Scientific Research Publishing

Advances in Bioscience and Biotechnology, 2019, 10, 86-97
http://www.scirp.org/journal/abb
ISSN Online: 2156-8502
ISSN Print: 2156-8456

Effect of Wild Marjoram (*Origanum vulgare*) Plant Extracts on Capacitation of Sheep Spermatozoa *in Vitro*

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How to cite this paper: Alenezy, E.S., Barakat, I.A.H. and Al Musayeib, N.M. (2019) Effect of Wild Marjoram (*Origanum vulgare*) Plant Extracts on Capacitation of Sheep Spermatozoa *in Vitro*. *Advances in Bioscience and Biotechnology*, **10**, 86-97.
https://doi.org/10.4236/abb.2019.104006

Received: March 12, 2019
Accepted: April 19, 2019
Published: April 22, 2019

Abstract

The purpose of this study was to evaluate the effect of the addition of *Origanum vulgare* extract to *in vitro* capacitation sperm medium (IVCSM). This study investigated the antioxidant and antimicrobial effects of *O. vulgare* extracts at different concentrations (0.3, 0.6, 1.2 µg/ml and 25.0, 50.0, 100.0 µg/ml, respectively) in IVCSM. Significant enhancements in semen quality parameters such as total motility, live and live capacitated sperm were found when *O. vulgare* extract was added as an antioxidant source (1.2 µg/ml). The treatment of spermatozoa with *O. vulgare* extract at the highest concentration (100 µg/ml) for 2 hrs without antibiotics improved sperm characteristics. In conclusion, incubation of sperm with *O. vulgare* extract in capacitation medium had beneficial effects on the characteristics of ram sperm.

Keywords

Sheep, Sperm, *Origanum vulgare*, *In Vitro*

1. Introduction

Globally sheep is important livestock providing both animal protein and milk, which are essential for their contribution to human nutritional needs and ability to provide food security. Therefore, increasing productivity using the appropriate modern technologies including *in vitro* embryo production (IVEP) techniques is necessary. In the future, IVP systems could play a crucial role in sheep production by accelerating sheep breeding and reinforcing the production po-

tency [1]. *In vitro* embryo production is a multi-step process consisting of: 1) *In vitro* maturation (IVM) of oocytes, 2) *In vitro* fertilization (IVF), and 3) *In vitro* culture (IVC) of zygotes up to the blastula stage [2]. In addition to the potential use of IVP technology in sheep breeding, embryos will also be needed for other purposes like cloning and transgenic animals production [3]. Although substantial progress has been achieved in the IVP field, the rate of *in vitro* embryo development is still lower than what is seen *in vivo* [4] [5].

Mahfouz *et al.* (2010) [6] proved that both quality (motility, viability) and functional integrity of sperm membranes decrease gradually during handling. It is also known that reactive oxygen species (ROS) are generated during sperm capacitation [7] [8]. Evans and Maxwell, (1987) [9] showed that ram sperm cell membranes are susceptible to the oxidative damage caused by ROS as they contain a high amounts of polyunsaturated fatty acids. Lamirande and Flaherty (2012) [10] showed that excessive ROS affects the genetic and functional integrity of spermatozoa. Oxidative stress (OS) results in reduced sperm quality and fertilization rates [9] [11], decreasing sperm motility [12] and reducing metabolic activity, longevity and viability [13]. *In vitro* spermatozoa have been preserved from the risk of OS by many different antioxidant sources [14].

In recent study, different plant extracts were used to improve ram semen quality [15] [16] [17]. The high oxygen concentrations *in vitro* lead to elevated ROS generation and, in turn, elevated OS [18]. Indeed, high levels of ROS cause deoxyribonucleic acid injury by disrupting the mitochondrial membranes causing the release of cytochromes and the activation of proteolytic enzyme cascades [19]. Recently, Kitagawa *et al.* (2004) [20] and Rocha-Frigoni *et al.* (2016) [21] confirmed that the increase in reactive oxygen species (ROS) levels during IVP induces oxidative stress in embryo cells, resulting in weak embryonic development and nonviable embryos. There are many studies where spices and herbs having high antioxidant activity have been used in semen processing [22] [23].

