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Effect of *Zataria multiflora* essential oil on rooster semen during storage at 4°C

Fahimeh Gerami Nezhad, Mohammad Roostaei-Ali Mehr

Animal Science Department, College of Agricultural Science, University of Guilan, Rasht, Iran

Keywords

antioxidant, essential oil, rooster sperm

Abstract

Experiment was conducted to determine effect of *Zataria multiflora* boiss essential oil on stored spermatozoa. Semen collection was performed by using 15 mature roosters twice a week at four times. In each session, ejaculates were pooled and split into seven parts. The amounts of 0 (EO0), 50 (EO50), 100 (EO100), 200 (EO200), 400 (EO400), 600 (EO600) and 1000 (EO1000) ng/ml *Zataria multiflora* boiss essential oil were added to each part. Samples were chilled to 4°C and maintained for 72 h. Sperm assessment was performed at 0, 24, 48 and 72 h. Lipid peroxidation was evaluated after 48 h. Results showed that there was no interaction between *Zataria multiflora* essential oil and incubation time on membrane integrity, sperm motility and viability ($p > 0.05$). The highest sperm progressive motility (80.43%), viability (86.31%) and functional membrane integrity (85.81%) was observed in EO200 ($p < 0.05$). The lowest sperm motility (61.31%) and viability (73.31%) was observed in EO1000 ($p < 0.05$). The concentrations of malondialdehyde was lowest in EO200 (0.17 nM/ml, $p < 0.05$). Therefore, addition of 200 ng/ml *Zataria multiflora* boiss essential oil to semen improved longevity of rooster spermatozoa at 4°C.

Abbreviations

RSA: radical scavenging activity
EO: essential oil
MDA: malondialdehyde
HOST: hypo-osmotic swelling test

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Tel: +98 51 3880 3742 *Fax:* +98 51 3876 3852
Web: ijvst.um.ac.ir *Email:* ijvst@um.ac.ir

Corresponding author:

Mohammad Roostaei-Ali Mehr
Animal Science Department, College of Agricultural Science, University of Guilan, Rasht, Iran

Email: roostaei@guilan.ac.ir
Tel +98 9111355262
Fax: +98 1333690281

Introduction

Nowadays, livestock breeders use artificial insemination to reduce costs, control sexual diseases and accelerate genetic improvement of the herd [1, 2-3]. Artificial insemination is well used in dairy cattle, pigs, sheep and commercial turkey breedings, but the use of this method is limited in poultry breeding due to cost and semen storage problems [4]. Artificial insemination may become cost-effective in broiler breeder management, since it would be possible to increase the insemination interval to 10-14 days (instead of 7 days) with a lesser concentration of sperm per insemination [5]. It is well known that fertility following the use of artificial insemination will be satisfactory if the quality of the stored sperm is kept well. However, there is greater willingness to develop liquid semen storage methods for economic and practical reasons in the poultry industry [6].

Diluting and cooling semen is necessary to store spermatozoa *in vitro*. Semen dilution decreases the concentration of seminal plasma antioxidants. Plasma membrane of birds' spermatozoa is rich in unsaturated fatty acids and phospholipids [7]. High concentration of polyunsaturated fatty acids in the sperm membrane causes extreme sensitivity to lipid peroxidation, being positively correlated with male infertility [8]. Supplementation of bird semen extenders with antioxidant may improve quality of the stored sperm.

Compounds in plant essential oils display antioxidant activity [9]. Moreover, these products seem to have lower side effects and can be regarded safer in cell biology analyses [10]. *Zataria multiflora*, known as Avishan Shirazi, is an aromatic plant whose antibacterial, antiviral, antifungal, acaricidal, and antioxidative activities have been demonstrated [11]. In addition, the essential oil of *Zataria multiflora* includes phenolic compounds, playing a role as scavengers of free radicals [12]. The objective of this experiment was to determine the effect of different levels of the essential oil of *Zataria multiflora* as a supplementation agent for standard extender (Sexton extender) on the longevity of refrigerated rooster spermatozoa.

