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In vitro Effects of *Salvia officinalis* on Bovine Spermatozoa

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Abstract*—In vitro* storage and processing of animal semen 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. This study compared the ability of different concentrations of the *Salvia officinalis* extract on the motility, mitochondrial activity, viability and reactive oxygen species (ROS) production by bovine spermatozoa during different time periods (0, 2, 6 and 24 h) of *in vitro* culture. Spermatozoa motility was assessed using the Computer-assisted sperm analysis (CASA) system. Cell viability was examined using the metabolic activity MTT assay, the eosin-nigrosin staining technique was used to evaluate the sperm viability and ROS generation was quantified using luminometry. The CASA analysis revealed that the motility in the experimental groups supplemented with 0.5-2 μg/mL *Salvia* extract was significantly lower in comparison with the control ($P<0.05$; Time 24 h). At the same time, a long-term exposure of spermatozoa to concentrations ranging between 0.05 μg/mL and 2 μg/mL had a negative impact on the mitochondrial metabolism ($P<0.05$; Time 24 h). The viability staining revealed that 0.001-1 μg/mL *Salvia* extract had no effects on bovine male gametes, however 2 μg/mL *Salvia* had a persisting negative effect on spermatozoa ($P<0.05$). Furthermore 0.05-2 μg/mL *Salvia* exhibited an immediate ROS-promoting effect on the sperm culture ($P>0.05$; Time 0 h and 2 h), which remained significant throughout the entire *in vitro* culture ($P<0.05$; Time 24 h). Our results point out to the necessity to examine specific effects the biomolecules present in *Salvia officinalis* may have individually or collectively on the *in vitro* sperm vitality and oxidative profile.

***Keywords*—**Bulls, CASA, MTT test, reactive oxygen species, sage, *Salvia officinalis*, spermatozoa.

I. Introduction

OXIDATIVE stress (OS) has become one of the main factors in the pathogenesis of male infertility [1], [2]. Spermatozoa were the first cell type reported to exhibit susceptibility to OS. The inability to restore the damage induced by OS coupled with cell membranes rich in polyunsaturated fatty acids (PUFAs), render spermatozoa to be highly susceptible to ROS-induced damage. Subsequently, a rapid loss of intracellular ATP causes mitochondrial and axonemal damage, decreased sperm viability, and increased mid-piece sperm morphological defects, all of which contribute to a decreased sperm motility [3], [4]. OS has become a great concern for clinicians and scientists as this programmed deterioration may lead to poor fertilization and embryonic development, pregnancy loss and birth defects [5]–[8].

Over the years, a great variety of medicinal plants have been used for the enhancement of male fertility [9]–[12]. Currently, medicinal plants are increasingly recognized worldwide as an alternative source of efficient and cost-effective biologically active compounds to synthetic medication, and used as primary health care remedies [13], [14].

Around 900 species of *Salvia* are known worldwide including *Salvia officinalis*, the most common species [15], which is native to Mediterranean countries and is commonly known as sage. Recent studies have found that Salvia has beneficial anxiolytic [16], anti-inflammatory [17], antioxidant [18] and antihyperglycemic properties [16]. Sage is used in the treatment of excessive lactation and salivation [19], profuse perspiration [15], anxiety, depression [15], [16], sterility and menopausal complications [20]. *Salvia* extracts furthermore exhibit disinfecting and antiseptic properties [15], and have antimicrobial effects against bacteria and fungi [21].

A detailed chemical examination of *Salvia* revealed that leaves collected from sage contain a vast diversity of chemical components including cineol, borneol, pinene, flavonoids, saponin, glycoside, resin, vitamin C and E, rosmarinic acid, chlorogenic acid, caffeic acid, steroids and tannin [15], [18]. The leaf extracts of *Salvia officinalis* have a significant antioxidant activity, largely attributable to various phenolic constituents including phenolic diterpenes carnosol and hydroxycinnamic acid derivatives, notably rosmarinic acid [18], [21], [22]. Such phytoconstituents are known to have protective effects on the structural integrity and functional activity in mammalian spermatozoa due to their energy-promoting and antioxidant properties [23]–[25]. Furthermore, *Salvia* species have been reported to improve male reproductive function in *in vivo* studies [26]–[28].

