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CURCUMIN IN MALE FERTILITY: EFFECTS ON SPERMATOZOA VITALITY AND OXIDATIVE BALANCE

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ABSTRACT

The aim of this study was to assess the dose- and time-dependent effects of curcumin on bovine spermatozoa during short-term (0h, 2h, 6h) and long-term (12h, 24h) *in vitro* culture periods. Semen samples were collected from 20 adult breeding bulls, and diluted in physiological saline solution containing 0.5% DMSO together with 0, 1, 5, 10, 50 and 100 μM/L of curcumin. Spermatozoa motion parameters were determined using the SpermVision™ and CASA (Computer Assisted Semen Analyzer) system. Cell viability was measured using the metabolic activity MTT assay, and the nitroblue-tetrazolium (NBT) test was used to assess the intracellular superoxide formation. The CASA analysis revealed that concentrations of 50 μM/L and 10 μM/L of curcumin were able to significantly prevent the decrease of motility and progressive motility ($P<0.001$ in case of group B and $P<0.01$ in case of group C) over all time periods of the *in vitro* incubation. At the same time, supplementation of concentrations ranging from 50 μM/L to 5 μM/L of curcumin led to a significant preservation of the cell viability in comparison to the control ($P<0.001$ in case of groups B and C; $P<0.05$ in case of group D). Concentrations in between 50 μM/L and 5 μM/L of curcumin demonstrated antioxidant properties, translated in a significant reduction of the intracellular superoxide production throughout the *in vitro* culture ($P<0.001$). The results indicate that the addition of curcumin, especially in concentrations between 50 μM and 10 μM to the culture medium could be beneficial for a complex enhancement of spermatozoa activity and protection against complications resulting from *in vitro* culture.

Keywords: Curcumin, spermatozoa, bulls, motility, viability, oxidative stress

INTRODUCTION

Over the last years, different studies have evaluated the beneficial effects of oral antioxidant administration on spermatozoa physiology and fertility in animals and humans **(Donnely *et al.*, 1999; Agarwal and Sekhon, 2010)**. However, data regarding the *in vitro* effects of antioxidants on the sperm cell is still very sparse or controversial. The *in vitro* data are however essential, as it has been systematically shown that diverse antioxidants may protect the male germ cells against oxidative injury and subsequent sperm dysfunction (i.e. loss of motility and viability). This is essential for spermatozoa processing protocols routinely performed in medical and veterinary laboratories for long-term spermatozoa preservation (cryoconservation) or procedures related to assisted reproductive techniques. The *in vitro* environment represents an additional danger to the sperm survival, as it provides suitable conditions for ROS (reactive oxygen species) overproduction and a subsequent structural and/or functional damage to the sperm cell **(Saleh and Agarwal, 2002)**.

The addition of synthetic antioxidants to cell cultures is one of the most efficient ways to prevent oxidative damage. However, the safety of synthetic additives has been questioned stimulating the evaluation of naturally occurring compounds with antioxidant properties. Moreover, the chemical diversity, structural complexity, availability, lack of significant toxic effects and intrinsic biologic activity of natural products make them ideal candidates for new therapeutics **(Alarcón de la Rastra, 2008)**.

The polyphenol curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] (CUR) is a major bioactive chemical component of turmeric power, found in the herbal remedy and dietary spice turmeric. This vibrant yellow spice is derived from the rhizome of the plant *Curcuma longa Linn* which is obtained by crushing the roots into powder **(Ammon and Wahl, 1991; Litwinienko and Ingold, 2004)**. Curcumin is the phytochemical that gives a yellow color to turmeric and is now recognized as being responsible for most of its therapeutic effects **(Aggarwal *et al.*, 2007)**.

Curcumin with its proven antioxidant properties has been shown to have several therapeutic advantages. It was shown to be a potent scavenger of a variety of reactive oxygen species including superoxide anion radicals, hydroxyl radicals **(Reddy and Lokesh, 1994)** as well as nitrogen dioxide radicals **(Unnikrishnan and Rao, 1995; Sreejayan and Rao, 1997)**. It was also shown to inhibit lipid peroxidation in different animal models **(Reddy and Lokesh, 1992; Sreejayan and Rao, 1994)**.