Results

There was no interaction between *Zataria multiflora* essential oil and incubation time on membrane integrity, sperm motility and viability ($p > 0.05$). Membrane integrity, sperm motility and viability were higher in the group with 200 ng/ml essential oil than the control group (Table 1; $p < 0.05$). The lowest membrane integrity, sperm motility and viability were observed in the group with 1000 ng/ml essential oil ($p < 0.05$). There was no difference between the effects of 0, 50 and 100 ng/ml essential oil on membrane integrity, sperm motility and sperm viability ($p > 0.05$). Membrane integrity, sperm viability and motility were lower in the presence of 600 ng/ml essential oil than in the control group ($p < 0.05$). There was no difference between 0 and 400 ng/ml essential oil on membrane integrity ($p > 0.05$). The lowest MDA concentration was observed in the group with 200 ng/ml essential oil (Table 2, $p < 0.05$). Concentration of MDA was lower in groups with 50 and 100 ng/ml essential oil than the control group ($p > 0.05$). Concentration of MDA was higher in the groups with 400, 600 and 1000 ng/ml essential oil than the control group ($p > 0.05$).

Table 1.
Effect of *Zataria multiflora* essential oil on rooster sperm during storage in liquid form

Variables		Sperm motility (%)	Sperm viability (%)	Membrane integrity (%)
Zataria multiflora essential oil (ng/mL)	0	74.3 ± 1.73^{bcd}	82.4 ± 1.20^{b}	82.8 ± 0.88^{bc}
	50	76.8 ± 1.73^{abc}	83.6 ± 1.20^{ab}	83.6 ± 0.88^{ab}
	100	77.4 ± 1.73^{ab}	83.6 ± 1.20^{ab}	84.5 ± 0.88^{ab}
	200	80.4 ± 1.73^{a}	86.3 ± 1.20^{a}	85.8 ± 0.88^{a}
	400	72.1 ± 1.73^{cd}	80.5 ± 1.20^{bc}	80.5 ± 0.88^{cd}
	600	67.2 ± 1.73^{e}	77.2 ± 1.20^{c}	78.3 ± 0.88^{d}
	1000	61.3 ± 1.73^{f}	73.3 ± 1.20^{d}	75.0 ± 0.88^{e}
Incubation time (h)	0	82.6 ± 1.30^{a}	88.5 ± 0.91^{a}	88.4 ± 0.66^{a}
	24	76.9 ± 1.30^{b}	85.3 ± 0.91^{b}	84.7 ± 0.66^{b}
	48	70.9 ± 1.30^{c}	79.1 ± 0.91^{c}	79.3 ± 0.66^{c}
	72	60.7 ± 1.30^{d}	71.0 ± 0.91^{d}	73.6 ± 0.66^{d}

Different letters (a-e) within a column shows significant differences ($p < 0.05$).

Discussion

The spermatozoa metabolism does not completely stop at sub-ambient temperature, but its rate declines. Free radicals as toxic products of metabolism, may accumulate and damage sperm

Table 2.
Effect of *Zataria multiflora* essential oil on lipid peroxidation of rooster sperm after 48 h storage at 4°C

	(ng/mL)	Malondialdehyde (nM/10×10^6 sperm)
Zataria multiflora essential oil	0	0.81 ± 0.09^c
	50	0.50 ± 0.09^b
	100	0.49 ± 0.09^b
	200	0.17 ± 0.09^a
	400	1.07 ± 0.09^d
	600	1.21 ± 0.09^d
	1000	1.48 ± 0.09^e

Different letters (a-e) within a column shows significant differences ($p < 0.05$).

structure and function during cold liquid storage. It would be expected that quality of stored spermatozoa improves through scavenging free radicals during semen storage [13].

