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The Effect of Curcumin on Cryopreserved Bovine Semen

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***Abstract*—**Oxidative stress associated with semen cryopreservation may result in lipid peroxidation (LPO), DNA damage and apoptosis, leading to decreased sperm motility and fertilization ability. Curcumin (CUR), a natural phenol isolated from *Curcuma longa* Linn. has been presented as a possible supplement for a more effective semen cryopreservation because of its antioxidant properties. This study focused to evaluate the effects of CUR on selected oxidative stress parameters in cryopreserved bovine semen. 20 bovine ejaculates were split into two aliquots and diluted with a commercial semen extender containing CUR (50 μmol/L) or no supplement (control), cooled to 4 °C, frozen and kept in liquid nitrogen. Frozen straws were thawed in a water bath for subsequent experiments. Computer assisted semen analysis was used to evaluate spermatozoa motility, and reactive oxygen species (ROS) generation was quantified by using luminometry. Superoxide generation was evaluated with the NBT test, and LPO was assessed via the TBARS assay. CUR supplementation significantly ($P<0.001$) increased the spermatozoa motility and provided a significantly higher protection against ROS ($P<0.001$) or superoxide ($P<0.01$) overgeneration caused by semen freezing and thawing. Furthermore, CUR administration resulted in a significantly ($P<0.01$) lower LPO of the experimental semen samples. In conclusion, CUR exhibits significant ROS-scavenging activities which may prevent oxidative insults to cryopreserved spermatozoa and thus may enhance the post-thaw functional activity of male gametes.

***Keywords*—**Bulls, cryopreservation, curcumin, lipid peroxidation, reactive oxygen species, spermatozoa.

I. Introduction

ARTIFICIAL insemination (AI), the first modern biotechnology method established in animal production, contributes significantly to the advancement of controlled reproduction in cattle. Short- or long-term semen preservation as an essential pillar of animal AI has shown numerous advantages, including preservation of genetic resources, improvement of the genetic progress and enhancement of the transportation of genetic material across countries [1], [2].

Unfortunately, the advancement of *ex vivo* semen preservation is slow and complicated, as a considerable proportion of spermatozoa loses their viability and activity during storage at low temperatures, rendering the semen sample useless for AI [2].

The unique structure and function renders spermatozoa to be exceptionally sensible to the freeze-thawing process. Such instability caused by temperature changes leads to cold shock and oxidative stress (OS)-induced cellular damage. The abundance of polyunsaturated fatty acids (PUFAs) in the sperm membranes causes susceptibility of the male gamete to LPO, and ROS generated during such process lead to further cellular damage during long-term storage of semen samples [3], [4]. Semen contains an array of antioxidant molecules which, under normal circumstances, provide protection against the detrimental effects of ROS [5], [6]. Nevertheless, following the freeze-thawing process, this system tends to fail to protect spermatozoa against oxidative insults [7], [8].

Many years of research have led to the establishment of a variety of methods in order to improve the process of sperm preservation, among which using antioxidants in the semen extenders could be a promising solution to prevent the detrimental structural and functional changes during the chilling, freezing and thawing process. Currently, the main goal is to define an antioxidant or a combination of different ones, with a high ROS scavenging activity, metal chelation properties and the ability to modulate the activity of antioxidant enzymes that could ultimately contribute to the alleviation of the undesirable effects of sperm chilling or freezing, and to prolong the sperm vitality [9].

CUR [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene -3,5-dione] is a bioactive chemical component found in the herbal remedy and dietary spice turmeric. CUR is ought to act as a potent scavenger of an array of ROS such as superoxide and hydroxyl radicals [10] as well as nitrogen dioxide [11]. The molecule is also an effective LPO inhibitor [12]. Controversial data are, however, available with respect to the role of CUR in male fertility. Diverse *in vivo* [13] and *in vitro* reports [14], [15] suggest energy-promoting and protective events of CUR on the testicular tissue, spermatogenesis, and sperm behavior. On the other hand, other studies emphasize on a negative involvement of CUR in signaling pathways related to spermatogenesis [16].

Specific questions related to the potential antioxidant activity of CUR supplemented to cryopreservation media on the post-thaw sperm viability still need to be answered. Therefore, we focused on evaluating the effects of CUR on the sperm motility, free radical production and LPO in cryopreserved bovine semen.

