IN VITRO EFFECTS OF THE CHLAMYDOMONAS REINHARDTII EXTRACT ON BOVINE SPERMATOZOA

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ABSTRACT

In vitro storage and processing of animal semen is represents a risk factor to spermatozoa vitality, potentially leading to reduced fertility. A variety of substances isolated from natural sources may exhibit protective or antioxidant properties on the spermatozoon, thus extending the lifespan of stored ejaculates. Chlamydomonas has been shown to possess antimicrobial, anti-inflammatory and antioxidant properties, turning the extract into a potential candidate for preserving liquid animal semen during in vitro storage. This study compared the ability of different concentrations of the Chlamydomonas reinhardtii extract on the motility, viability and superoxide production of bovine spermatozoa during different time periods (0, 2, 6, 12 and 24h) of in vitro culture. Spermatozoa motility was assessed using the SpermVision™ CASA (Computer aided sperm analysis) system. Cell viability was examined using the metabolic activity MTT assay and the nitroblue-tetrazolium (NBT) test was applied to quantify the intracellular superoxide formation. The CASA analysis revealed that the Chlamydomonas extract supplementation was able to prevent a rapid decline of spermatozoa motility, especially in the case of concentrations ranging between 1 and 5 µg/mL (P<0.01 with respect to Times 6h, 12h and 24h). At the same time, concentrations ranging between 5 and 10 µg/mL of the extract led to a significant preservation of the cell viability throughout short-term (P<0.05 in case of Time 6h) as well as long-term periods of the experiment (P<0.01 with respect to Time 12h, and P<0.01 in case of Time 24h). 5 and 10 µg/mL of the extract exhibited antioxidant characteristics, translated into a significant reduction of the intracellular superoxide production, particularly notable at Times 12h (P<0.01 with respect to10 µg/mL and P<0.05 in case of 5 µg/mL) and 24h (P<0.01). The results indicate that the Chlamydomonas extract is capable of delaying the damage inflicted to the spermatozoa by the in vitro environment.

Keywords: Chlamydomonas, spermatozoa, motility, viability, superoxide production

INTRODUCTION

Over the last decade, diverse studies have reported about the beneficial effects of oral supplementation of energetic or antioxidant substances on semen quality and male fertility in animals and humans (Donnelly et al., 1999; Agarwal and Sekhon, 2010). Nevertheless, knowledge concerned with the in vitro effects of stimulating or protective molecules on the spermatozoon is still substantially limited or controversial. Meanwhile, in vitro data are crucial for further progress in practical andrology, as it has been systematically shown that diverse biologically active compounds may protect the spermatozoon against the loss of motility or viability. Subsequently, this information may be viable for spermatozoa handling protocols in medical and veterinary laboratories for long-term semen preservation (cryoconservation) or artificial insemination. The in vitro environment represents a hazard 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 cell (Saleh and Agarwal, 2002). Administration of synthetic supplements to cell cultures is an effective way to prevent structural or functional alterations to spermatozoa. However, the safety of synthetic additives has been questioned leading to the renaissance of naturally occurring substances with numerous beneficial properties. The chemical diversity, structural complexity, availability or lack of substantial toxic effects of natural products converts them into ideal candidates for new therapeutic strategies (Alarcón de la Rastra, 2008).

Algae, such as Chlamydomonas, Chlorella or Gelidiella are a rich source of novel biologically active metabolites with various application in pharmaceutical industries. These are a pool of antioxidants such as carotenoids, astaxanthin, phenol and flavonoid derivatives. Many research studies suggest that the biological composition of microalgae including proteins, carbohydrates, minerals and bioactive compounds has a potential medical value (Fuentes et al., 2000; Kightlinger et al., 2014). Correspondingly to the present urge to discover novel and effective biologically active agents, algal derived compounds have a broad range of antibiotic, antiviral, antioxidant, antifouling, anti-inflammatory, cytotoxic and antimitotic activities which could be explored further (Salvador, 2007). Bioactive compounds such as polyphenols, catechin, flavonols, glycosides, and phlorotannins discovered from methanol extract of red, green and brown algae have been reported to have a uniqueness in their molecular skeleton and structures contributing to the strong antioxidant activity (Khodami et al., 2013). Based on this body of evidence, this in vitro study was aimed to assess the efficacy of the Chlamydomonas reinhardtii extract on bovine spermatozoa motility, viability and superoxide radical formation during a 24 hour in vitro cultivation, in order to provide information on its behavior in the male reproductive cell, as well as to define optimal concentrations of this extract for further experiments in veterinary andrology.