We observed that supplementation of semen extender with *Z. multiflora* essential oil was effective on concentration of MDA in a dose-dependent manner. *Z. multiflora* essential oil up to 200 ng/ml inhibited sperm lipid peroxidation, while the amount of ≥ 400 ng/ml of essential oil increased the MDA concentration. Incubation of mammalian cells with compounds obtained from other plants such as epigallocatechin-3-gallate [14], quercetin [15], and silymarin [16], was accompanied by similar results. *Z. multiflora* contains high levels of phenolic compounds [11]. These polyphenols play important role in absorption and neutralization of free radicals, quenching singlet oxygen [17-18]. Moreover, it was reported that the *Z. multiflora* essential oil had a potent radical scavenging activity [11]. However, it was reported that the free radicals absorbing capacity of antioxidants increased with concentration, only when their concentration was low, thus, acting as a strong oxidation stimulator at high concentrations [19]. It seems that *Z. multiflora* essential oil may act as prooxidant affecting inner cell membranes and organelles such as mitochondria at high concentrations [11].

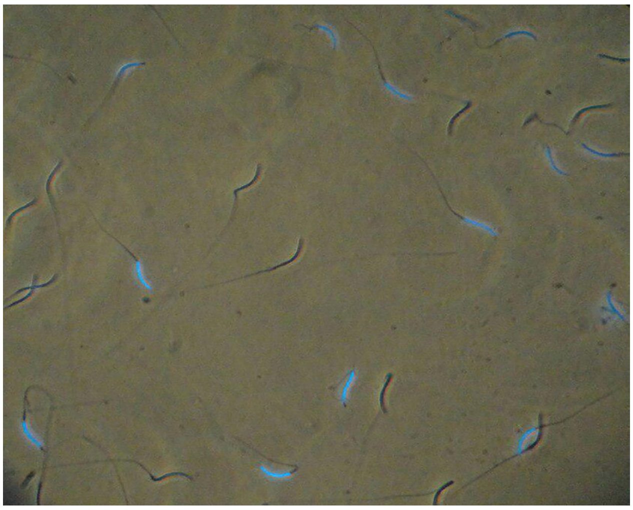

Figure 1.
Spermatozoa viability assessed by the fix vital stain method.

During the time of storage, membrane integrity, sperm viability and motility were declined, which was in agreement with study performed by other investigators [20]. Decrease in quality of stored spermatozoa in liquid form related to the accumulation of the toxic products of metabolism [21].

Sperm assessment showed that membrane integrity, sperm viability and motility were higher in 200 ng/ml *Z. multiflora* than the control group. Furthermore, the sperm quality decreased in the presence of ≥600 ng/ml *Z. mutiflora* essential oil during cooled liquid storage. In the present study, we found a dose dependence of the effects of *Z. mutiflora* essential oil on stored rooster spermatozoa in a cool liquid form. It has been shown that the exposure to essential oils could induce mitochondrial damage involving mitochondrial membranes and DNA [22]. However, it was reported that the use of the low level of herbal antioxidants such as resveratrol and quercetin improved sperm quality rate [23]. Furthermore, cell viability was reduced at high concentration (1000ng/ml) of *Z. multiflora* essential oil [11]. The main components of the essential oil of *Z. multiflora* were reported to be thymol (16%) and carvacrol (52%) and p-cymene (10%) [11]. It is well known that carvacrol has anti-oxidative and anti-apoptotic properties [24]. Low concentrations of carvacrol protected DNA from oxidative damage mediated by hydroxyl radicals from hydrogen peroxide, while its high concentrations increased DNA damage [25]. Additionally, thymol can interact with several proteins, phospholipids, cell membranes affecting membrane permeability, membrane potential and potassium fluxes [26]. Moreover, it was reported that some herbal volatile oils containing high level of thymol had spermicidal effects [27-28]. Thymol does not have protective effects on spermatozoa, and acts as potent immobilizing and spermicidal agent [29], whereas carvacrol, as one of the major components of the essential oil *Z. multiflora*, can protect sperm during storage. It has been suggested that the effects of thymol on sperm are partly masked by the effects of other compounds present in the essential oil [29]. It has also been mentioned that the low concentration of flavonoid improved cell survival and reduced apoptotic function, while higher concentrations increased apoptosis [15]. Therefore, it is possible that protective effects of *Z. mutiflora* essential oil at low concentration (up to 200 ng/ml) may be associated with proper concentration of monoterpenic phenol such as carvacrol. Moreover, it has been speculated that the toxic effect of thymol might decrease sperm quality at high levels of essential oil of *Z. multiflora*.