Controversial data are however available with respect to the effects of CUR on male fertility parameters. Several *in vivo* **(Salahshoor *et al.*, 2012)** as well as *in vitro* studies **(Bucak *et al.*, 2012; Hendin *et al.*, 2013)** strongly emphasize on the involvement of CUR in energy-promoting and protective effects of CUR on the testicular tissue, spermatogenesis as well as oxidative balance of the sperm cell. Furthermore, CUR has been shown to reverse male toxicity caused by a variety of physiological **(Wei *et al.*, 2009)** or environmental factors **(El-Wakf *et al.*, 2011; Dev *et al.*, 2013; Khorsandi *et al.*, 2013)**. At the same time, other studies implicate the negative involvement of CUR in pathways related to human and murine spermatogenesis **(Naz, 2011; Xia *et al.*, 2012)** suggesting a cautious and thorough investigation of CUR toxicity as well as contraceptive effects.

The present *in vitro* study is therefore aimed to find out the efficacy of CUR on bovine spermatozoa motility, viability and superoxide radical formation during a 24 hour in vitro cultivation, in order to define an optimal concentration of this compound for further experiments.

MATERIAL AND METHODS

Bovine semen samples were obtained from 20 adult breeding bulls (Slovak Biological Services, Nitra, Slovak Republic). The samples had to accomplish the basic criteria given for the corresponding breed. The samples were obtained on a regular collection schedule using an artificial vagina. After collecting the samples were stored in the laboratory at room temperature (22–25°C).

Each sample was diluted in physiological saline solution (PS; sodium chloride 0.9 % w/v; Bieffe Medital, Italia) containing 0.5% DMSO (Dimethyl sulfoxide; Sigma-Aldrich, St. Louis, USA), with various concentrations of CUR (Sigma-Aldrich, St. Louis, USA; A – 100; B – 50; C – 10; D – 5; E – 1μM/L) using a dilution ratio of 1:40. The samples were cultured at room temperature (22–25°C). We compared the control (Ctrl) group (medium without CUR supplementation) with the experimental groups.

Motility and progressive motility analysis was carried out using the CASA (Computer Assisted Semen Analyzer) system equipped with the SpermVision™ program (MiniTub, Tiefenbach, Germany) and the Olympus BX 51 microscope (Olympus, Japan) at cultivation Times 0 h, 2h, 6h (models suitable for a short-term *in vitro* culture) as well as 12 h and 24 h (models suitable for a long-term *in*

vitro culture). Each sample was placed into the Makler Counting Chamber (depth 10 μm, Sefi-Medical Instruments, Israel) and the percentage of motile (motility>5μm/s; MOT) and progressively motile spermatozoa (motility > 20 μm/s; PROG) was evaluated. 1000–1500 cells were assessed in each analysis **(Massányi *et al.*, 2008)**. Viability of the cells exposed to CUR in vitro was evaluated by the metabolic activity (MTT) assay **(Mosmann, 1983; Knazicka *et al.*, 2012)**. This colorimetric assay measures the conversion of 3-(4,5-dimetylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, USA) to purple formazan particles by mitochondrial succinate dehydrogenase of intact mitochondria of living cells. Formazan can then be measured spectrophotometrically at a measuring wavelength of 570 nm against 620 nm as reference by a microplate ELISA reader (Multiskan FC, ThermoFisher Scientific, Finland). The data are expressed in percentage of control (i.e. optical density of formazan from cells not exposed to CUR). Results from the analysis were collected during five repeated experiments at each concentration.

The nitroblue-tetrazolium (NBT) test was used to assess the intracellular formation of superoxide radical **(Esfandiari *et al.*, 2003)**. 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-colored, nitroblue tetrazolium chloride (2,2′-bis(4-Nitrophenyl)-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-diphenylene)ditetrazolium chloride; Sigma-Aldrich, St. Louis, USA) and superoxide radical. Formazan can be measured spectrophotometrically at a measuring wavelength of 620 nm against 570 nm as reference by a microplate ELISA reader (Multiskan FC, ThermoFisher Scientific, Finland). The data were expressed in percentage of control (i.e. optical density of formazan from cells not exposed to CUR). Results from the analysis were collected during five repeated experiments at each concentration **(Tvrdá *et al.*, 2013)**.

