured nitroblue tetrazolium chloride (2,2'-bis(4-nitrophenyl)-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'diphenylene) ditetrazolium chloride; Sigma-Aldrich) and superoxide radical.

The NBT salt was dissolved in Dulbecco's PBS containing 1.5% DMSO and added to the cell suspension. After a 1 h incubation (shaker, 37 °C, 95% air atmosphere, 5% CO_2), the cells were washed twice with PBS and centrifuged at 300 × *g* for 10 min. Lastly, the cells and formazan crystals were dissolved in 2 M potassium hydroxide (KOH; Centralchem) in DMSO. The optical density was determined at a wavelength of 620 nm against 570 nm as a reference using the Multiskan FC microplate photometer (Thermo Fisher Scientific Inc.). Data were obtained from five independent experiments and expressed in percentage of the control, which was set to 100% (Tvrda et al. 2016b).

Reactive oxygen species measurement. ROS levels in the samples were assessed by the chemiluminescence assay using luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma-Aldrich) as the probe (Kashou et al. 2013). Test samples consisted of luminol (10 µl, 5 mM) and 400 µl of control or experimental sample. The negative control was prepared by replacing the sperm suspension with

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400 µl of PBS. The positive control included 400 µl PBS and 50 µl hydrogen peroxide (30%; 8.8 M; Sigma-Aldrich) in triplicates. Chemiluminescence was measured on a 48-well plate for 15 min using the Glomax Multi$^+$ Combined Spectro-Fluoro Luminometer (Promega Corporation, Madison, USA; Tvrda et al. 2016a). The results are expressed as relative light units (RLU)/s/10^6 sperm.

Oxidative DNA damage assessment. Sperm DNA was isolated using the GenElute^{TM} Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich) and quantified using the Genesys 10 spectrophotometer (Thermo Fisher Scientific Inc.) at 260/280 nm. The obtained DNA was subsequently processed with the EpiQuik^{TM} 8-OHdG DNA Damage Quantification Direct Kit (Epigentek Group Inc., Farmingdale, USA). This commercial kit enables a direct colorimetric quantification of 8-hydroxy-2'-deoxyguanosine (8-OHdG) 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. Thawed semen samples were centrifuged (800 × *g*, 25 °C, 10 min) and washed twice with PBS. Supernatants were removed and the resulting pellet was sonicated at 28 kHz for 30 s on ice using RIPA buffer (Sigma-Aldrich) with a protease inhibitor cocktail suitable for mammalian cell and tissue extracts (Sigma-Aldrich). The samples were subsequently centrifuged at 11 828 × *g* at 4 °C for 15 min to purify the lysates from the residual cell debris. The resulting supernatants comprising the intracellular content were stored at –80 °C for further assessment.

Carbonyl group quantification was performed using the traditional 2,4-dinitrophenylhydrazine (DNPH) method. One ml of the pre-treated sample was added to 1 ml DNPH (10 mM in 2 N HCl; Sigma-Aldrich), mixed, and incubated at 37 °C in the dark for 1 h. After the addition of 1 ml trichloroacetic acid (20% w/v; Sigma-Aldrich), the mixture was incubated at 4 °C for 10 min and subsequently centrifuged at 11 828 × *g* for 15 min. The supernatant was discarded without disturbing the pellet which was subsequently washed three times with 1 ml ethanol/ethyl acetate (1/1; v/v) to remove any free DNPH reagent. Lastly, the pellet was resuspended in 1 ml 6 M guanidine-HCl (Sigma-Aldrich) before absorbance measurement at 360 nm, using 6 M guanidine-HCl as a blank solution. The molar absorption coefficient of 22 000 $M^{-1}cm^{-1}$ was used to quantify the concentration of protein carbonyls. The results are expressed as nmol/mg protein (Weber et al. 2015).

The extent of LPO, expressed as malondialdheyde (MDA) concentration was quantified with the help of the TBARS assay, modified for a 96-well plate and ELISA reader. The final product created by the reaction of MDA and thiobarbituric acid (Sigma-Aldrich) under high temperature (90–100 °C) and acidic conditions was measured at 530–540 nm (Tvrda et al. 2016a). MDA concentration is expressed as µmol/g protein.