Conclusion

There was no interaction between *Zataria multiflora* essential oil and incubation time on the quality of rooster spermatozoa. *Z. multiflora* essential oil was effective on rooster spermatozoa in a dose-dependent manner. Supplementation of sperm extender with *Z. multiflora* essential oil up to 200 ng/ml improved quality of stored sperm, while the amount of ≥ 400 ng/ml of essential oil had detrimental effects.

Material and methods

Zataria multiflora essential oil compounds

Table 3.
Content of total polyphenols, flavonoids and antioxidant activity of *Zataria multiflora* essential oil

content/activity	
Total polyphenols content (mg of Gallic acid/mL of essential oil)	2.229 ± 0.003
Flavonoid content (mg of catechin/ mL of essential oil)	12.982 ± 0.04
Free radical scavenging activity (RSA %)	91.424 ± 0.034

Values are means of three replicates ± SD.

analysis

Zataria multiflora essential oil (Barij Essence Pharmaceutical Co, Iran) compounds analysis was performed using T80+ V/Visspectrometer (PG Instrument, Ltd). Determination of each compound was performed in three replicates. Table 3 presents the results of essential oil compounds analysis. Total polyphenols content of *Zataria multiflora* essential oil was determined using the Folin-Ciocalteu method [30]. Total flavonoids content of *Zataria multiflora* essential oil was determined by the following procedure of Park et al. [31].

The free radical scavenging activity (RSA) of essential oil was determined by using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay based on the method of Du et al. [11] with minor modifications. Briefly, 75 µL of essential oil was added to 925 µL of a 0.1 mM solution of DPPH in methanol. After the reaction was allowed to occur in the dark for 30 min, the absorbance at 517 nm was recorded to determine concentration of remaining DPPH. Inhibition of DPPH in percent (RSA %) of essential oil sample was calculated by the following formula:

$RSA\ (\%) = (Acont - Asamp)/Acont \times 100$ where Acont is the absorbance of control reaction, and Asamp is the absorbance of the tested sample.

Semen collection and preparation

Fifteen healthy fertile native Guilan roosters at the age of 32 weeks were used. The birds were kept in individual cages (1×1×1.5 m) for 10 weeks. They were also kept under uniform husbandry conditions with 14 h light/day, 80 (at beginning) to 90 (up to end) g/day food (protein: 12.7 %; energy metabolism: 2760 Kcal/Kg; Ca: 1.2%; P: 0.4 %) and water ad libitum. The animals kept and cared for under experimental procedures and protocols approved by the Veterinary Organization of Iran and were housed at the University of Guilan, Faculty of Agricultural Sciences, Education Research and Practice Farm, South of Rasht (it is located at 37°12´north latitude and 49°39´east longitude).

Zataria multiflora essential oil was dissolved in absolute ethanol (1 mg/ml) and then diluted up to 2 pg/ml by Sexton extender (0.64 g potassium citrate tribasic monohydrate, 8.07 g sodium-L-glutamate, 0.34 g magnesium chloride anhydrous, 5 g d-(−)-Fructose, 12.7 g potassium phosphate dibasic trihydrate, 0.65 g potassium phosphate monobasic, 3.95 g TES, 4.3 g sodium acetate trihydrate, 1 l distilled water, pH 7.3–7.4).

Semen samples were collected by abdominal massage with three-day intervals between sessions over six consecutive weeks. After ejaculation, the semen was diluted 0.5: 1 (v/v) with Sexton extender. The samples were immersed in 39°C water and transferred to the laboratory by Styrofoam box within 10 min after collection. Upon reaching the laboratory, evaluation of the samples was performed immediately. All diluted ejaculates were tested to possess acceptable progressive motility (>70%) and concentration (>3 × 10^9 sperm/ml).