Statistical analysis was carried out using the GraphPad Prism program (version 3.02 for Windows; GraphPad Software, La Jolla California USA, www.graphpad.com). Descriptive statistical characteristics (mean, standard error) were evaluated at first. One-way ANOVA with Dunnett's post test was used for statistical evaluations. The level of significance was set at *** ($P<0.001$); ** ($P<0.01$); * ($P<0.05$).

RESULTS AND DISCUSSION

The CASA assessment of the motion parameters showed a gradual decrease of spermatozoa motility and progressive motility in all groups over the course of a 24h *in vitro* culture (Table 1, Table 2). The initial (Time 0h) MOT was higher in all experimental groups when compared to the control group (0 μM/L CUR), although without any statistical significance ($P>0.05$). Although statistically insignificant, a motion-promoting effect of CUR remained visible after 2h, specifically in experimental groups B (50 μM/L CUR), C (10 μM/L CUR) and D (5 μM/L CUR). At the same time, 100 μM/L CUR (group A) caused a non-significant decrease of the spermatozoa motility parameters. After 6h, the decline of spermatozoa motion characteristics was significantly ceased in the group B ($P<0.001$ in case of MOT and $P<0.05$ in case of PROG) in comparison with the control. Examination at 12h of *in vitro* culture showed that the spermatozoa motility and progressive motility were significantly increased in groups B ($P<0.001$) and C ($P<0.05$) when compared to the control. At the same time, an insignificantly higher ($P>0.05$) motion parameters were recorded in the group D, while a non-significant ($P>0.05$) decline was observed in groups A and E (1 μM/L CUR) in a comparison with the control group. At the end of the experiments (24h), the highest motility parameters were observed in the experimental groups B and C, being significantly higher in comparison with the control ($P<0.001$ in case of B and $P<0.01$ in case of C). Meanwhile, both MOT as well as PROG were significantly decreased ($P<0.05$) in the group A, supplemented by the highest concentration of CUR, after a comparison with the Ctrl group (Table 1, Table 2).

Table 1 Spermatozoa motility (%) in the absence (Ctrl) or presence (A-E) of curcumin during different time periods (Mean±SEM; n=20)

Groups	Ctrl	A	B	C	D	E
Time 0h						
	87.36±1.16	90.08±1.27	92.41±1.30	88.17±0.88	89.13±1.27	87.50±0.96
Time 2 h						
	84.02±1.72	81.55±2.29	89.73±1.70	87.38±1.67	86.31±1.32	84.04±1.10
Time 6 h						
	75.99±3.51	70.28±2.09	86.68±1.57***	80.84±1.68	77.21±1.98	75.92±1.54
Time 12 h						
	71.47±1.87	65.79±1.12	83.82±1.03***	76.93±2.04*	73.30±1.87	69.48±2.91
Time 24 h						
	52.41±2.67	44.12±1.41*	62.75±2.74***	60.10±3.02**	57.20±3.06	54.42±2.75

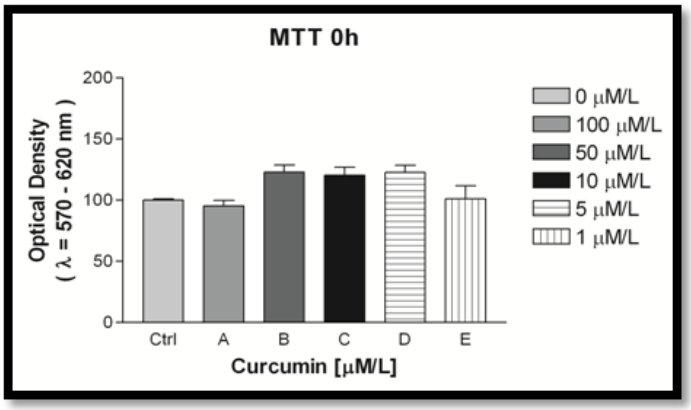
*** ($P<0.001$); ** ($P<0.01$); * ($P<0.05$)