This study investigated the *in vitro* effects of *Salvia officinalis* L. on the motility, viability and oxidative profile of bovine spermatozoa.

II. Material and Methods

A. Plant Material

Leaves from *Salvia officinalis* L. were obtained from the Botanical Garden at the Slovak University of Agriculture in Nitra. After drying, the plant tissues were crushed, weighed and soaked in ethanol p.a. (96%, Centralchem, Bratislava, Slovak Republic) during two weeks at room temperature in the

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dark. Exposure to sunlight was avoided to prevent the degradation of active components. The ethanolic plant extracts were subjected to evaporation under reduced pressure at 40 °C in order to remove any residual ethanol (Stuart RE300DB rotary evaporator, Bibby Scientific Limited, UK, and vacuum pump KNF N838.1.2KT.45.18, KNF, Germany). Crude plant extracts were dissolved in DMSO (Dimethyl sulfoxide; Sigma-Aldrich, St. Louis, USA) to equal 100.4 mg/mL as a stock solution.

B. Sample Collection and Processing

Bovine semen samples were obtained from 10 adult Holstein breeding bulls (Slovak Biological Services, Nitra, Slovak Republic). The animals were of similar age and were kept under uniform feeding and housing conditions. Two samples were obtained from each bull on a regular collection schedule with the help of an artificial vagina. Subsequently, sperm concentration and motility was evaluated using phase-contrast microscopy (200 x). Only semen samples with a minimum 70% progressive motility and $1x10^9$ sperm/mL were used for the scheduled experiments.

Each sample was diluted in physiological saline solution (PS; sodium chloride 0.9 % w/v; Bieffe Medital, Italia) containing different concentrations of the Salvia extract (0.01, 0.05, 0.1, 0.5, 1 and 2 µg/mL) using a dilution ratio of 1:40. The samples were cultured at laboratory temperature (22-25°C). The control (Ctrl) group (medium without *Salvia* supplementation, containing 0.5% DMSO) was compared with the experimental groups.

C. Spermatozoa Motion Analysis

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

D. Spermatozoa Viability

Eosin-nigrosin staining method was used to evaluate the functional integrity of the sperm membrane, based on the ability of eosin to penetrate into non-viable cells [29]. Ten µL of each sample were placed on a tempered glass slide, mixed with 20 µL 5% eosin (Sigma-Aldrich), followed by 20 µL 10% nigrosin (Sigma-Aldrich). The mixture was smeared on a glass slide and let air dry at 37 °C. The slides were observed using bright filed microscopy at 1,000 x magnification and with oil immersion. At least 200 spermatozoa per slide were evaluated and identified as either dead (with red heads) or live (with white heads) and expressed as a percentage rate. All slides were labeled and assessed by one observer.

E. Mitochondrial Activity (MTT Test)

Viability of the cells exposed to Salvia was evaluated by the metabolic activity (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT test. This colorimetric assay measures the conversion of a yellow tetrazolium salt (MTT) to blue formazan particles by mitochondrial succinate dehydrogenase of intact mitochondria within living cells. Formazan can be measured spectrophotometrically.

The MTT tetrazolium salt (Sigma-Aldrich) was dissolved in phosphate-buffered saline (Dulbecco's PBS; Sigma-Aldrich) at 5 mg/mL. Ten microliters of the solution was added to the cells (in 100 µL medium per well). After 2 h of incubation (shaker, 37 °C, 95% air atmosphere, 5% CO_2), the cells and the formazan crystals were dissolved in 150 µL of acidified (0.08 M HCl; Centralchem) isopropanol (Centralchem). The optical density was determined at a wavelength of 570 nm against 620 nm as reference by a microplate ELISA reader (Anthos MultiRead 400, Austria). The data were expressed as percentage of the control, set to 100% [30].

