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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 ($P<0.001$ 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 $P<0.001$ 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 spermatozoon 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 (**Donnely *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 (**Khoddami *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 vaccum pump KNF N838.1.2KT.45.18, KNF, Germany). Crude extracts were dissolved in DMSO (Dimethyl sulfoxide; Sigma-Aldrich, St. Louis, USA)

to equal 100.4 µg/mL as a stock solution (**Michalko and Matušíková, 2012**; **Tvrdá *et al.*, 2015**).

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* extact (A – 50; 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, 37°C; Sefi Medical Instruments, Haifa, Israel) and immediately assessed. At least 1000 cells were evaluated in each sample (**Massanyi *et al.*, 200**8).

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-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, Thermo Fisher Scientific, Finland). The data are 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 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,) 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 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

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-inflamatory 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).

Table 1 Spermatozoa motility (%) in the absence (Ctrl) or presence (A-F) of the *Chlamydomonas reinhardtii* extract during different time periods (Mean±SEM; n=10)

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

*** ($P<0.001$); ** ($P<0.01$); * ($P<0.05$). Ctrl – 0; A – 50; B – 10; C – 5; D – 1; E – 0.5; F – 0.1 µg/mL *Chlamydomonas reinhardtii* extract

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).

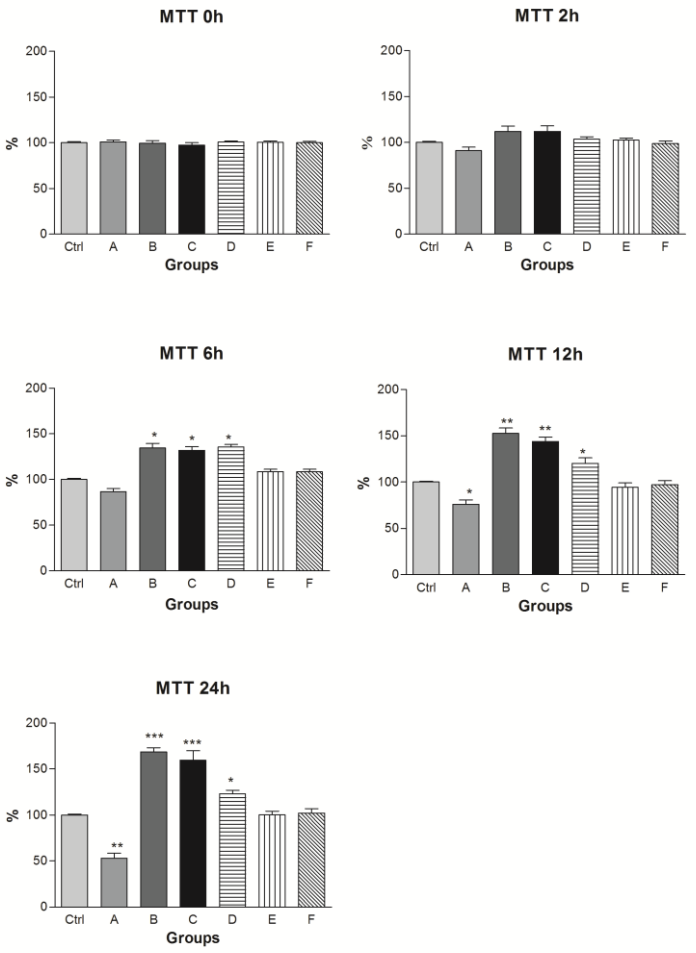

Figure 1 The effect of various concentrations of the *Chlamydomonas reinhardtii* extract on the viability of bovine spermatozoa (n=10) at 0h, 2h, 6h, 12h and 24h. Each bar represents mean (±SEM) optical density as the percentage of controls, which symbolize 100%. The data were obtained from five independent experiments. The level of significance was set at *** P<0.001; ** P<0.01; * P<0.05. Ctrl – 0; A – 50; B – 10; C – 5; D – 1; E – 0.5; F – 0.1 µg/mL *Chlamydomonas reinhardtii* extract.

At 6h it was revealed that 1-10 µg/mL extract (groups B-D) had a viability promoting effect on the bovine spermatozoon, alongside with statistically significant results (P<0.05) when compared to the control group (Figure 1). These stimulating effects remained visible and statistically relevant throughout time periods of 12h (P<0.01 in relation to groups B and C; P<0.05 with respect to group D), as well as 24h (P<0.05 in case of group D; P<0.001 with respect to groups B and C). Similar to the CASA analysis, the MTT test revealed an inhibition of the cell viability followed by the administration of 50 µg/mL *Chlamydomonas* extract (group A), particularly during long-term timeframes of the in vitro culture (P<0.05 with respect to Time 12h; P<0.01 with regards to Time 24h).