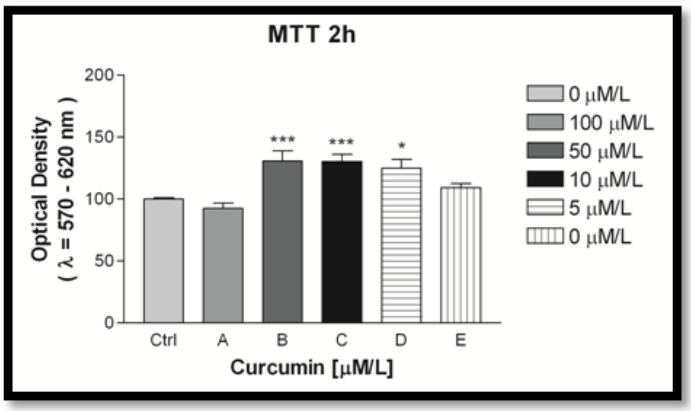
Table 2 Spermatozoa progressive motility (%) in the absence (Ctrl) or presence (A-E) of curcumin during different time periods (Mean±SEM; n=20)

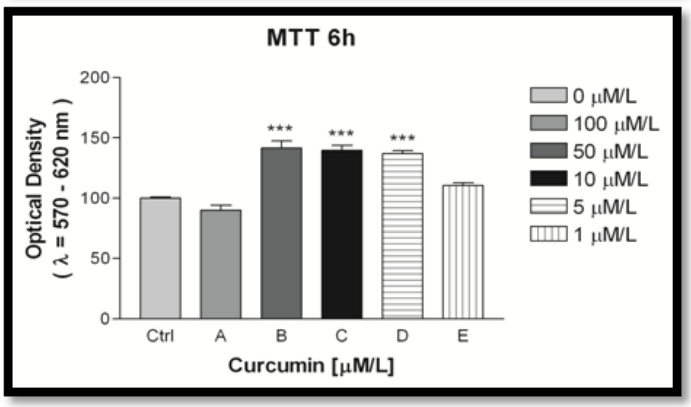
Groups	Ctrl	A	B	C	D	E
Time 0h						
	78.79±1.38	82.51±1.38	82.87±1.25	76.50±1.44	75.62±1.45	74.19±1.61
Time 2 h						
	77.08±1.74	79.44±3.47	78.97±1.73	75.50±1.58	73.70±1.41	73.62±1.25
Time 6 h						
	69.06±3.44	67.67±2.13	73.37±1.62*	72.11±1.64	72.10±1.93	67.24±1.52
Time 12 h						
	61.84±1.76	58.98±1.95	71.94±1.12***	67.03±2.08*	64.02±1.72	60.14±3.06
Time 24 h						
	44.79±2.69	37.73±3.11*	56.10±2.80***	51.14±2.97**	45.73±1.71	44.98±2.94

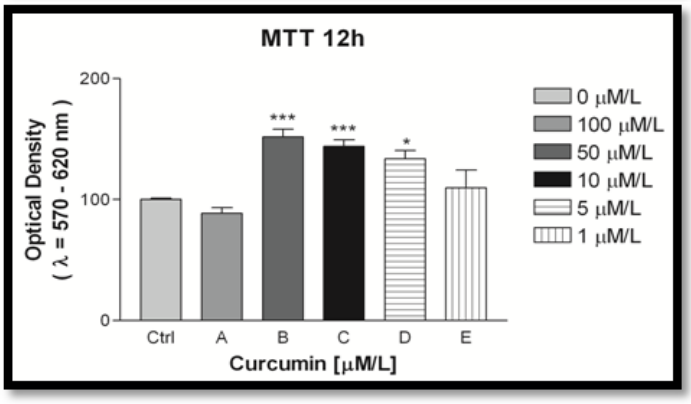
*** ($P<0.001$); ** ($P<0.01$); * ($P<0.05$)