O. vulgare is a member of the plant family Lamiaceae (Labiatae) [24]. *O. vulgare* L. is widely known as a healthy and flavorful herb, and contains a large array of medicinally active compounds, as well as phenolic glucosides, flavonoids, tannins, sterols and a large concentration of terpenoids [25]. *O. vulgare* is the preferred spice plant for creating natural antioxidants [26]. Previous studies have shown that *O. vulgare* has vital chemicals and biological activities, including antibacterial, antifungal and anti-genotoxic effects [27] [28] [29] [30] [31] [32]. Moreover, several recent studies have shown that *O. vulgare* is one of the most important medicinal plants used in the treatment of many diseases due being rich in minerals and vitamins and containing a high percentage of vegetable estrogen [33] [34]. Sedigheh *et al.* (2015) [35] studied the effects of *O. vulgare*, Luteinizing Hormone-Releasing Hormone (LHRH)-A2, and estradiol-17β on the ultrastructure of gonadotroph cells and ovarian oogenesis in immature *Trichogaster trichopterus*, finding faster oogenesis in fish treated with *O. vulgare* after estradiol 17β treatment. In addition, *O. vulgare* extract affects the early

embryonic stages in pregnant rats [36]. Moreover, using *O. vulgare* as a dietary supplement, improves reproductive activity in sows [37]. *In vivo* treatment of mouse embryos during pre-implantation the stages with high doses of *O. vulgare* extract showed no toxic effects [38]. Luno *et al.* (2014) [39] also used *O.* vulgare extract as a source of antioxidants in boar sperm cryopreservation and found that it had beneficial effects on improved sperm function, fertilizing capacity, preventing lipid peroxidation and DNA oxidation of frozen boar sperm.

There are no reports on the use of *O. vulgare* extract as a source of antioxidant and antimicrobial in the *in vitro* capacitation sperm medium (IVCSM) for sheep sperm. Thus, this study is the first report to investigate the effects of *O. vulgare* in a supplemented capacitation medium for sperm in sheep.

2. Materials and Methods

2.1. Chemicals and Reagents

All chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sterile plastic culture dishes and Millipore membrane filters syringe were purchased from Nunclon, Nalge Nunc International, Roskide, Denmark.

2.2. Experimental Design

An *O. vulgare* stock solution was diluted with double distilled water (1 mg/ml) and stored at 4˚C until use. The *O. vulgare* extract was added to the capacitation medium of sperm at different concentrations. So, fresh semen was collected from a fertile healthy ram and was divided into six treatment groups plus a control group designated group one (G1). *O. vulgare* extract was added to the first three treatment groups (G2, G3, and G4) at different concentrations (0.3, 0.6, and 1.2 µg/ml) to be evaluated as an antioxidant source, and higher concentrations (25.0, 50.0, and 100.0 µg/ml) were added to the other three treatment groups (G5, G6, and G7) to evaluate its use as an antimicrobial.

2.3. Preparation of *O. vulgare* Extract

Flowering *O. vulgare* plants (**Figure 1**) were collected from Medina, Saudi Arabia. The taxonomic identification of plant materials was confirmed by a senior plant taxonomist (Dr. Mona Al Wahaibi, Herbarium Division, King Saud University, Riyadh, Saudi Arabia). The extract was prepared according to Braho *et al.* (2013) [40]. The obtained extracts were kept in sterile sample tubes and stored at −20˚C until use.

2.4. Sperm Capacitation

Fresh semen was collected from a ram with confirmed fertility. For *in vitro* capacitation, sperm was incubated for 2 hrs at 38.5˚C, with 5% CO_2 humidified air in Brackett and Oliphant (B.O) medium [41] with or without plant extract as

shown in the experimental design. After capacitation, the sperm were assessed using nigrosin-eosin staining. Spermatozoa for each treatment were counted for dead/live and capacitated/non-capacitated spermatozoa. This method is based on the increased membrane permeability of dead spermatozoa for the stain which leads to partial or complete purple stain in their heads, whereas the low permeability of live sperm excludes eosin and therefore their heads maintain a whitish color [42] Spermatozoa were classified into the following four categories (**Figure 2**):

1) Live capacitated spermatozoa (LCS)—light rose postacrosomal regions and white "acrosomal regions".