II. Material and Methods

A. Sample Collection and Processing

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

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Two semen samples were collected from each bull on a regular collection schedule using an artificial vagina. Immediately after collection, sperm concentration and motility was estimated using phase-contrast microscopy (200 x). Only fresh semen with the required quality (minimum 70 % progressive motility and concentration of $1x10^9$ sperm/mL) was used for the subsequent processing of samples. All semen samples met the criteria set by the common standards used for producing AI doses.

Each semen sample was divided into two equal fractions and diluted to a final concentration of $11x10^6$ sperm/mL in an extender consisting of Triladyl (Minitüb GmbH, Tiefenbach, Germany), containing 20 % (w/v) fresh egg yolk, TRIS, citric acid, sugar, buffers, glycerol and antibiotics (Tylosin, Gentamicine, Spectinomycine and Lincomycine) and diluted with distilled water. In case of the experimental group, the extender additionally contained 50 µmol/L CUR, previously dissolved in DMSO, while the control groups contained an equal amount of DMSO. The selected CUR concentration was chosen upon the previous studies in our laboratory [17], [18]. Diluted semen samples were loaded into 0.25 mL French straws, cooled down to 4 °C in 2 h and subsequently frozen at a pre-programmed rate in a digital freezing machine (Digitcool 5300 ZB 250; IMV). Finally, the straws were plunged into liquid nitrogen. At least after 24 h, frozen straws were thawed in a 37 °C water bath for 20 s immediately before use. Three replicates from every sample were evaluated in each experiment.

B. Spermatozoa Motion Analysis

Spermatozoa motion (%) was assessed by using the computer-aided sperm analysis (CASA, Version 14.0 TOX IVOS II.; Hamilton-Thorne Biosciences, Beverly, MA, USA). 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 elongation – 20-100; default cell size - 4 pixels; default cell intensity - 40. Ten µL of each sample were placed into the Makler counting chamber (depth 10µm, 37 °C; Sefi Medical Instruments, Haifa, Israel) and immediately assessed. 10 microscopic fields were subjected to each analysis in order to include at least 300 cells.

C. ROS Generation

ROS levels in samples were assessed by the chemiluminescence assay using luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione; Sigma, St Louis, MO) as the probe [12]. The test samples consisted of luminol (10 µL, 5 mM) and 400 µL of control or experimental sample. Negative controls were prepared by replacing the sperm suspension with 400 µL of the extender. Positive control included 400 µL of the extender and 50 µL of hydrogen peroxide (30%; 8.8 M; Sigma-Aldrich, St. Louis, USA) in triplicates. Chemiluminescence was measured on a 48-well plate for 15 min by using the Glomax Multi+ Combined Spectro-Fluoro-Luminometer (Promega). The results were expressed as relative light units (RLU)/sec/10^6 sperm [18], [19].

D. Superoxide Production

The nitroblue-tetrazolium (NBT) test was used to quantify the intracellular formation of the superoxide radical, by assessing blue NBT formazan deposits, generated by the reduction of the membrane permeable, yellow-colored, nitroblue tetrazolium chloride (2, 20-bis(4-nitrophenyl)-5,50-diphenyl-3, 30-(3, 30-dimethoxy-4, 40-diphenylene) ditetrazolium chloride; Sigma-Aldrich) by the superoxide radical. The NBT salt was dissolved in PBS containing 1.5% DMSO (dimethyl sulfoxide, Sigma-Aldrich) to a final concentration of 1 mg/mL and added to the cells (100 µL per well). 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 KOH (potassium hydroxide; Centralchem) in DMSO. Optical density was determined at a wavelength of 620 nm against 570 nm as reference by a microplate ELISA reader (Anthos MultiRead400). Data are expressed in percentage of the SC Control (Control 1) set to 100% [18]. Semen extender was run as a negative control in order to exclude possible cross-reactions with the tetrazolium and false-positive results.

E. LPO

The extent of LPO by means of malondialdehyde (MDA) content was quantified with the help of the TBARS assay, modified for a 96-well plate and ELISA reader. The final product formed by the reaction of MDA and thiobarbituric acid (TBA; Sigma-Aldrich, St. Louis, USA) under high temperature (90–100°C) and acidic conditions was measured at 530–540 nm [18]. Semen extender was run as a negative control in order to assure the objectivity of the results. MDA concentration was expressed as µmol/L.