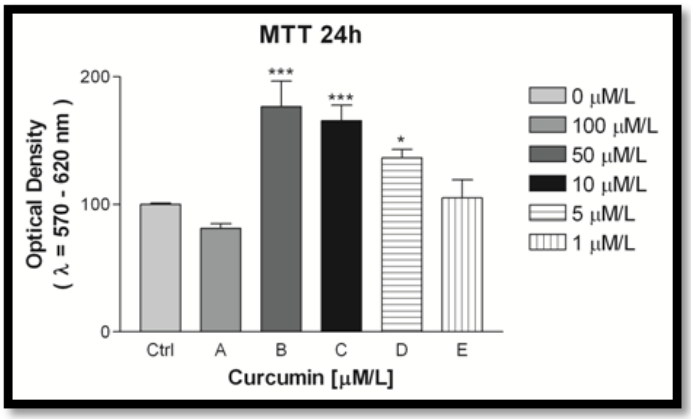
According to the MTT assay, the immediate CUR administration (Time 0h) had no significant effects on the sperm cell viability in any of the experimental groups ($P>0.05$; Figure 1). At 2h it was revealed that 50 μM/L CUR (group B), 10 μM/L CUR (group C) as well as 5 μM/L CUR (group D) had a stimulating and vitality-promoting effect on the bovine spermatozoon, alongside with statistically significant results ($P<0.001$ in case of groups B and C; $P<0.05$ in case of group D) when compared to the control group (0 μM/L CUR; Figure 2). These beneficial effects remained visible and statistically relevant throughout the entire *in vitro* culture, as shown in Figure 3 (Time 6h; $P<0.001$ in groups B, C and D), Figure 4 (Time 12h; $P<0.001$ in case of groups B and C; $P<0.05$ in case of group D) as well as Figure 5 (Time 24h; $P<0.001$ in case of groups B and C; $P<0.05$ in case of group D). Similar to the CASA analysis, the MTT test revealed an inhibition in the cell viability followed by the administration of 100 μM/L CUR (group A), however the differences were non-significant at full length of the *in vitro* spermatozoa culture ($P<0.05$; Figures 1 – 5). Moreover it was shown that the administration of 1 μM/L CUR (group E) had no specific effects on the spermatozoa survival during the entire experiment ($P<0.05$; Figures 1 – 5).