2) Live uncapacitated spermatozoa (LUCS)—the entire sperm head appeared light white with acrosomal regions.

Figure 1. Wild Marjoram (*O. vulgare*) plant after collection from the field.

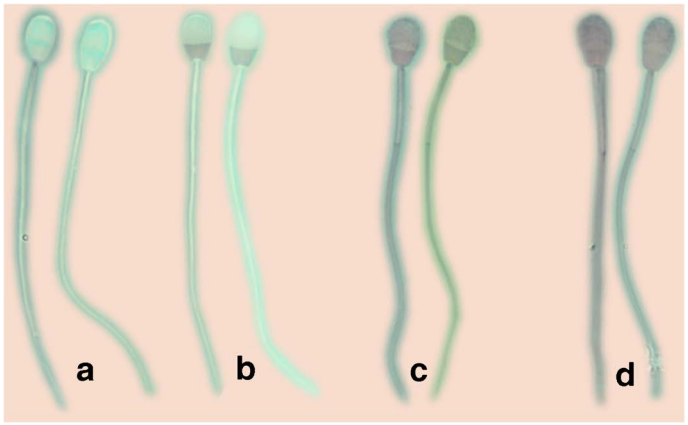

Figure 2. Different types of spermatozoa after *in vitro* capacitation in medium supplemented with *O. vulgare* extract.

3) Dead capacitated spermatozoa (DCS)—dark postacrosomal regions and pink acrosomes.

4) Dead uncapacitated spermatozoa (DUCS)—dark postacrosomal regions with an acrosomal region.

2.5. Statistical Analysis

Replicates of the experiments were performed on different days. Statistical analysis for all data was analyzed as a completely randomized design using IBM SPSS statistic program for windows, version 23.0 (IBM corporation. Armonk, NY, USA). Groups were compared using one way analysis of variance (One Way ANOVA). Statistical differences were considered significantly at $P \leq 0.05$ levels by using Duncan's Multiple Range Test. Results were expressed as mean ± SEM (Standard Error of Mean).

3. Results

3.1. Effects of *O. vulgare* Extract as an Antioxidant on the *in Vitro* Capacitation of Sheep Sperm

All the treatments produced significantly different live, dead, live un-capacitated, dead capacitated and sperm motility parameters (Table 1). Treatment with 1.2 µg/ml of *O. vulgare* extract in the sperm capacitation medium, had the highest rates of live, live capacitated, live un-capacitated and most motile sheep spermatozoa, followed by the 0.6 µg/ml treatment. The same concentration also gave the lowest means of dead, dead capacitated and dead un-capacitated spermatozoa as shown in Table 1.

3.2. Effect of *O. vulgare* Extract Concentrations as an Antimicrobial on the *in Vitro* Capacitation of Sheep Sperm

Sperm motility, viability, and capacitation after 2 hrs incubation in capacitation medium supplemented with different concentrations of *O. vulgare* extract are shown in Table 2. The mean number of live (269.00 ± 2.309) and live capacitated (127.67 ± 1.453) spermatozoa and the percentage that were motile (90.00 ±

Table 1. Effect of various concentrations of *O. vulgare* as an antioxidant on *in vitro* capacitation of sheep sperm (mean ± SEM).