MATERIAL AND METHODS

Chlamydomonas reinhardtii was grown aseptically on an agar medium (1/2 Murashige- Skoog, 10 % sucrose, 0.6 % plant agar, pH 5.2) in 225 ml plastic boxes and growth chamber under a 16/8 day/night period at 22°C (Michalko and Matušíková, 2012). After collection and drying, the algal tissues were crushed, weighed and soaked in ethanol p.a. (96 %, Sigma-Aldrich, St. Louis, USA) during two weeks at room temperature in the dark. Exposure to sunlight was avoided to prevent the degradation of active components. The ethanolic algal extracts were subjected to evaporation under reduced pressure at 40 °C in order to remove ethanol (Stuart RE300DB rotary evaporator, Bibby Scientific Limited, UK, and vacuum pump KNF N858.1.2KT A518, KNF, Germany). Crude extracts were dissolved in DMSO (Dimethyl sulfoxide; Sigma-Aldrich, St. Louis, USA).
Bovine semen samples were obtained from 10 adult breeding bulls (Slovak Biological Services, Nitra, Slovak Republic) on a regular collection schedule using an artificial vagina. The ejaculates had to accomplish the basic criteria given for the corresponding breed. 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, Italy) containing various concentrations of the *Chlamydomonas* extract (A – 0.5; B – 10; C – 5; D – 1; E – 0.5; F – 0.1 μg/mL) 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 *Chlamydomonas* supplementation, containing 0.5% DMSO) with the experimental groups.

Spermatozoa motility (percentage of spermatozoa with a motility >5 μm/s; %; MOT) was examined with the help of the Computer-aided sperm analysis (CASA) system using the SpermVision™ program (Minitube, Tiefenbach, Germany) and Olympus BX 51 phase contrast microscope (Olympus, Tokyo, Japan). The samples were placed into the Makler Counting Chamber (depth 10 μm, 57°C; Sefi Medical Instruments, Haifa, Israel) and immediately assessed. At least 1000 cells were evaluated in each sample (Masonyl et al., 2008). Viability of the cells exposed to *Chlamydomonas in vitro* was evaluated by the metabolic activity (MTT) assay. 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 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). The data were expressed in percentage of control (i.e. optical density of formazan from cells not exposed to *Chlamydomonas*). Results from the analysis were collected during five repeated experiments at each concentration (Knazicka et al., 2012). The nitroblue-tetrazolium (NBT) test was used to assess the intracellular formation of superoxide radical. This assay is conducted by counting the cells containing blue NBT formazan deposits, which are formed by reduction of the soluble, yellow colored, nitroblue tetrazolium (3,3′,4,4′-diphenylazonitrophenyl)bis(4-nitrophenyl)dinitrophenylated tetrazolium chloride; Sigma-Aldrich,) 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). The data were expressed in percentage of control (i.e. optical density of formazan from cells not exposed to *Chlamydomonas*).

Results of the analysis were collected during five repeated experiments at each concentration (Tvrda et al., 2013).

### Table 1

<table>
<thead>
<tr>
<th>Time</th>
<th>Ctrl</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>0h</td>
<td>90.11±2.17</td>
<td>83.02±1.19</td>
<td>90.90±1.17</td>
<td>90.20±1.44</td>
<td>91.06±1.29</td>
<td>88.25±1.98</td>
<td>89.53±2.15</td>
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<tr>
<td>2h</td>
<td>82.48±3.15</td>
<td>75.40±1.46</td>
<td>86.64±1.58</td>
<td>91.91±1.79</td>
<td>87.23±1.41</td>
<td>84.15±1.60</td>
<td>83.01±1.13</td>
</tr>
<tr>
<td>6h</td>
<td>62.61±1.14</td>
<td>38.65±3.73***</td>
<td>68.01±1.32</td>
<td>83.92±1.52***</td>
<td>80.55±1.66***</td>
<td>66.52±1.98</td>
<td>64.46±2.12</td>
</tr>
<tr>
<td>12h</td>
<td>53.08±3.04</td>
<td>21.88±2.07***</td>
<td>55.80±2.37</td>
<td>73.92±2.11***</td>
<td>71.88±2.22***</td>
<td>56.73±3.14</td>
<td>54.57±1.54</td>
</tr>
<tr>
<td>24h</td>
<td>41.15±1.81</td>
<td>10.45±1.67***</td>
<td>49.82±2.61</td>
<td>66.99±3.24***</td>
<td>65.34±2.23***</td>
<td>57.12±2.12***</td>
<td>52.03±2.27***</td>
</tr>
</tbody>
</table>