In each session, the ejaculates (at least 10 collected ejaculates) were pooled and diluted to 4000 × 10^6 sperm/ml by Sexton extender. Diluted semen was split into seven parts and 0, 100, 200, 400, 600 and 1000 ng/ml *Zataria multiflora* essential oil were added. The final concentration of spermatozoa was 2000 × 10^6 cell/ml. The samples were cooled by Test Chamber (EG53AH, KATO, Saitama-ken, Honshu, Japan) to 4°C over 2 h (0.25°C/min) and incubated for 72 h. Sperm viability, motility and membrane integrity were evaluated at 0, 24, 48 and 72 h. After 48-hour incubation, lipid peroxidation level of sperm was measured by determining the malondialdehyde (MDA) production, using thiobarbituric acid [32]. Quantification of thiobarbituric acid reactive substances was determined by comparing the absorption with the standard curve of MDA equivalents generated by the acid catalyzed hydrolysis of 1,1,3,3-tetramethoxypropane (Sigma Aldrich, USA). The values of MDA were expressed as nM/10 × 10^6 sperm.

Sperm assessment

The concentration of spermatozoa was determined by means of a Neubauer haemocytometer. Spermatozoa viability was assessed by the fix vital stain method [13]. The sample was mixed with an equal volume of glutaraldehyde fixative solution (glutaraldehyde at 2% in phosphate buffered saline). Then, it was mixed with an equal volume of 20 µg/ml bisbenzimide H33258. A smear was prepared after 10 min of incubation at room temperature. Two hundred spermatozoa per smear were evaluated in three to seven different microscopic fields for each sample using an Olympus IX70 phase-contrast microscope (high-pressure mercury illuminator, UG1 excitation filter, U dichroic mirror, L420 barrier filter; Olympus, Tokyo, Japan). The procedure was performed by epifluorescence microscopy combined with bright-field illumination. Light intensity of the microscope was set at an optimum to visualize both spermatozoa and fluorescence of H33258-labelled nuclei. Sperm showing partial or complete blue color were considered dead, and colorless sperm were considered to be alive (Figure 1).

The percentage of sperm motility was assessed by phase-contrast microscopy (400× magnification) at a warm stage at 37°C. The samples were diluted with sexton extender up to 300×10^6 sperm/ml, and a wet mount was made using a 5 µL drop of semen placed directly on a microscope slide and covered by a cover slip. Sperm motility was estimated at least 5 different microscopic fields for each semen sample. The subjective estimations were approximated to the nearest 10 % by single technician. The mean of the successive estimations was recorded as the final motility score.

The hypo-osmotic swelling test (HOST) was used to evaluate functional integrity of the sperm membrane. HOST was performed by incubating 5 µL of semen with 500 µL of a 100 mOsm hypo-osmotic solution (7.35 g sodium citrate dihydrate and 13.51 g fructose in 1 L distilled water) at 37°C for 30 min. One drop of the mixture was placed on a pre-warmed slide, covered with a cover slip and examined under a phase-contrast microscope (400 × magnification). The sperm with swollen tails were considered intact. To assess the percentages of intact sperm, a total of 200 sperm in at least five different microscopic fields were evaluated.

Statistical analysis

Analysis of variance was performed to study effects of the treatments on motility, viability and plasma membrane integrity of spermatozoa using MIXED procedure of SAS (2002) with repeated measures data. The samples taken from pooled semen were considered as subjects in these experiments. Statistical model included concentration of *Zataria multiflora* essential oil, time and their interaction effect. Results of MDA concentration were analyzed by using the GLM procedure. When the analysis revealed a significant difference, comparison of treatment means was performed by Tukey's test. For all statistical tests, the level of statistical significance was chosen at $p < 0.05$.

Author contributions

FG performed the experiments and MRAM designed the research project and wrote the draft of manuscript.

Conflict of interest

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

None of the authors have any conflict of interest to declare.

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