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 as well as frozen goat, mouse and human semen (**Purdy *et al.*, 2004**; **Mazzi *et al.*, 2011**; **Tung *et al.*, 2014**). Furthermore, **Ibrahim *et al.* (2014)** have shown that flavonoids isolated from diverse natural sources possess a protective effect against DNA damage in murine sperm. At the same time, a sulfono glycolipid (S-ACT-1) isolated from *Gelidiella acerosa* has shown to possess a potent human sperm motility stimulating activity *in vitro* with the potential to be developed into a sperm stimulant (**Premakumara *et al.*, 2001**). The analysis of sperm parameters in the study of **Sobhani *et al.* (2015)** demonstrated that the general and advanced motility of frozen-thawn human spermatozoa significantly increased following incubation with the extract of the *Sargassum* brown algae. On the other hand, twelve seaweeds were screened for *in vitro* spermicidal activity in the report by **Prakash *et al.* (2014)**. Among these twelve seaweeds, *Halimeda gracilis* showed 100% inhibition of human spermatozoa at 10 µg/ml in 20 s. Furthermore, dose- and time-dependent spermicidal assay revealed that the sperm was completely immobilised for 20 s. Plasma membrane of sperm was damaged due to the exposure of *H. gracilis* extract. MTT assay with *H. gracilis* extract showed 88.5% of cytotoxic incidence.

Although the *Chlamydomonas* extract had no instant effects on the oxidative balance within the *in vitro* spermatozoa culture (P>0.05; Time 0h and 2h; Figure 2), experiments following a 6h cultivation revealed that the administration of 5 and 10 µg/mL extract led to a significant decline of the superoxide formation in comparison to the control (P<0.05). *Chlamydomonas* extract concentrations ranging from 1 to 10 µg/mL (groups B-D) exhibited a long-term and statistically significant antioxidant protection of spermatozoa and a subsequent prevention of the escalating intracellular superoxide production, considered to be the first step in the generation of oxidative stress (P<0.01 in case of 10 µg/mL, and P<0.05 with respect to 5 µg/mL at Time 12h; P<0.01 in terms of 5-10 µg/mL, P<0.05 with respect to 1 µg/mL at Time 24h). On the other hand, high (group A) concentrations of *Chlamydomonas* exhibited pro-oxidant properties reflected in a significant superoxide overgeneration, staring at Time 12 (P<0.05) and deepening the detrimental effects in a time-dependent manner (P<0.01 with respect to Time 24h; Figure 2). Numerous studies have emphasized on the fact that algae possess significant antioxidant activities (**Annamalai and Nallamuth, 2014**; **Kightlinger *et al.*, 201**4). The antioxidant ability could be attributed to the exceptionally high content of phenolic compounds, particularly flavonoids with potent ROS-scavenging activities (**Anandakumar *et al.*, 2009**). Thus, *Chlamydomonas* extracts could be a promising natural source of antioxidants, possibly used in nutritional or pharmaceutical industry for the prevention of ROS-mediated diseases. Our NBT results complement the report by **Sobhani *et al.* (2015)** showing that following in vitro administration of 250 or 500 pg/ml of *Sargassum* extract the level of ROS notably declined in frozen-thawn human semen. Phenolic compounds have been repeatedly shown to have beneficial effects of the oxidative balance in male reproductive tissues and cells.