*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 Dunnet’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**

Over the past years, algae have emerged exhibiting a complex biological activity. Due to their broad range of effects, particularly with respect to antibacterial, anti-inflammatory protection and antioxidant mechanisms, *Chlamydomonas*, *Chlorella* or *Gelidiella* have attracted a widespread scientific and consumer interest (Fuentes et al., 2000; Annamalai and Nallamuth, 2014; Kightlinger et al., 2014). Different studies have reported that *Chlamydomonas* extracts are well absorbed and rapidly metabolized, while being well tolerated and no distinct toxicity was reported (Annamalai and Nallamuth, 2014; Kightlinger et al., 2014; Sobhani et al., 2015).

The CASA assessment showed a continuous decrease of spermatozoa motility in all groups over the course of a 24h *in vitro* culture (Table 1). The initial (Time 0h) MOT was higher in the experimental groups B-D (0.5-5 μg/mL *Chlamydomonas* extract) when compared to the control group (0 μg/mL *Chlamydomonas* extract), although without any statistical significance (P>0.05). A statistically significant motion-promoting effect of the *Chlamydomonas* extract became visible after 2h, specifically in the group C (P<0.05). Moreover, 50 μg/mL *Chlamydomonas* extract (group A) caused a non-significant decrease of the spermatozoa motility (P>0.05). After 6h, the decline of spermatozoa MOT became significant in the group A (P<0.001) in comparison with the control, while we observed a significantly higher spermatozoa motion in the experimental groups C and D (P<0.001).

Examination at 12h of *in vitro* culture showed that the spermatozoa motility was significantly increased in groups C and D (P<0.001) when compared to the control. At the same time, a significantly decreased motion was detected in the group A (P<0.001) in comparison to the control. At the end of the experiment (24h), the motility observed in the experimental groups supplemented with 0.1-10 μg/mL *Chlamydomonas* extract (experimental groups B-F) was significantly higher in comparison with the control (P<0.05 in case of group B; P<0.001 with respect to groups C-E; P<0.01 in relation to the group F).

Meanwhile, MOT was significantly decreased in the group A (P<0.001), supplemented with the highest concentration of the *Chlamydomonas* extract (50 μg/mL) after a comparison with the Ctrl group (Table 1).

According to the MTT assay, instant *Chlamydomonas* supplementation (Time 0h and 2h) had no significant effects on the sperm cell viability in any of the experimental groups (P>0.05; Figure 1).
chlorophyll-containing algae on sperm motility and mitochondrial activity after flavonoid administration has shown to possess a potent human sperm motility promoting effect on the bovine spermatozoon, alongside with statistically significant results (P<0.05). It has been previously stated that Chlamydomonas contains a variety of flavonoids, such as isoflavones, flavanones, flavonols and dihydrochalcon (Khoddami et al., 2013), all of which have been extensively studied for their potential roles on spermatogenesis or in vitro sperm survival. Improved spermatozoa motility and mitochondrial activity after flavonoid administration was recorded in different studies on fresh and as frozen-thawed human semen. As shown by Ateşşahin et al. (2015) showing that following in vitro administration of 250 or 500 µg/ml of Sargassum extract the level of ROS notably declined in frozen-thaw human semen. Phenolic compounds have been repeatedly shown to have beneficial effects of the oxidative balance in male reproductive tissues and cells.
polyphenol administration led to significantly increased total antioxidant capacity, superoxide dismutase levels, as well as sperm percentage, viability, motility, accompanied by a decrease of malondialdehyde in rats, hence suggesting that flavonoids could be effective in enhancing healthy semen parameters.

**CONCLUSION**

Our results, although preliminary, support the evidence for the dose-dependent in vitro biological activity and scavenger potential of the *Chlamydomonas reinhardtii* extract against oxidative stress induced in bovine spermatozoa. The development of new culture media offering a better protection to spermatozoa from the oxidative damage and improve their energy requirements is absolutely necessary. *Chlamydomonas* extracts, in small amounts, could be used as a ROS scavenger and a metabolic promoting supplement, especially in routine andrology techniques such as in vitro fertilization, artificial insemination or spermatozoa cryopreservation. These results obviously cannot foresee a definitive *in vivo* outcome, since a direct impact of *Chlamydomonas* supplementation on male subfertility needs to be explored further. To translate our findings into routine practice, studies on the toxicity, pharmacokinetics and bioavailability of *Chlamydomonas* extracts in male reproduction are critical.

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