Traits / Groups	Live (%)	Dead (%)	Live Capacitated (%)	Live un-Capacitated (%)	Dead Capacitated (%)	Dead un-Capacitated (%)	Motility (%)
Control (G I)	161.67 ± 4.91^{a} (53.89)	138.33 ± 4.91^{d} (46.11)	75.00 ± 2.89^{a} (25)	86.67 ± 2.03^{a} (28.89)	123.33 ± 2.03^{d} (41.11)	15.00 ± 2.89^{ab} (5)	25.00 ± 0.00^{a} (25)
0.3 µg/ml (G II)	174.00 ± 0.58^{b} (58)	126.00 ± 0.58^{c} (42)	80.00 ± 2.89^{a} (26.67)	94.00 ± 2.31^{b} (31.33)	106.33 ± 3.76^{c} (35.44)	19.67 ± 3.18^{b} (6.5)	32.67 ± 1.45^{b} (32.67)
0.6 µg/ml (G III)	188.00 ± 1.73^{c} (62.76)	112.00 ± 1.73^{b} (37.33)	89.00 ± 2.31^{b} (29.67)	99.00 ± 0.58^{b} (33)	94.33 ± 3.18^{b} (31.44)	17.67 ± 1.45^{b} (5.89)	40.00 ± 0.00^{bc} (40)
1.2 µg/ml (G IV)	213.67 ± 0.88^{d} (71.22)	86.33 ± 0.88^{a} (28.78)	107.00 ± 1.12^{c} (35.67)	106.67 ± 2.03^{c} (35.56)	80.33 ± 2.60^{a} (26.68)	6.00 ± 3.46^{a} (2)	47.33 ± 1.45^{d} (47.33)

*Mean values in the same columns with different superscripts (a, b, c, d) differ significantly ($p \leq 0.05$).

Table 2. Effect of *O. vulgare* extracts concentrations as antimicrobial source on the *in vitro* capacitation of sheep sperm (mean ± SEM).

Traits / Groups	Live (%)	Dead (%)	Live Capacitated (%)	Live un-Capacitated (%)	Dead Capacitated (%)	Dead un-Capacitated (%)	Motility (%)
Control (G I)	161.67 ± 4.91^{a} (53.89)	138.33 ± 4.91^{d} (46.11)	75.00 ± 2.89^{a} (25)	86.67 ± 2.03^{a} (28.89)	123.33 ± 2.03^{d} (41.11)	15.00 ± 2.89^{d} (5)	25.00 ± 0.00^{a} (25)
25 µg/ml (G II)	226.33 ± 4.91^{b} (75.44)	73.67 ± 4.91^{c} (24.56)	107.33 ± 4.33^{b} (35.78)	119.00 ± 58^{b} (39.67)	65.00 ± 2.89^{c} (21.67)	8.67 ± 2.03^{c} (2.89)	65.00 ± 0.00^{b} (65)
50 µg/ml (G III)	241.67 ± 4.91^{c} (80.56)	58.67 ± 4.91^{b} (19.44)	110.00 ± 5.77^{b} (36.67)	131.67 ± 0.88^{c} (43.89)	50.67 ± 3.48^{b} (16.89)`	7.67 ± 1.45^{b} (2.56)	72.67 ±1.45^{c} (72.67)
100 µg/ml (G IV)	269.00 ± 2.31^{d} (89.67)	31.00 ± 2.31^{a} (10.33)	127.67 ± 1.45^{c} (42.56)	141.33 ± 0.88^{d} (47.11)	27.00 ± 1.73^{a} (9)	4.00 ± 0.58^{a} (1.33)	90.00 ± 0.00^{d} (90)

*Mean values in the same columns with different superscripts (a, b, c, d) differ significantly at $p \leq 0.05$.

0.000) were highly significantly higher in group IV (100 µg/ml) than the other groups while the mean number of dead and dead capacitated spermatozoa (31.00 ± 2.309 and 27.00 ± 1.732, respectively) were significantly lower. There were however no significant differences in the number of dead uncapacitated spermatozoa between the treatments Table 2.