F. ROS Generation

ROS levels in samples were assessed by the chemiluminescence assay using luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione; Sigma-Aldrich) as the probe [31]. The test samples consisted of luminol (10 µL, 5 mM) and 400 µL of control or experimental sample. Negative controls were prepared using 400 µL Dulbecco's PBS (Sigma-Aldrich). Positive control included 400 µL Dulbecco's PBS and 50 µL of hydrogen peroxide (30%; 8.8 M; Sigma-Aldrich) in triplicates. Chemiluminescence was measured on a 48-well plate for 15 min using the Glomax Multi$^+$ Combined Spectro-Fluoro-Luminometer (Promega, Madison, WI, USA). The results were expressed as relative light units (RLU/sec/10^6 sperm) [31], [32].

G. Statistical Analysis

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. As we focused to study the impact of different Salvia concentrations on the spermatozoa activity (experimental groups) in comparison to the control at a specific time frame, thus taking one factor into consideration, one-way ANOVA was used for specific statistical evaluations. Dunnett test was used as a follow-up test to ANOVA, based on a comparison of every mean to a control mean, and computing a confidence interval for the difference between the two means. The level of significance was set at $P<0.05$.

III. Results and Discussion

Over the past years, natural compounds isolated from plants 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, plant extracts have attracted a widespread scientific and consumer interest [12]–[15].

Different in *vivo* studies have reported that *Salvia* extracts are well absorbed and rapidly metabolized, while being well tolerated and no distinct toxicity was reported [23], [26]–[28]. As such, we focused on the *in vitro* impact of *Salvia* extracts

on the structural and functional competence of male reproductive cells.

The CASA assessment showed a continuous decrease of spermatozoa motility in all groups over the course of a 24h in vitro culture (Fig. 1). The initial (Time 0h) MOT was higher in the experimental groups supplemented with 0.05-1 µg/mL *Salvia* extract when compared to the control group, although without any statistical significance (P>0.05). Similarly, non-significant differences among the control and experimental groups were recorded at Time 2h. A statistically significant motion-inhibiting effect of the *Salvia* extract became visible after 6h, specifically in the groups supplemented with 1 µg/mL and 2 µg/mL. At the end of the experiment (Time 24h), the motility observed in the experimental groups supplemented with 0.5-2 µg/mL *Salvia* extract was significantly lower in comparison with the control (P<0.05). *Salvia* concentrations ranging between 0.01 µg/mL and 0.1 µg/mL had no significant impact on the sperm MOT when compared to the control (Fig. 1).

According to the MTT assay, instant *Salvia* supplementation (Time 0h and 2h) had no significant effects on the sperm mitochondrial activity in any of the experimental groups (P>0.05; Fig. 2). At 6h it was revealed that 1 µg/mL and 2 µg/mL extract had a viability inhibiting effect on the bovine spermatozoon, alongside with statistically significant results (P<0.05) when compared to the control group. These inhibiting effects became more profound at 24h, as concentrations ranging between 0.05 µg/mL and 2 µg/mL had a negative impact on the mitochondrial metabolism (P<0.05).

Our results show an undesirable decrease in the sperm functional activity associated with the treatment by *Salvia officinalis* extracts which is in accordance with Al-Malaly *et al.* [24] who tested the effects of *Salvia officinalis* hot water extract on Awassi ram sperm activity. Three different concentrations were used (0.001, 0.005, and 0.1 mg/mL) and the sperm activity was evaluated at 0, 24, 48 and 72h. The results showed significantly decreased sperm activity associated with *Salvia* administration and a progressive decline in sperm vitality following 48h and 72h. On the other hand, the results are contrary to *in vivo* studies reporting on a significant enhancement of male fertility [23], [25], [26]. Such observations may be related to the direct effects of *Salvia* on the structure and function of the male reproductive system including an increased testicular and epididymal weight, increased germinal epithelium in the seminiferous tubules, increased sperm count, motility and fertilizing ability, as well as a decrease in morphological abnormalities. We may therefore hypothesize that the Salvia extract may have a direct inhibiting effect on the mitochondrial energy metabolism, a crucial factor supporting key spermatozoa motion. Inhibition of the mitochondrial function has a direct impact on ATP depletion which may lead to a decreased sperm motility, and subsequently male fertilizing capacity.