Protein concentration was assessed using the DiaSys Total Protein (DiaSys, Holzheim, Germany) commercial kit and the Randox RX Monza clinical chemistry analyzer (Randox Laboratories, Crumlin, Great Britain). The measurement is based on the Biuret method, according to which copper sulphate reacts with proteins to form a violet blue colour complex in alkaline solution, and the intensity of the colour is directly proportional to the protein concentration when measured at 540 nm.

Statistical analysis. Results are expressed as mean ± SEM. The statistical analysis was carried out using the GraphPad Prism program (version 5.0 for Windows; GraphPad Software, La Jolla California USA, www.graphpad.com). Initially, descriptive statistical characteristics were evaluated. The paired *t*-test was used for specific statistical evaluations, based on the assumption that values in each row represent paired observations. The level of significance was set at $P < 0.001$, $P < 0.01$, and $P < 0.05$.

RESULTS

The effects of lycopene on the structural and functional characteristics of cryopreserved bull sperm are displayed in Table 1. Addition of lycopene to the semen extender led to higher sperm motility and progressive motility in comparison with the control ($P < 0.001$) following freezing and thawing. Significant differences were additionally observed for all secondary motion characteristics ($P < 0.001$ in the case of VAP, VCL, VSL, BCL, STR and LIN; $P < 0.01$ with respect to ALH; Table 1). At the same time, LYC promoted higher sperm membrane stability ($P < 0.001$) and acrosomal integrity ($P < 0.001$) when compared to the control

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Table 1. The effect of lycopene supplementation on the motility characteristics, mitochondrial activity, membrane and acrosome integrity of frozen-thawed bovine spermatozoa. Values are presented as mean ± SEM

Parameter	Control (n = 30)	Lycopene (n = 30)
Motility (%)	47.99 ± 0.78	62.97 ± 1.05***
PM (%)	30.19 ± 0.74	43.01 ± 0.91***
VAP (μm/s)	68.88 ± 0.87	78.90 ± 0.89***
VSL (μm/s)	59.00 ± 0.97	66.54 ± 0.58***
VCL (μm/s)	100.19 ± 0.92	108.80 ± 0.72***
ALH (μm)	3.89 ± 0.16	4.46 ± 0.36**
BCF (Hz)	17.55 ± 0.69	23.88 ± 0.85***
STR (%)	62.02 ± 1.11	68.07 ± 0.91***
Linearity (%)	54.55 ± 0.71	62.05 ± 0.93***
MI (%)	77.00 ± 1.28	84.90 ± 0.55***
AI (%)	76.80 ± 0.44	85.20 ± 0.42***
MA (%)	100.00 ± 0.00	147.50 ± 6.26***

AI = acrosomal integrity, ALH = amplitude of lateral head displacement, BCF = beat cross frequency, MA = Mitochondrial activity, MI = membrane integrity, PM = progressive motility, STR = path straightness, VAP = average path velocity, VCL = velocity of curvilinear motion, VSL = linear velocity
*P < 0.05; **P < 0.01; ***P < 0.001

(Table 1). Moreover, the presence of LYC in the semen extender led to a higher mitochondrial activity of frozen-thawed bovine sperm (P < 0.001) in comparison with the control group.

As shown in Table 2, intracellular superoxide generation was decreased in the experimental group supplemented with LYC when compared to the control (P < 0.001). Furthermore, LYC was effective in decreasing the ROS generation by cryopreserved bovine sperm (P < 0.01; Table 2). The assessment of oxidative damage to proteins, lipids and DNA revealed that LYC supplementation to the cryopreservation extender led to decreased levels of 8-OHdG (P < 0.001), protein carbonyls (P < 0.01) as well as MDA (P < 0.001), when compared to the control group (Table 2).