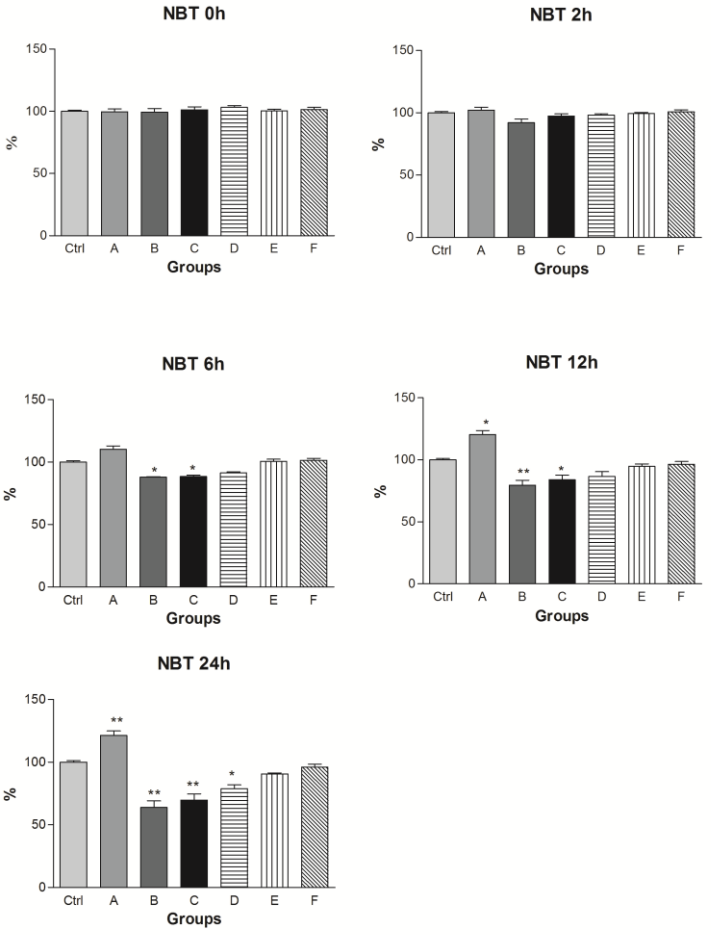

Figure 2 The effect of various concentrations of the *Chlamydomonas reinhardtii* extract on the spermatozoa (n=10) superoxide production at 0h, 2h, 6h 12h and 24h. Each bar represents the mean (±SEM) optical density as the percentage of controls, which symbolize 100 %. The data were obtained from five independent experiments. The level of significance was set at *** P<0.001; ** P<0.01; * P<0.05. Ctrl – 0; A – 50; B – 10; C – 5; D – 1; E – 0.5; F – 0.1 µg/mL *Chlamydomonas extract*.

As shown by **Ateşşahin *et al.* (2010)** biologically active compounds frequently found in *Chlamydomonas* were able to significantly decrease lipid peroxidation, restore glutathione synthesis and catalase activity, associated with normal spermatogenesis and sperm viability. In a different study (**Çeribaş *et al.*, 2012**),

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 scavenging 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* extract 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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REFERENCES

Agarwal, A. & Sekhon, L. H. (2010). The role of antioxidant therapy in the treatment of male infertility. *Human Fertility Cambridge*, 13(4), 217-225. http://dx.doi.org/10.3109/14647273.2010.532279

Alarcón de la Lastra, C. (2008). Curcumin: a promising spice for therapeutics. *Molecular Nutrition & Food Research*, 52, 985. http://dx.doi.org/10.1002/mnfr.200890036

Anandakumar, A. M., Paulsamy, S., Sathish Kumar, P. & Senthilkumar, P. (2009). Preliminary phytochemical studies for the quantification of secondary metabolites of medicinal importance in the plant *Acalypha fruticose* Forssk. *Journal of Applied and Natural Science* 1(1), 41-43.

Annamalai, J. & Nallamuthu, T. (2014). Antioxidant potential phytochemicals from methanol extract of *Chlorella vulgaris* and *Chlamydomonas reinhardti*. *Journal of Algal Biomass Utilization*, 5(4), 60-67.

Ateşşahin, A., Türk, G., Yilmaz, S., Sönmez, M., Sakin, F. & Ceribasi, A. O. (2010). Modulatory effects of lycopene and ellagic acid on reproductive dysfunction induced by polychlorinated biphenyl (Aroclor 1254) in male rats. *Basic & Clinical Pharmacology & Toxicology*, 106(6), 479-489. http://dx.doi.org/10.1111/j.1742-7843.2009.00529.x

Çeribaşı, A. O., Sakin, F., Türk, G., Sönmez, M. & Ateşşahin, A. (2012). Impact of ellagic acid on adriamycin-induced testicular histopathological lesions, apoptosis, lipid peroxidation and sperm damages. *Experimental and Toxicologic Pathology*, 64(7-8), 7171-724. http://dx.doi.org/10.1016/j.etp.2011.01.006

Donnelly, E. T., McClure, N. & Lewis, S. E. (1999). The effect of ascorbate and α-tocopherol supplementation *in vitro* on DNA integrity and hydrogen peroxide-induced DNA damage in human spermatozoa. *Mutagenesis*, 14(5), 502-512.