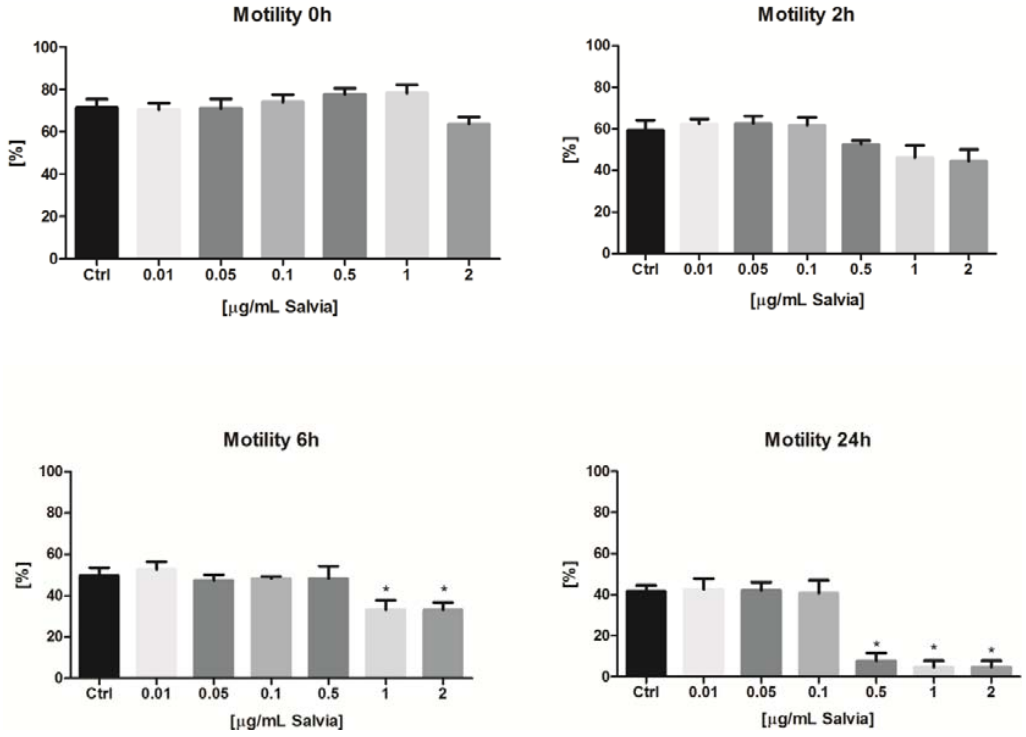

Fig. 1 Spermatozoa motility (%) in the absence (Ctrl) or presence of the *Salvia officinalis* extract in different time periods. Mean±SEM; *- P < 0.05