DISCUSSION

Based on a preliminary body of evidence indicating favourable effects of lycopene on the quality of chilled or cryopreserved semen, this study was designed to assess the potential beneficial effects of lycopene on (1) the structural integrity and functional activity, and (2) selected oxidative markers of frozen-thawed bovine sperm.

Table 2. The effect of lycopene supplementation on oxidative stress markers in frozen-thawed bovine spermatozoa. Values are presented as mean ± SEM

Parameter	Control (n = 30)	Lycopene (n = 30)
Superoxide production (%)	100.00 ± 0.00	42.58 ± 3.47***
Reactive oxygen species (RLU/s/10^6 sperm)	39.19 ± 3.22	25.75 ± 2.91***
Protein carbonyls (nmol/mg prot)	4.68 ± 0.51	2.22 ± 0.30**
Oxidative DNA damage (%)	12.35 ± 0.26	4.19 ± 0.20***
Lipid peroxidation (μmol/g prot)	7.45 ± 0.81	2.43 ± 0.56***

*P < 0.05; **P < 0.01; ***P < 0.001

The experimental data depicted in Table 1 suggest that LYC supplemented to the semen extender acts as an effective motion-promoting and membrane-protecting molecule, significantly improving sperm motility, membrane integrity and mitochondrial activity following cryopreservation.

It is widely acknowledged that the cryo- and oxidative damage resulting from freezing and thawing may lead to a decline in sperm motility, viability and membrane fluidity (Ball 2008; Bucak et al. 2010). Sperm cryopreservation has been repeatedly associated with axonemal alterations, leading to disruptions to sperm motion behaviour and mitochondrial membrane potential, ATP depletion, premature acrosome reaction and increased morphological alterations (de Lamirande and Gagnon 1992; Ball 2008). Important associations have been reported to exist between sperm motility, stability of the membranous structures and mitochondrial metabolism (Martinez-Pastor et al. 2004). At the same time, we must keep in mind that motility is an essential prerequisite for the male gamete to penetrate the cumulus cells and fuse with the ovum (de Lamirande and Gagnon 1992).

Our data are in agreement with previous *in vitro* reports on chilled or frozen rabbit, turkey and fowl semen (Rosato et al. 2012a; Rosato et al. 2012b; Mangiagalli et al. 2007) suggesting that LYC may have beneficial effects on sperm motion characteristics as a result of its ROS-quenching abilities and protective effects against oxidative damage to

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structures crucial for the functional activity of male reproductive cells. Furthermore, the protective effects of LYC on mitochondrial metabolism recorded in our study complement the findings of Bucak et al. (2015) who observed a marked improvement in the preservation of the sperm mitochondrial membrane potential following LYC supplementation. Using a broader range of LYC concentrations, Uysal and Bucak (2007) reported that, similarly to our data, 800 μg/ml (approximately 1.5 μM/l) LYC supplemented to a ram semen extender prevented typical detrimental effects of cryopreservation on sperm motility, morphology, acrosome integrity and cell survival. In contrast, Zini et al. (2010) observed that pre-incubating human sperm with LYC did not reverse the loss of motility caused by subsequent addition of H_2O_2 to the culture. Failure of LYC to stabilise sperm motility in this case could be attributed to the presence of H_2O_2, a notorious ROS able to quickly escape the ROS-quenching abilities of LYC and to curb sperm activity through numerous oxidative mechanisms (Aitken et al. 1989).

Assessment of the selected oxidative stress markers in our study indicates that LYC has the ability to at least partially stabilise the delicate oxidative balance within male reproductive cells subjected to temperature fluctuations during freezing and thawing. Our data provide evidence for the ROS-trapping properties of LYC, which are associated with a subsequent protection of critical sperm structures prone to oxidative insults.

Cryopreserved semen is more susceptible to oxidative damage than fresh ejaculates. Intracellular antioxidant capacity fails to provide protection against the oxidative stress associated with the toxic effects of ROS following freezing and thawing (Ball 2008; Rosato et al. 2012a; Rosato et al. 2012b). The cold shock inflicted on cryopreserved sperm is linked to oxidative damage to crucial structural and functional biomolecules, followed by alterations to intracellular signalling pathways and engagement of apoptosis (Ball 2008). Membranous structures containing high levels of polyunsaturated fatty acids, sulfhydryl-containing proteins and DNA are exceptionally sensitive to the cryopreservation process.

