

DNA Integrity, Acrosomal Integrity and Semen Characteristics Following Supplementation of Some Additives to Chilled and Frozen Rabbit Semen

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Abstract: The objective of the present study was to evaluate the impact of methionine and aqua extract of *Nigella sativa* on the preservation, acrosomal integrity, sperm DNA integrity and post-thawing motility of rabbit bucks. Methionine at the level of 1mM and 2mM and *Nigella sativa* at the level of 200 µl/ml extended semen were added to the extended semen and the success of preservation was checked daily, in a period of 3-days-long, for sperm motility (SM%), alive sperm (AS%), sperm abnormalities (SA%), acrosomal integrity and sperm DNA integrity. Furthermore, the influence of methionine and *Nigella sativa* extract on post-thawing motility, acrosomal and sperm DNA integrities were assessed. Results elaborated that the addition of methionine and *Nigella sativa* significantly ($P < 0.05$) improved SM% and AS% and reduced SA %. Adding methionine at both levels improved post-thawing motility ($46.0 \pm 0.58\%$ and $42.0 \pm 1.16\%$, respectively) vs. $41.67 \pm 1.67\%$ in the control group while the addition of *Nigella sativa* extract reduced that motility to $36.67 \pm 3.33\%$. The current investigation showed that the sperm DNA integrity % of rabbit spermatozoa in both chilled and frozen-thawed semen was higher and the frequency of acrosomal defects was lower in treated groups than in control. In conclusion, the addition of methionine and *Nigella sativa* extract to extended rabbit semen induced remarkable physiological effects on semen quality during conservation for 3-days-long period at 5°C, improved its motility, viability, DNA integrity and freezability of rabbit semen.

Key words: Methionine • *N. sativa* • DNA • Acrosome • Rabbit • Semen

INTRODUCTION

Conservation of the fertilizing capacity of fresh semen for the longest possible time is essential in the practice of artificial insemination (A.I) which becomes more favorable and most suitable for large commercial Rabbitries [1].

A basic problem with semen preservation is the high unsaturated fatty acid content of spermatozoal membrane which tends to bind oxygen resulting in the formation of numerous peroxide bonds. Lipid peroxidation induced by reactive oxygen species (ROS) directly damages the phospholipids components of cell membrane [2].

Mammalian semen contains antioxidants, such as glutathione [3] but these endogenous antioxidants may be inadequate to stop lipid peroxidation during cooled storage of spermatozoa [4].

During the processing of semen, the addition of antioxidants such as glutathione and ascorbic acid to equine sperm [4] and superoxide dismutase and catalase to ram sperm [5], melatonin hormone to extended goat semen [6] has been shown to protect sperm against harmful effects of reactive oxygen species and to improve sperm motility and membrane integrity during sperm liquid storage.

Methionine is a sulfur-containing amino acid that represents one of the total free amino acids in seminal plasma [7]. It acts also as a precursor amino acid for glutathione in the protection of cells from oxidative damage and plays a vital role in detoxification [8]. In addition, the thiol group of methionine was shown to chelate lead and remove it from tissues [9].

Singh *et al.* [10] recorded that feeding of extra methionine and lysine to buffalo bulls produced a

beneficial effect on semen quality and freezability. Furthermore, Nizza *et al.* [11] found that dietary supplementation with lysine and methionine increased alive sperm concentration and the motility of rabbit bucks' spermatozoa.

In vitro incubation of ram spermatozoa with selenomethionine significantly improved sperm motility and oxygen consumption [12]. Cryopreservation of ram semen in extenders fortified with methionine improved post-thaw motility and fertility of spermatozoa [13]. In a recent study, Khalifa [14] indicated that *in vitro* supplementation of buffalo semen extenders with methionine resulted in pronounced enhancement in post-thaw buffalo sperm motility, viability and plasma membrane integrity besides a clear reduction in the post-thaw sperm abnormalities.

Nigella sativa (Sativa) commonly known as black seed belongs to the botanical family of Ranunculaceae. It has been in use in Middle Eastern and Far Eastern countries as a natural remedy for over 2000 years. It was found that *N. sativa* improved the reproductive performance of rabbits [15]. The current study was designed to investigate the physiological effect of methionine and *N. sativa* on the quality of rabbit semen-preserved in both liquid and frozen conditions.

MATERIALS AND METHODS

This investigation was carried out at an Industrial Rabbitry near El-Badrashan city, Giza Province, Egypt.

Experimental Animals: Thirty six sexually mature Californian rabbit bucks (12 months age) were used in two experiments. Animals were fed *ad libitum* a commercial diet according to NRC [16] recommendations. All animals were kept under the same managerial and hygienic conditions.