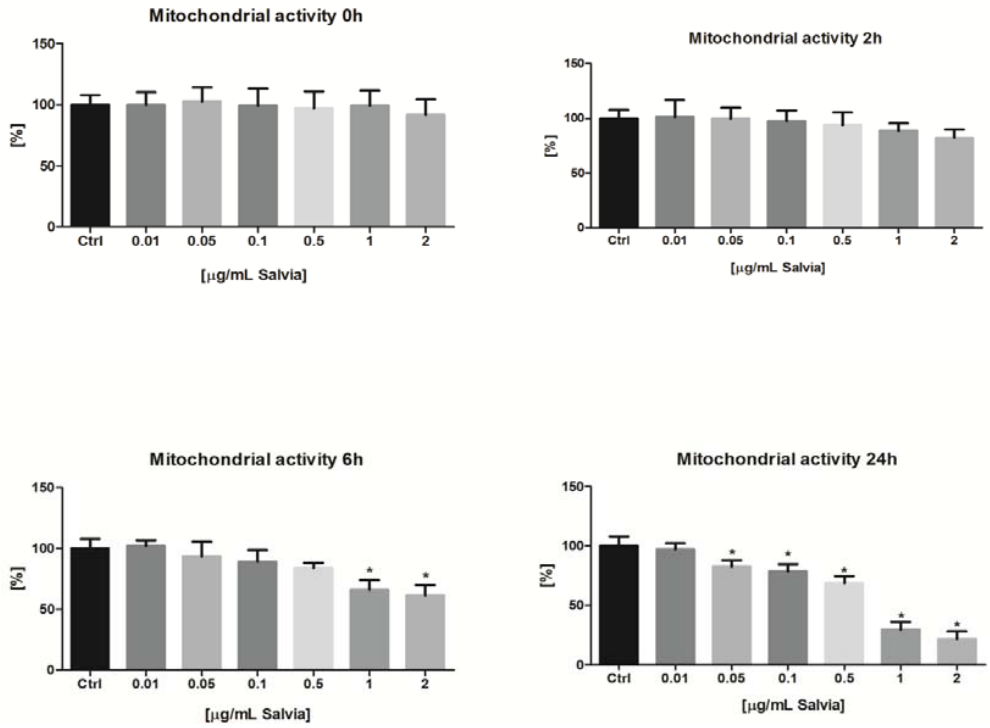

Fig. 2 Spermatozoa mitochondrial activity (%) in the absence (Ctrl) or presence of the *Salvia officinalis* extract in different time periods. Each bar represents mean (±SEM) optical density as the percentage of controls, which symbolize 100%. The data were obtained from five independent experiments. Mean±SEM; *- $P < 0.05$

The viability staining showed that 0.001-1 µg/mL *Salvia* supplementation to the sperm culture had no effects on the membrane integrity of bovine spermatozoa (Fig. 3). The vitality of spermatozoa decreased in a time-dependent manner however none of these concentrations caused any significant positive or negative effect on the structural integrity of the rabbit sperm membranes ($P>0.05$; Fig. 3). At the same time, 2 µg/mL *Salvia* had an instant negative effect on the membrane integrity of spermatozoa, which remained significant throughout all assessment times when compared to the control ($P<0.05$).

The luminometric analysis revealed that 0.05-2 µg/mL *Salvia* exhibited an instant ROS-promoting impact on the sperm culture ($P>0.05$; Time 0h and 2h; Fig. 4). Further experiments following a 6h and 24h cultivation confirmed this pro-oxidant properties of the plant extract, as the ROS production was significantly higher in the experimental groups administered with 0.05-2 µg/mL *Salvia*. At the same time, concentrations lower than 0.01 µg/mL *Salvia* had no significant effect on the ROS production by bovine spermatozoa ($P>0.05$; Fig. 4).

Oxidative stress (OS) has become one of the leading causes related to the loss of viable spermatozoa during cryopreservation. ROS over generation is nowadays accepted as a notable side effect of *in vitro* processing and handling protocols of semen, leading to major disruptions in the cellular oxidative metabolism. The resulting OS may subsequently lead to irreversible alterations of membrane structures via LPO, as well as oxidative degradation of proteins or DNA, followed by apoptotic activation [33].

Salvia extracts have been extensively studied for a broad range of biological properties, particularly its antioxidant characteristics. Hamdy *et al.* [18] evaluated the antioxidant properties and total phenolic composition of *Salvia* extracts. The study revealed that the most abundant biocomponents of the extract included rosmarenic acid, methyl rosmarenate, caffeic acid, cinnamic acid, chlorogenic acid and quinic acid ferulic acid, apigenin, luteolin and quercetin. Such phenolic acids and flavonoids possess a high antioxidant activity, which may be better than vitamin C, α-tocopherol and butylated hydroxyanisole.