4. Discussion

Healthy embryos require good quality oocytes [1]. Three of the key issues encountered within culture systems are the intrinsic quality of the immature oocytes [43] oxidative stress [44] and substandard culture medium [45]. We are unaware of any reports on the effects of adding *O. vulgare* extract to IVCSM for sheep sperm. Our results showed that the addition of *O. vulgare* extract to the capacitation medium had a positive effect on sperm quality parameters, including motility, viability, and capacitation (acrosome integrity) of sheep sperm. Various cellular changes occur during sperm cell capacitation, including the activation of adenylyl cyclase increasing cAMP concentration, the inflow of Ca^{2+} ions and the generation of reactive oxygen species (ROS) [7] [10]. The high unsaturated fatty acid content within the cytomembrane and as well as the low anti-oxidant capability of seminal plasma makes boar spermatozoa particularly sensitive to the harmful effects of ROS [46] [47]. Extreme ROS leads to lipid peroxidation [48] resulting in a loss of motility and viability, injury to sperm DNA, insufficient oocyte penetration and sperm-oocyte fusion [49] [50]. Various components have been added as supplements to maintain motility and fertilization capability and to preserve the integrity of the sperm membrane [51] [52] [53] [54] these components have antioxidant activity and also reduce the process of oxidation [55]. We have shown that the addition of *O. vulgare* extract as an antioxidant source had a positive effect on sperm quality parameters, increasing their motility, viability, and number of live capacitated sperm. The methanol extract of *O. vulgare* is rich in phenolics, they comprise 22% of the extract, and phenolic acids e.g., rosmarinic acid and po-

lyphenols as well as other chemicals such as flavonoids [56]. Zhang *et al.* (2014) [57] isolated 21 phenolic compounds from *O. vulgare.* These components possess antioxidant activity and function as scavengers for free radicals and as chelators for metals [58]. Our results were supported by the findings of several studies on ram sperm quality after supplementation with extracts as antioxidants. Motlagh *et al.* (2014) [16] demonstrate that the addition of rosemary aqueous extract on post-thawed ram sperm at 2%, 4%, 6%, and 8% concentrations has a useful effect on post-thawed ram sperm characteristics. Furthermore, the addition of *Camellia sinensis* extract at 10 mg/L improves post-thawing quality of ram semen cryopreserved more than 5 or 15 mg/L [17]. The addition of different concentrations of *Syzygium aromaticum* extract (0, 35, 75, and 115 μg/ml) to ovine semen extenders has a useful effect on semen characteristics with 75 μg/ml of the extract having the best effect [59]. Our results indicated that the treatment of sheep spermatozoa with high concentrations (100 μg/ml) of *O. vulgare* extract improved various sperm parameters, these findings are in agreement with previous studies on the addition of antioxidants to ram semen, the addition of rosmarinic acid (RA) to cryopreserved sperm in a lactose-egg yolk buffer improved both the post thaw quality of boar spermatozoa and their ability to fertilize oocytes and at a higher concentration (105 μM/ml) the breakthrough rate [39]. Additionally, Malo *et al.* (2012) [60] found that using high concentrations of *Foeniculum vulgare* extract in a lactose-egg-yolk extender produced a significant improvement in total sperm motility and viability. The concentrations of the extracts differs due to different factors affecting the efficacy of the extract, such as plant source, collection season, air temperature, pH, type of solvent and extraction method [61]. Therefore, further studies are needed to determine the active compounds in the *O. vulgare* extract and which of their functions are responsible for its beneficial effects on sperm. During semen collection, it is difficult to avoid contamination with saprophytic bacteria from the prepuce or with bacteria from the surroundings [62] [63] [64]. Otter, (2008) [65] isolated bacteria from ram seminal samples with suspected infertility. *Escherichia coli* was found to have an effect on sperm cell motility due to adhesion and agglutination [66] [67] or by the induction of spermatozoon structural changes in the midpiece, membrane, and acrosome [68]. Several varieties of antibiotics, notably streptomycin and penicillin are added to seminal extenders to control bacterial growth [69]. The results of this study revealed that the motility, viability and the integrity of the acrosome membrane were significantly higher in fresh ram semen with various concentrations of *O. vulgare* extract than in the control group. Both aqueous and ethanolic *O. vulgare* leaf extracts have immunostimulant, cytotoxic, antibacterial and antioxidant properties [70]. In the light of our results, *O. vulgare* extract may also be a good alternative antibiotic to be included in culture media in *in vitro* embryo production systems, although its impact on sheep ram spermatozoa needs to be evaluated more fully.

5. Conclusion

The addition of *O. vulgare* extract to maturation medium for sheep spermatozoa leads to an increase in the number living sperm as well as an increase in the number of capacitated sperm.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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