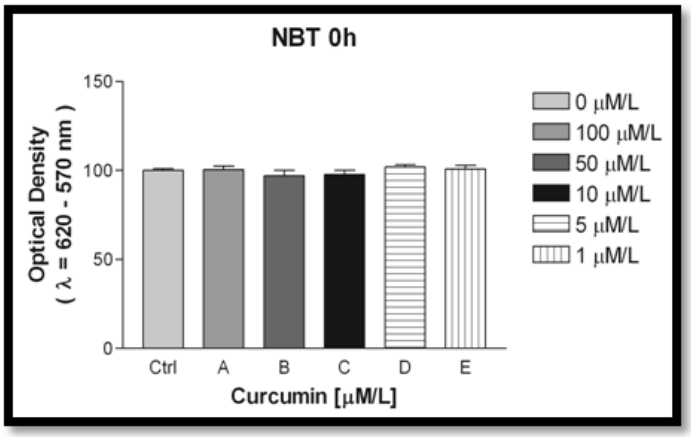


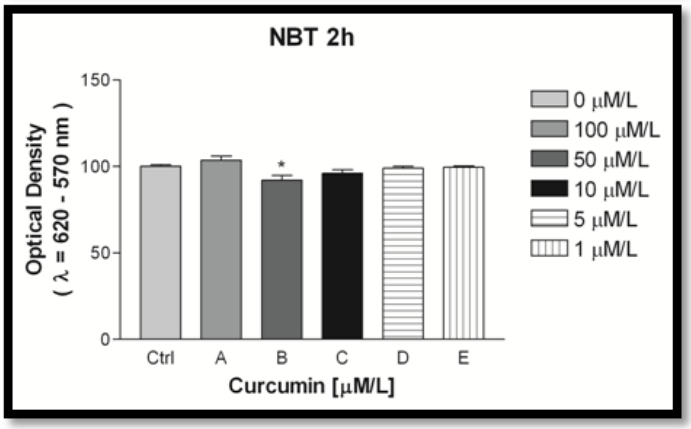


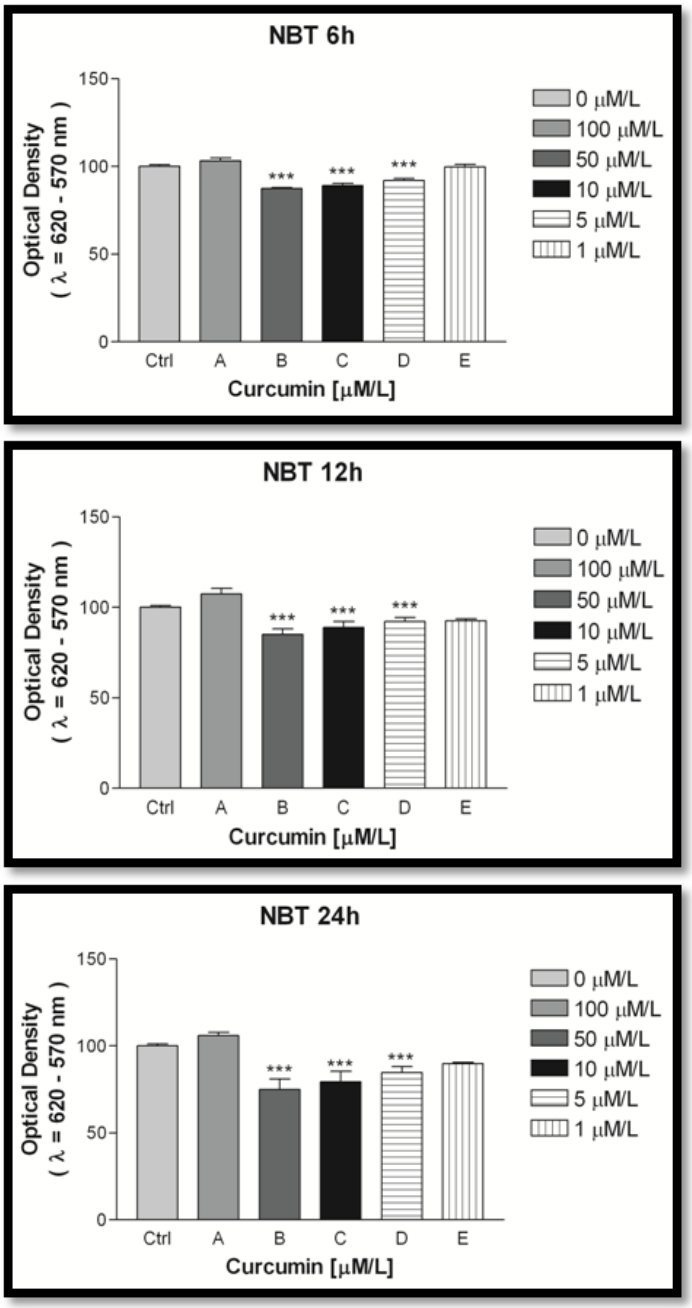


Figures 1, 2, 3, 4, 5 The effect of various doses of curcumin on the viability of bovine spermatozoa at 0h, 2h, 6h, 12h and 24h.
Each bar represents mean (±SEM) optical density as the percentage of controls (n=20), which symbolize 100%. The data were obtained from five independent experiments. The level of significance was set at *** P<0.001; ** P<0.01; * P<0.05.

Although CUR had no instant effects on the oxidative balance within the *in vitro* spermatozoa culture (P>0.05; Figure 6), experiments following a 2h cultivation revealed that administration of 50 μM CUR led to a significant decline of the superoxide formation in comparison to the Control (0 μM CUR; Figure 7). Starting with Time 6h and following the entire in vitro incubation it was shown that CUR concentrations ranging from 50 μM to 5 μM (groups B, C and D) exhibited a long-term and statistically significant (P<0.001) antioxidant protection of the male germ cells and a subsequent prevention of the escalating intracellular superoxide production, considered to be the first step in the creation of oxidative stress (Figures 7, 8, 9 and 10). On the other hand, high (group A) as well as very low (group E) concentrations of CUR had no significant effects (P>0.05) on the superoxide generation within the spermatozoon neither during short-term (Figures 6, 7 and 8) nor during long-term timeframes (Figures 9 and 10) of the *in vitro* culture.