Despite the rich antioxidant compounds constituting the *Salvia* extract, the concentrations used in this study had either no effects or a negative impact on the sperm oxidative balance, as well on the integrity of the plasma membrane – the key structure of spermatozoa most prone to oxidative insults. These observations may be attributed to the fact that perhaps high concentrations of the extract were used in the experiments.

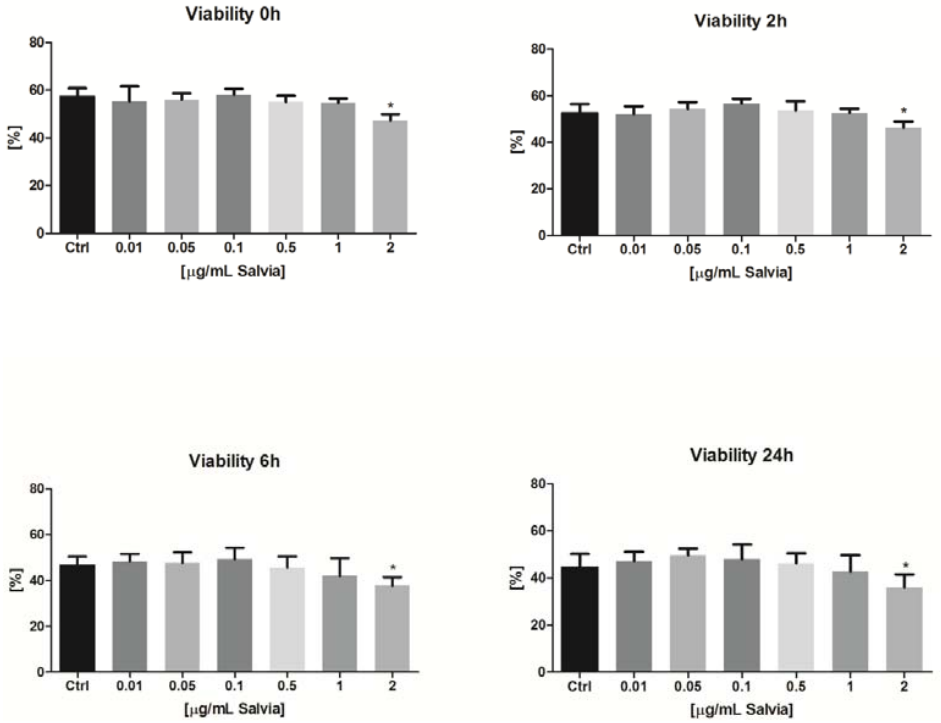

Fig. 3 Spermatozoa viability (%) in the absence (Ctrl) or presence of the *Salvia officinalis* extract in different time periods. Mean±SEM; n=20; *- P < 0.05