F. Statistical Analysis

Results are expressed as mean±SEM. Statistical analysis was carried out using the GraphPad Prism program (version 5.0 for Windows; GraphPad Software, La Jolla California USA, www.graphpad.com). Descriptive statistical characteristics were evaluated at first. 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 $^{*}P<0.05$.

III. Results and Discussion

OS has become one of the leading causes related to the loss of viable spermatozoa during cryopreservation. ROS overgeneration is nowadays accepted as a notable side effect of temperature fluctuations during freeze-thaw cycles, leading to major disruptions in the cellular oxidative metabolism [20]. The resulting OS subsequently leads to irreversible alterations of membrane structures via LPO, as well as oxidative degradation of proteins or DNA, followed by apoptotic activation [21].

Based on previous evidence about the possible useful *in vitro* effects of CUR on male gametes [18], [22], [23], this

study was performed to investigate specific ROS-quenching and protective properties of CUR in relation to the functional activity of frozen-thawed bovine spermatozoa.

The effects of CUR on the spermatozoa motion are presented in Fig. 1. CUR administration to the semen extender led to a significantly ($P<0.001$) higher spermatozoa motility (63.62±0.85%) in comparison with the control (50.54±0.54%) following freezing and thawing.

Spermatozoa motility is an essential prerequisite to secure their passage through the female reproductive system, followed by an effective and penetration into the egg [24]. In the present study, CUR supplementation to the cryopreservation medium led to a significant maintenance of sperm motility, being in accordance with [14], [23] as well as [15] who demonstrated a significant improvement in the motion characteristics of post-thawed ram, Angora goat and rat spermatozoa supplemented with 2.5 mmol/L CUR. On the other hand, [14] reported that administration of 2.5 mmol/L CUR into a semen extender for Holstein bulls led to nonsignificant differences in the sperm motility parameters, except for linearity. Moreover, [16] found that the incubation of human or murine spermatozoa with 125 μmol/L CUR led to a decrease in the spermatozoa forward motility, capacitation and acrosome reaction. At CUR concentrations higher than 250 μmol/L, a complete inhibition of spermatozoa motility was observed. We may therefore hypothesize, that CUR may exhibit a dual biological activity – while lower CUR concentrations may protect and stimulate the sperm functional activity, higher CUR doses may have 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, and may be affected by the semen processing protocol, time of exposure and animal species.

The improvement of bovine spermatozoa motility following CUR administration was accompanied by a decreased ROS and superoxide production (Figs. 2 and 3). The presence of CUR in the experimental group was very effective in decreasing both ROS (69.80±1.51 RLU/s/10^6spem; $P<0.001$) and superoxide concentrations (61.48±3.89%; $P<0.01$) when compared to the control group (90.19±1.62 RLU/s/10^6 sperm and 100.0±5.89%; respectively; Figs. 2 and 3).

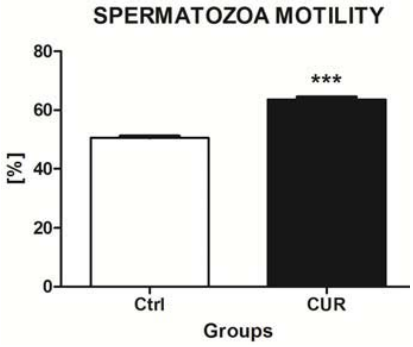

Fig. 1 The effect of CUR on the motility of cryopreserved bovine spermatozoa. Mean ± SEM. *** $P<0.001$

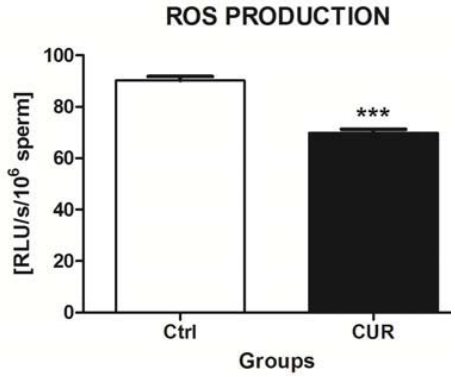

Fig. 2 The effect of CUR on the ROS production in cryopreserved bovine semen Mean ± SEM. *** $P<0.001$