Sobhani, A., Eftekhaari, T. E., Shahrzad, M. E., Natami, M. & Fallahi, S. (2015). Antioxidant effects of brown algae *Sargassum* on sperm parameters. *Medicine (Baltimore)*, 94(52), e1938. http://dx.doi.org/10.1097/MD.0000000000001938

Fuentes, M. M. R., Acién Fernández, G. G., Sánchez Pérez, J. A. & Guil Guerrero, J. L. (2000). Biomass nutrient profiles of the microalga *Porphyridium cruentum*. *Food Chemistry*, 70, 345-353. http://dx.doi.org/10.1016/S0308-8146(00)00101-0

Ibrahim, N. A., Arabi, O. & Kahil, M. A. (2014). The role of some medicinal plants in reducing sperm DNA damage of male mice. *Global Journal of Biology, Agriculture & Health Sciences*, 3(4), 7-10.

Khoddami, A., Wilkes, M. A. & Roberts, T. H. (2013). Techniques for analysis of plant phenolic compounds. *Molecules*, 18, 2328-2375. http://dx.doi.org/10.3390/molecules18022328

Kightlinger, W., Chen, K., Pourmir, A., Crunkleton, D. W., Price, G. L. & Johannes, T. W. (2014). Production and characterization of algae extract from *Chlamydomonas reinhardtii*. *Electronic Journal of Biotechnology*, 17, 14-18. http://dx.doi.org/10.1016/j.ejbt.2013.12.003

Knazicka, Z., Tvrda, E., Bardos, L. & Lukac N. (2012). Dose- and time dependent effect of copper ions on the viability of bull spermatozoa in different media. *Journal of Environmental Science and Health. Part A, Toxic/Hazardous Substances & Environmental Engineering*, 47, 1294-1300. http://dx.doi.org/10.1080/10934529.2012.672135

Massanyi, P., Chrenek, P., Lukáč, N., Makarevich, A. V., Ostro, A., Živčák, J. & Bulla, J. (2008). Comparison of different evaluation chambers for analysis of rabbit spermatozoa motility using CASA system. *Slovak Journal of Animal Science*, 41, 60-66.

Mazzi, L., Geminiani, M., Collodel, G., Iacoponi, F., Martini, S., Bonechi, C., Rossi, C. & Moretti, E. (2011). Quercetin and rutin: effects of two flavonoids on induced oxidative stress in human ejaculated sperm. *Journal of the Siena Academy of Sciences*, 3(1), 22-26. http://dx.doi.org/10.4081/jsas.2011.22

Michalko, J. & Matušíková, I. (2012). The role of glucanases in digestion of carnivorous plant *Drosera rotundifolia* L. *Journal of Microbiology, Biotechnology and Food Sciences*, 1(February Special Issue), 671-678.

Prakash, S., Ravikumar, S., Reddy, K. V. R. & Kannapiran, E. (2014). Spermicidal activity of Indian seaweeds: an *in vitro* study. *Andrologia*, 46, 408-416. http://dx.doi.org/10.1111/and.12096

Premakumara, G. A. S., Ratnasooriya, W. D., Tillekeratne, L. M. V., Amarasekare, A. S. & Atta-Ur-Rahman, A. (2001). Human sperm motility stimulating activity of a sulfono glycolipid isolated from Sri Lankan marine red alga *Gelidiella acerosa*. *Asian Journal of Andrology*, 3, 27-31.

Purdy, P. H., Ericsson, S. A., Dodson, R. E., Sternes, K.L. & Garner, D. L. (2004). Effects of flavonoids, silibinin and catechin on the motility of extended cooled caprine sperm. *Small Ruminant Research*, 55(1-3), 239-243. http://dx.doi.org/10.1016/j.smallrumres.2004.02.005

Saleh, R. A. & Agarwal, A. (2002). Oxidative stress and male infertility: from research bench to clinical practice. *Journal of Andrology*, 23(6), 737-752.

Salvador, N., Garreta, A. G., Lavelli, L. & Ribera, M. (2007). Antimcrobial activity of Iberian macroalgae. *Scientia Marina*, 71, 101-113.

Tung, N. H., Shoyama, Y., Wada, M. & Tanaka, H. (2014). Improved *in vitro* fertilization ability of mouse sperm caused by the addition of Licorice extract to the preincubation medium. *The Open Reproductive Science Journal*, 6, 1-7. http://dx.doi.org/10.2174/1874255601406010001

Tvrdá, E., Lukáč, N., Lukáčová, J., Kňažická, Z. & Massányi, P. (2013). Stimulating and protective effects of vitamin E on bovine spermatozoa. *Journal of Microbiology, Biotechnology and Food Sciences*, 2(1), 1386-1395.

Tvrdá, E., Michalko, J., Matušíková, I. & Lukáč, N. (2015). The *Drosera* extract as an alternative in vitro supplement to animal semen: effects on bovine spermatozoa activity and oxidative balance. *Scientific Papers: Animal Science and Biotechnologies*, 48(1), 68-75.