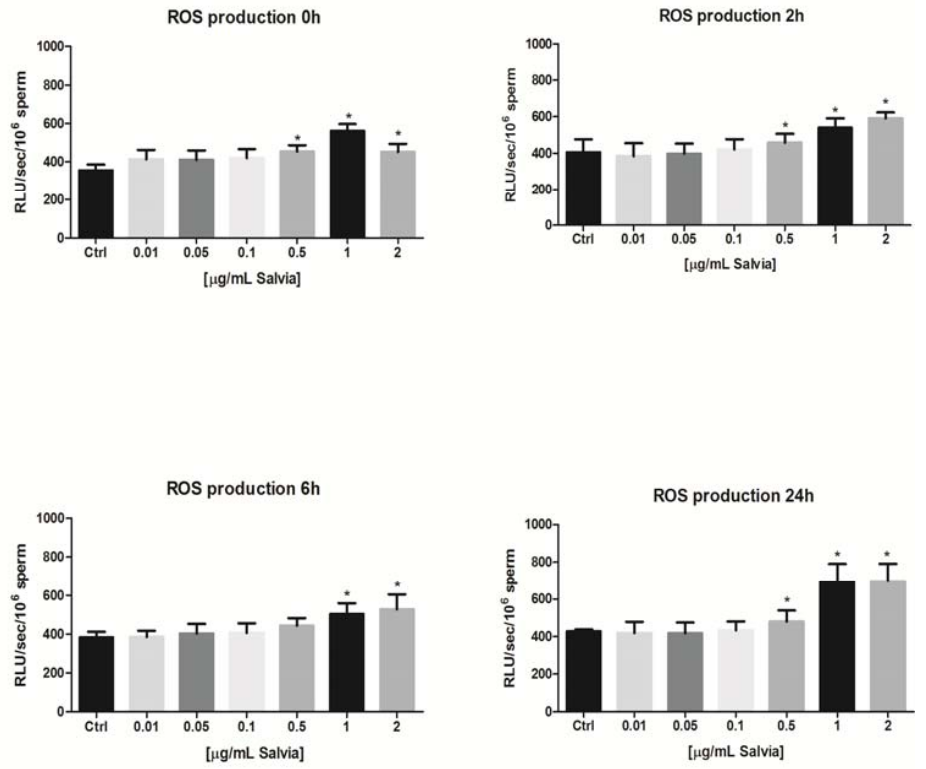

Fig. 4 ROS production by bovine spermatozoa (RLU/sec/10^6 sperm) in the absence (Ctrl) or presence of the *Salvia officinalis* extract in different time periods. Mean±SEM; n=20; *- P < 0.05

Aherne et al. [34] aimed to determine the effects of sage extract on the viability, membrane integrity, antioxidant status and DNA integrity of Caco-2 cells, as well as to investigate the cytoprotective and genoprotective effects of the extract against oxidative stress in Caco-2 cells followed by exposure to hydrogen peroxide (H_2O_2). *Salvia* extract revealed to be relatively toxic however it affected the antioxidant status of the cells by increasing the glutathione (GSH) content. At the same time, sage protected against H_2O_2-induced cytotoxicity and DNA damage.

The toxic and antioxidant protective effects of *Salvia officinalis* essential oil were evaluated on freshly isolated rat hepatocytes in the report by Lima *et al.* [35]. Cell viability, lipid peroxidation and GSH status were measured in cells exposed to the essential oil and in cells exposed to the essential oil and an oxidative compound (tert-Butyl hydroperoxide; t-BHP) together for 30 min. The results showed that the essential oil was not toxic when present at concentrations below 200 nL/mL; it was only at 2,000 nL/mL when a significant apoptosis and GSH decrease were observed indicating cell damage. On the other hand, in the range of concentrations tested, the essential oil did not show protective effects against t-BHP-induced toxicity.

Our data highly emphasize on the need to further examine the exact impact the biomolecules present *Salvia* extracts have individually or collectively on the *in vitro* sperm survival and oxidative balance. We may suggest that high concentrations of the *Salvia* extract may stimulate the activity of the mitochondrial respiratory chain of complex II, thus significantly increasing the risk of ROS overproduction. At the same time, high ROS concentrations often lead to lipid peroxidation, causing detrimental changes to the sperm plasma membrane, alongside with mitochondrial structures. These insults may lead to mitochondrial dysfunction and rupture, resulting in an increased ROS release into the surrounding environment. Lastly, the decreasing mitochondrial viability measured by the MTT test was mirroring the increasing ROS production detected by luminometry, based on which we may assume that a significant amount of ROS could be leaking from dysfunctional mitochondria.

IV. Conclusions

Our results, although preliminary, provide more evidence for the biological activity of the *Salvia officinalis* extract on male reproductive cells. The development of new culture media offering a better protection to spermatozoa against oxidative damage and improving their energy requirements is absolutely necessary. Our results obviously cannot foresee a definitive *in vivo* or *in vitro* outcome since a direct impact of *Salvia* extract supplementation on male subfertility needs to be explored further. To translate our findings into practice, studies on the toxicity, pharmacokinetics and bioavailability of *Salvia officinalis* extracts in male reproduction are critical.

Acknowledgment

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