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 of curcumin. Spermatozoa motion parameters were determined using the SpermVision™ and CASA (Computer Assisted Sperm 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 (Donnelly *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 powder, 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; Litwinenko and Ingold, 2004). Curcumin is the phytochemical that gives a yellow color to Curcuma 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 (Bucka *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-Walit *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 (Noz, 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 Sperm 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>5 μm/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; Knazieka et al., 2012). This colorimetric assay measures the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, USA) to purple formazan deposits 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 (MultiASA 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., 2013). 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 (2,3-bis(2-nitrophenyl)-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-diphenylamino)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 (MultiASA 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 5). One-way ANOVA with Dunnett’s post test was used for statistical evaluations. The level of significance was set at “∗” (P<0.01); “∗∗” (P<0.001); “∗∗∗” (P<0.0001).

RESULTS AND DISCUSSION

The CSA 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 of 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)

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

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

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

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.
agreed 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 doses 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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REFERENCES


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 levels, 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.

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 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 of this study.