Experimental Materials: Methionine and the other chemical reagents used for the preparation of extender were purchased from Sigma-Aldrich Co., Deisenhofen, Germany while *N. sativa* was bought from Haras shop for medicinal plants. *N. sativa* extract was prepared according to Riad *et al.* [17].

Semen Collection and Evaluation: Semen was collected twice weekly by means of an artificial vagina. After collections, the ejaculates were transferred to the laboratory of the farm within 2-3 minutes for evaluation by means of conventional methods.

Semen Extender: Tris glucose glycerol egg yolk (TGGY) was used for preserving the rabbit semen and it was prepared according to Roca *et al.* [18] for rabbit semen. It consisted of Tris-hydroxymethyl amino methane (3.801 g), glucose (0.6 g), citric acid monohydrate (2.166 g), glycerol (6.7 ml), fresh chicken egg yolk (10 ml), penicillin (100,000 IU), streptomycin (100 mg) and bi-distilled water to 100 ml.

Semen Processing and Experimental Design: Only ejaculates of >70% initial motility and 2000×10^6 sperm cells/ml were pooled and extended with TGGY at 1:4 extension rate and used in the following experiments:

Experiment I: This experiment was designed to explore the influence of methionine at 1 and 2 mM concentrations [19] on the preservation, acrosomal integrity and DNA integrity of chilled rabbit semen in TGGY extender. After dilution, the extended semen was incubated at 5°C to be examined daily for three successive days for:

SM%: A drop of semen was placed on a pre-warmed (37°C) glass slide and cover slipped. Visual motility was microscopically assessed (x 400; Olympus BX20, Japan) at 37°C with closed circuit television [20].

AS% and SA%: An Eosin-Nigrosin stained smear was prepared and total sperm abnormalities and viability were evaluated by examining 200 sperm cells according to the criteria described by Barth and Ako [21].

Acrosomal Integrity Examination: Acrosomal staining procedure followed the method of Kovacs and Foote [22]: equal drops of trypan blue and diluted semen at room temperature were mixed on slides with the edge of another slide and smeared; semen smears were air dried, slides were fixed for two minutes and then rinsed with tap and distilled water. The spermatozoa were stained in Geimsa for at least 3.5 h. Slides were rinsed with tap and distilled water and then held for two min in a jar of distilled water for the best differentiation. Finally the slides were dried in air and then examined after being covered with a cover slide. A total of 200 spermatozoa/ smear were evaluated with light microscopy at x 1000 magnifications.

Dna Integrity Using Acridine-Orange Staining Assay (AO): Sperm DNA integrity was assessed using the A fluorescence method [23]. Air-dried slides were fixed overnight in freshly prepared Carney's solution (three parts methanol and one part glacial acetic acid)

and allowed to air dry for a few minutes. Dried slides were restained for 3 min with AO. The stained slides were evaluated immediately under a fluorescence microscope. Normal DNA content showed green fluorescence over the head region, while DNA abnormalities showed varying fluorescence (from yellow-green to red). At least 100 spermatozoa per smear were evaluated for DNA abnormalities [23]. Sperm cells with changes in fluorescence from yellow-green to red were recorded as sperm with abnormal DNA content.

Experiment II: This experiment was established to find out the impact of *N. sativa* extract on the conservation and acrosomal integrity of chilled rabbit semen in TGGY extender. Semen samples were processed as previously mentioned in experiment I then *N. sativa* extract at the level of 200 µl/ml extended semen [17] was added to the extended semen.

Experiment III: This experiment was designed to investigate influence of methionine (1 and 2 mM) and *N. sativa* extract (at the level of 200 µl/ml extended semen) on the post-thawing motility, DNA and acrosomal integrity of rabbit semen extended in TGGY. Semen samples were split, diluted 1:4 and then left to be cooled at the rate of 0.3°C/min at the cooling cabinet to reach 5°C within 60 min. Semen was then filled in 0.25 ml French

straws and automatically sealed. Straws were placed on a rack and left at a cooled cabinet at 5°C for an equilibration time of 2 h. They were then frozen, at a height of 4 cm above the liquid nitrogen (in nitrogen vapor, -120°C) for 15 min. before being plunged into liquid nitrogen for storage.

After few weeks, frozen rabbit semen was thawed in a water bath at 39°C for 30 seconds. The thawed semen was emptied in pre-warmed tubes and incubated in water bath at 30°C for assessment of sperm motility [6].

Statistical Analysis: Data were expressed as mean \pm standard errors of the mean then were analyzed using ANOVA test at a confidence limit not less than 95% using SPSS Version 16. LSD test was used to evaluate the significant difference between means at $P < 0.05$.

RESULTS

The current results showed that the addition of methionine to the extended buck semen, significantly increased ($P < 0.05$) the SM % (Table 1) and AS % (Table 2) and decreased the SA % (Table 3) during the liquid storage of extended rabbit semen for 3 days while adding *N. sativa* extract improved previous parameters non significantly as compared with the control group.