Figures 6, 7, 8, 9, 10 The effect of various doses of curcumin on the spermatozoa superoxide production at 0h, 2h, 6h 12h and 24h.
Each bar represents the mean (±SEM) optical density as the percentage of controls (n=20), which symbolize 100 %. The data were obtained from five independent experiments. The level of significance was set at *** P<0.001; ** P<0.01; * P<0.05.

Past data available on the impact of CUR on male fertilization potential are controversial. In a study by **Salashoor *et al.* (2012)**, increasing doses of CUR significantly increased reproductive indices such as sperm concentration, motility and testosterone content in rats. Inversely, **Naz (2011)** showed that incubation of human or murine sperm with CUR caused a concentration-dependent decrease in sperm forward motility, capacitation and acrosome reaction. At high CUR concentrations, there was a complete block of spermatozoa motility and function. Our results agree with **Salashoor *et al.* (2012)** and suggest stimulating and protective effects of CUR on spermatozoa motility and antioxidant status.

The first report on the useful effects of curcumin **(Bucak *et al.*, 2008)** on post-thawed sperm quality (motility and morphology) and antioxidant activities of cooled ram spermatozoa has given rise to its use as an antioxidant additive and has been shown to have cryoprotective effects when added to the freezing extender. Motility parameters recorded by our CASA system are in accordance with previous findings by **Bucak et al. (2008; 2010)** demonstrating a marked improvement in the motion of post-thawed ram spermatozoa supplemented with the curcumin. Inversely, their later study based on the CUR supplementation to the cryopreservation medium for bovine semen led to nonsignificant differences in the sperm motion characteristics **(Bucak et al., 2012)**. The spermatozoon motility and viability generally declines during *in vitro* incubation. We observed a gradual decrease in viability and motility copied by an increase in superoxide production resulting in low conception rate and a low success in ART techniques, as seen by **Soleimanzadeh and Saberivand (2013)** as well as **Marcus-Braun et al. (2004)**. Our results correlate with the report by **Soleimanzadeh and Saberivand (2013)** in frozen-thawed semen, where CUR addition had a positive impact on both motility and viability.

One of the possible ameliorative mechanisms of curcumin on the above mentioned parameters is to scavenge the free radicals and thereby act as an antioxidant. Another reason for the enhancement of sperm motility in spermatozoa observed in this study may be due to the increasing levels ROS-scavenging molecules, being in accordance with total antioxidant level of semen in curcumin supplemented group by by **Soleimanzadeh and Saberivand (2013)**. The antioxidant capacity of sperm is closely related to male fertility, as an appropriate antioxidant status provides a favorable environment for sperm swimming. The decreased level of antioxidants in the sample may be one of the causes of male infertility **(Agarwal et al., 2003)**. Semen processing, cryopreservation and induction of OS may decrease the antioxidant defense capacity of semen. Based on our results, the addition of curcumin to fresh sperm of bulls significantly preserved the antioxidant defense mechanisms in the sperm cells, agreeing with the conclusions drawn by **Soleimanzadeh and Saberivand (2013)**. Furthermore, a significant preservation of DNA integrity and post-thaw motility in curcumin supplemented group of rats compared to the control, was most probably due to increase in its antioxidant capacity, as **Soleimanzadeh and Saberivand (2013)** conclude.

CONCLUSION

In the present study, *in vitro*-mediated damage to sperm cell with a subsequent loss of spermatozoa fertilizing potential could be reduced using various doses of curcumin. We may conclude that all doses of curcumin increase the percentage of motile and viable spermatozoa but decreased and prevented the intracellular overproduction of free radicals within the sperm mitochondrial membrane. The most effective concentrations of curcumin seem to vary between 50 and 10 μM/L, although it may be important to further investigate specific doeses of curcumin specifically within the selected range.

According to our results, curcumin seems to protect bovine spermatozoa against the damage caused by the hostile *in vitro* environment. Supplementing the semen samples with curcumin could therefore be of scientific importance for extending the time of spermatozoa processing and storage before further designated andrology experiments and clinical procedures, such as artificial insemination, cryopreservation or *in vitro* fertilization techniques.

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