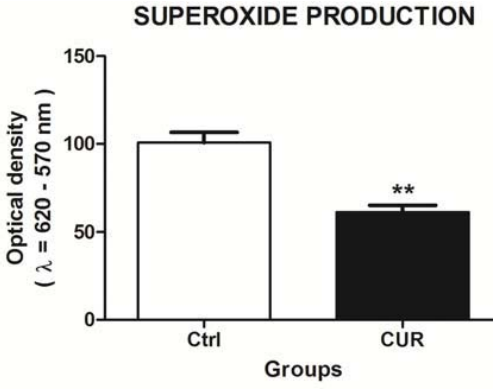

Fig. 3 The effect of CUR on the superoxide production in cryopreserved bovine semen. Each bar represents mean (±SEM) optical density as the percentage of the Control, which was set to 100% and the data are expressed as a percentage of the Control. ** $P<0.01$

The first scientific evidence about the beneficial effects of CUR on the quality and antioxidant capacity of semen led to its exploration as a possible antioxidant supplement and cryoprotective agent in spermatozoa cultures [22]. One of the possible ameliorative mechanisms of CUR on the spermatozoa functional activity is its ability to scavenge ROS. Another possible explanation for the enhancement of spermatozoa motility following CUR administration may lay in its stimulating activities of the internal antioxidant system, consistent with previous reports emphasizing on changes to the total antioxidant activity of male reproductive cells cultured in media enriched with CUR [15], [17].

The oxidative milieu of spermatozoa is closely related to male reproductive performance, as an appropriate antioxidant balance provides a favorable environment for spermatozoa function. Any shifts in this delicate environment in favor of free radical production may be an important cause of male infertility [25]. *In vitro* storage and cryopreservation of semen may cause such severe alterations to the oxidative balance, either through ROS overgeneration or through aberrations in the antioxidant defense capacity of spermatozoa. Based on the results of this study, the addition of CUR to the semen

extender may provide a higher protection to the natural oxidative balance of bovine semen, and these findings are consistent with the previous results of [15] and [18]. More specifically, CUR decreased the free radical concentrations as a result of its ROS activities and induction of detoxification enzymes. Finally, [26] suggested that CUR exhibits the ability to reverse oxidative damage, probably through its capacity to scavenge lipid peroxyl radicals before these can reach the membrane lipids.

The TBARS assay revealed that CUR administration to the semen extender resulted in a significantly ($P<0.01$) lower LPO of the spermatozoa membranes (17.93±1.11 μmol/L) in comparison with the control group (25.99±1.14 μmol/L; Fig. 4).

The experimental outcomes suggest that CUR was able to maintain the spermatozoa membrane integrity accompanied by a significant decrease of MDA. As such, CUR may prevent peroxidative changes to the sperm membrane structures, thus may maintaining motility and may decrease structural or functional alterations to the gametes. Such conclusions are in agreement with [27] as well as [28] hypothesizing that CUR provides protection against LPO in male reproductive structures. Data from the TBARS assessment are, however, contradicting findings from other studies performed on frozen-thawed mammalian spermatozoa [14], [22], [23] where there were no significant changes in the MDA content of male reproductive cells following CUR supplementation. Hence, we may argue that CUR could protect the sperm structural integrity against oxidative damage, even without the inhibition of LPO.

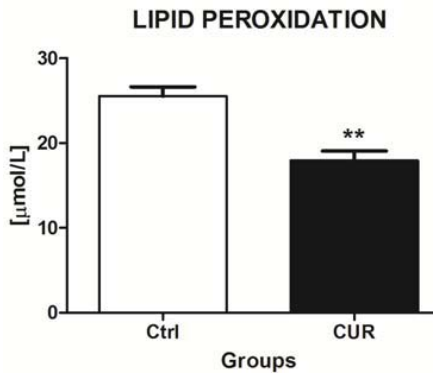

Fig. 4 The effect of CUR on the LPO in cryopreserved bovine semen. Mean ± SEM. ** $P<0.01$

IV. Conclusions

In conclusion, results of this study emphasize on the beneficial impact of CUR against the cryoinjury of bovine spermatozoa *in vitro*. The data obtained suggest that CUR supplementation to the semen extender offers protection to the sperm motility, oxidative profile, and membrane stability. Thus, CUR supplementation may be recommended to facilitate the improvement of semen preservation in bovine breeding industry.

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