Table 1: Effect of various additives on motile sperm% during incubation of rabbit semen at 5°C in tris-glucose-glycerol-egg yolk extender (TGGY)

Type of Additives	Time				Overall mean treatment
	Days				
	0	1	2	3	
Control	86.67±1.67 ^b	70.0±2.89 ^c	55.0± 2.89 ^d	43.33±1.67 ^d	63.75
1 mM methionine	91.67±0.88 ^a	83.33±3.33 ^b	66.67±1.66 ^c	60.0±2.88 ^c	75.42
2 mM methionine	93.67±1.86 ^a	81.67±4.42 ^b	65.0±2.88 ^c	60.0±2.89 ^c	75.08
Nigella sativa	88.33±1.68 ^a	75.0±2.88 ^c	63.33±1.67 ^c	58.33±1.66 ^d	71.24
Overall mean days	90.08	77.5	62.5	55.42	

Different superscript in rows are significantly different at least at $P < 0.05$

Table 2: Effect of various additives on dead sperm% during incubation of rabbit semen at 5°C in tris-glucose-glycerol-egg yolk extender (TGGY)

Type of Additives	Time				Overall mean treatment
	Days				
	0	1	2	3	
Control	7.33±1.45 ^a	30.0±2.89 ^c	41.67± 1.67 ^d	53.33±1.67 ^d	33.08
1 mM methionine	5.67±1.20 ^a	16.67±3.3 ^b	33.33±1.67 ^c	40.0±2.88 ^d	27.59
2 mM methionine	6.33±1.86 ^a	18.33±4.1 ^b	35.0±2.89 ^c	40.0±2.89 ^d	23.92
Nigella sativa	7.0±1.0 ^a	25.0±2.88 ^b	36.67±1.66 ^c	41.67±1.67 ^d	24.91
Overall mean days	6.58	22.5	36.67	43.75	

Different superscript in rows are significantly different at least at $P < 0.05$

Table 3: Effect of various additives on abnormal sperm% during incubation of rabbit semen at 5°C in tris-glucose-glycerol-egg yolk extender (TGGY)

Type of additives	Time			Overall mean treatment
	Days			
	1	2	3	
Control	8.0±1.53 ^b	13.33±0.88 ^c	18.0±0.58 ^d	13.11
1 mM methionine	6.33±0.88 ^a	9.33±0.88 ^b	11.33±0.88 ^c	10.11
2 mM methionine	7.0±0.58 ^a	8.0±0.58 ^b	11.67±0.67 ^c	8.97
Nigella sativa	7.0±1.0 ^a	11.33±0.33 ^c	12.0±0.58 ^c	8.89
Overall mean days	7.08	10.5	13.25	

Different superscript in rows are significantly different at least at P<0.05

Table 4: Effect of various additives on post-thawing motility and acrosomal integrity of cryopreserved rabbit semen

Type additives	Post-thawing	
	Motility % of cryopreserved rabbit semen	Acrosomal damage of cryopreserved rabbit semen
Control	41.67±1.67 ^b	13.0±1.53 ^b
1 mM methionine	46.0±0.58 ^a	9.0±1.0 ^a
2 mM methionine	42.0±1.16 ^b	10.0±1.0 ^a
Nigella sativa	36.67±3.33 ^c	14.33±2.03 ^b
Overall mean treatment	41.58	11.58

Different superscript in rows are significantly different at least at P<0.05

Table 5: Sperm DNA integrity %of chilled and frozen-thawed rabbit semen supplemented with methionine&Nigella sativa and stained with 1.0% Acridine orange (AO)

Type of additives	Type of semen			
	Chilled extended rabbit semen			Post-thawed rabbit semen
	Day 1	Day 2	Day 3	-
Control	84.20±2.89 ^b	77.0±2.89 ^c	76.0±1.67 ^c	69.60±1.33 ^b
1 mM methionine	94.2±2.61 ^a	81.60±1.67 ^b	79.20±1.66 ^c	80.40±1.72 ^a
2 mM methionine	91.60±3.33 ^a	79.60±1.66 ^b	78.80±2.88 ^c	77.80±1.62 ^a
Nigella sativa	85.80±4.42 ^b	79.20±2.88 ^c	76.60±2.89 ^c	72.20±2.06 ^b

Different superscript indicates a significant difference (P<0.05) among additives

On comparing the impacts of the two concentrations (1mM methionine and 2mM) on the trends of SM%, AS% and SA%, it is obvious that the addition of 1Mm methionine achieved better results than 2Mm methionine which persists along the 72h of liquid semen storage. Meanwhile, supplementation of 1Mm methionine to extended semen gave better significant (P<0.05) results compared with the addition of *N. sativa* extract, during the incubation period.