To protect male reproductive cells against the deleterious effects of freezing and thawing, a variety of experimental studies using antioxidant substances have been designed (Uysal and Bucak 2007; Bucak et al. 2008; Bucak et al. 2010). Carotenoids, particularly lycopene, are reported to act as effective scavengers of singlet oxygen ($^1O_2$) and other ROS. Due to its 11 conjugated double bonds, LYC contains numerous electrons which can be donated to ROS, resulting in their neutralisation (Atasoy 2012). As such, LYC is able to trap ROS before these can initiate oxidative chain reactions (Choi and Seo 2013).

The data collected from the NBT and luminometric assays reveal effective ROS-quenching properties of LYC, and complement earlier studies on its antioxidant potential in male reproductive structures determined via the total antioxidant capacity and/or activities of prominent antioxidant enzymes (Atessahin et al. 2006a; Turk et al. 2007; Tvrda et al. 2016a). On the other hand, Bucak et al. (2015) who studied the antioxidant activity of cryopreserved bovine spermatozoa following LYC addition, recorded no significant differences between the control and experimental group. The disparity between the outcomes related to the oxidative balance in our study may be attributed to the different extender composition and LYC doses used.

The assessment of 8-OHdG, a direct marker of oxidative damage to DNA, reveals that LYC has the ability to provide protection to the male genetic material against ROS-associated DNA fragmentation when compared to the control. These results are in agreement with previous reports on mammalian germ cells in which the extent of damage to the sperm DNA was assessed using the Comet assay, acridine orange test or the sperm chromatin structure assay (Devaraj et al. 2008; Zini et al. 2010; Rosato et al. 2012; Bucak et al. 2015), and indicate that sperm DNA was more intact following exposure to LYC. At the same time, our data reveal that LYC was able to decrease the amount of protein carbonyls in bovine sperm probably as a direct result of its ROS-quenching ability; this ability ensured that the residual ROS could not interact with the structure and/or function of protein molecules present in the male gamete. Previous data concerning the effects of LYC on sperm proteins are limited, and primarily focus on peptides exhibiting antioxidant activities, particularly reduced gutathione (GSH), which plays an important role as a redox sensor and a protective agent against ROS-induced cellular damage (Meseguer et al. 2007). Uysal and Bucak (2007) concluded that the addition of 800 μg/ml LYC to a cryopreservation extender maintained the total protein amount and stabilised

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the ratio of oxidized to reduced GSH following ram semen cryopreservation.

Our experimental data suggest that LYC has the ability to prevent peroxidative damage to sperm lipids when compared to the untreated control. Various earlier studies have reported a significant decrease in LPO following LYC supplementation (Goyal et al. 2006; Aly et al. 2012; Sarkar et al. 2012; Filipcikova et al. 2015). Our findings are, however, inconsistent with Zribi et al. (2011) who reported that although oxidative stress was linked to DNA damage, no correlations were recorded between nuclear alterations and MDA levels. Moreover, several reports have suggested that although LYC is a ROS scavenger, it failed to achieve any success in decreasing LPO in bull (Bucak et al. 2010; Bucak et al. 2012) and ram (Coyan et al. 2011) sperm following cryopreservation. Due to the conflicting data on the potential usefulness of LYC in preventing oxidative damage to lipids, it may be speculated that LYC may not play a primary role in counteracting LPO following mammalian sperm cryopreservation.

In conclusion, our results emphasise the protective and antioxidant effects of lycopene against the cryodamage inflicted on bovine sperm. As such, we suggest that lycopene may protect the structural stability, activity and oxidative profile of bovine gametes. Accordingly, lycopene supplementation to semen extenders may facilitate an improvement of semen handling and storage protocols in cattle breeding. However, further research is necessary to understand the specific molecular mechanisms underlying the beneficial effects of lycopene on mammalian reproductive cells.

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