Table 5 indicated that methionine supplementation at both concentrations (1 and 2 mM) to the extended rabbit semen significantly improved (P<0.05) percentage sperm DNA integrity of chilled rabbit semen during the 1st day of incubation period (94.2±2.61 and 91.60±3.33%, respectively) as compared to adding *N. sativa* extract (85.80±4.42 %) or that of the control (84.20±2.89%). This trend was persisting along the 3 days storage period.

Data presented in Table 4 elucidated that the addition of 1Mm methionine, to the extended buck semen, significantly increased (P<0.05) the post-thawing motility (46.0±0.58%) as compared with supplementation of other concentration of methionine (42.0±1.16 %), *Sativa* extract (36.67±3.33%) or the control (41.67±1.67%). The acrosome integrity in case of addition of 1 and 2 mM methionine significantly increased (P<0.005) than the addition of *N. sativa* extract and also it increased significantly than the control group (Table 4).

The percentage of sperm DNA integrity of post-thawed rabbit semen was significantly (P<0.05) higher (80.40± 1.72 and 77.80± 1.62%, respectively) with methionine supplementation at both concentrations (1 and 2mM, respectively) as compared to adding *N. sativa* extract (72.20± 2.06 %) or that of the control (69.60± 1.33%).

DISCUSSION

This is the first study to report the effects of methionine on the quality of liquid and frozen rabbit semen and demonstrated an improvement in sperm motility with the addition of methionine to the extender at all the used concentrations. The current study showed that the supplementation of methionine to the extended buck semen, significantly improved the SM%, AS% and post thaw motility while it reduced the SA% when compared to the control results. These results are in agreement with those of Nizza *et al.* [11] and Singh *et al.* [10] who reported that dietary supplementation with methionine produced a beneficial effect on semen quality and freezability in buffalo-bulls and rabbit bucks, respectively. Moreover, our results are compatible with those of Smirnov *et al.* [13] Khalifa [14] and Coyan *et al.* [19] who recorded that *in vitro* supplementation of ram and buffalo semen extenders with methionine resulted in pronounced enhancement in post-thaw sperm motility and plasma membrane integrity. On the contrary, Scholkamy [24] reported a negative relationship between the concentration of methionine in buffalo seminal plasma and sperm motility.

The results of the present study are in contrast with studies showing that methionine did not have any beneficial effect in different organs of the rat [25, 26].

The beneficial impact of methionine on semen preservation and freezability may be resulted from the following physiological mechanisms:

- Its ability to maintain a high level of alpha-tocopherol in seminal plasma and spermatozoa [27]. Alpha tocopherol was proved to be a very potent antioxidant that could inhibit lipid peroxidation in sperm membrane [28].
- Its tendency to stabilize the integrity of sperm plasma membrane and acrosomal membrane by keeping the sulphhydryl groups of the membrane in a reduced state [29].
- An increase in glutathione level within 72 h. Methionine, which is a thiol-containing antioxidant, acts as a precursor amino acid for glutathione [30].

The results of the present study declared that methionine supplementation at both concentrations (1 and 2mM) to the extended rabbit semen significantly improved the acrosomal integrity of frozen semen as well as the percentage of sperm DNA integrity of both chilled and frozen rabbit semen. To our knowledge, no available literature was found considering the influence

of the addition of methionine on those parameters. The protective effect of methionine on sperm functional and structural characteristics when included in the pre-freeze preparation may be attributed to that methionine penetrates the cell membrane easily, enhancing intracellular glutathione biosynthesis *in vivo*. This phenomenon may lead to a cryoprotective effect on the functional integrity of the membrane and cytoplasmic components such as the axosome and mitochondria of the sperm cells [9].

Regarding, the addition of *N.sativa* extract to the extended semen, the current study showed that *N. sativa* significantly increased ($P<0.05$) the SM % and AS %, decreased the SA % and improved the percentage of sperm DNA integrity of chilled and frozen rabbit semen. However, its supplementation induced no effect on the protection of acrosomal intactness. These findings are in agreement with Daader *et al.* [31] who revealed that feeding pellets contained 5% *N. sativa* seeds improved semen quality of rabbit bucks.

The positive influence of *N. sativa* extract on semen preservation and freezability may be attributed to the following physiological mechanisms:

- It acts as an anti-oxidant [32].
- It is a source of calcium, iron, sodium and potassium which are essential cofactors in various enzyme functions that protect semen.
- It acts as anti-bacterial agent [33].

In conclusion, the addition of methionine and *Nigella sativa* extract to extended rabbit semen induced remarkable physiological effects on semen quality during conservation for 3-days-long period at 5°C, improved its motility, viability, DNA integrity and freezability of rabbit semen.

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