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Cryopreservation of buck semen with emphasis on its chilling and its characteristics after some amino acids supplementation

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ABSTRACT

The aim of the present investigation was to evaluate the impact of some amino acids (methionine of various concentrations) on the preservation, acrosomal integrity and post thawing motility of extended goat semen.

Semen samples were diluted with a Tris-based extender containing the additive methionine 1.5, 2.5 and 5mM then the diluted samples were kept in glass tubes and cooled from 37°C to 5°C in a cold cabinet, and maintained at 5°C.

Sperm motility (SM%), alive sperm (AS%), sperm abnormalities (SA%) and acrosomal integrity were determined at 5°C for seven days of chilling storage. Furthermore, the influence of methionine on post-thawing motility was assessed.

The results elaborated that the addition of methionine particularly 2.5mM of methionine significantly improved SM% and reduced dead sperm %. Furthermore the addition of 2.5mM methionine improved post-thawing motility (43.75 ± 1.25% vs. 32.50 ± 3.23 in the control group). Moreover, the frequency of acrosomal defects was lower in treated groups than in control.

In conclusion, the addition of methionine induced remarkable physiological effects on goat semen quality during conservation for 7-days-long period at 5°C and improved its freezability.

***Keywords*:** Goat, methionine, sperm, motility, acrosome

Introduction

Conservation of the fertilizing capacity of fresh semen for the longest possible time is essential in the practice of artificial insemination for all farm animals' species including goat. A basic problem with the preservation of goat semen 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 compounds directly damage the phospholipids components of cell membrane (Cheeseman, 1993).

Mammalian semen contains antioxidants, such as glutathione (Kantola *et al.,* 1988) but this endogenous antioxidantes may be inadequate to stop lipid peroxidation during cooled storage of spermatozoa (Aurch *et al.,* 1997).

During the processing of semen, the addition of antioxidants such as several amino acids to fish milt (Kundu *et al.,* 2001 and He and Woods, 2003), glutathione and ascorbic acid to equine sperm (Aurich *et al.,* 1997) and superoxide dismutase and catalase to ram sperm (Maxwell and Stojanov, 1996) has been shown to protect sperm against harmful effects of reactive oxzgen 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 (Khilo, 1986). 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 (Reed, 1990). In addition, the thiol group of methionine was shown to chelate lead and remove it from tissues (Patra *et al.,* 2001).

Singh *et al.* (2000) recorded that feeding of extra methionine and lysine to buffalo bulls produced a beneficial effect on semen quality and freezability. Furthermore, Nizza *et al*. (2000) 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 sleno-methionine significantly improved sperm motility and oxygen consumption (Alabi *et al.,* 1985). Cryopreservation of ram semen in extenders

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fortified with methionine improved post-thaw motility and fertility of spermatozoa (Smirnov *et al.,* 1978). In a recent study, Khalifa (2001) indicated that in a 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. To our knowledge, few literature was found considering the influence of *in vivo* methionine supplementation on the male fertility and *in vitro* incubation of spermatozoa with methionine.

Consequently, the current study was designed to investigate the physiological effect of methionine on the quality of goat semen-preserved in both liquid and frozen conditions.

Materials and Methods

This investigation was carried out at private goat farm nearby Cairo-Alexandria desert road where six male zaraibi goat bucks aged 19 months approximately and weighed 30 – 40 kg were used. Each buck was fed one kg balanced concentrate and barseem hay ad libidium. Water was offered to those animals ad libidium all the day using manual water through system.

A-Experimental Materials:

Methionine and the other chemical reagents used for the preparation of extender were purchased from Sigma-Aldrich Co., Deisenhofen, Germany.

B. Semen Collection and Evaluation:

Semen was collected twice weekly by means of an artificial vagina using an estrous doe as a teaser. Only ejaculates of >70% initial motility and 2000 x 10^6 sperm cells/ml were used in the experiments. After collections, the ejaculates were transferred to the laboratory of the farm within 2-3 minutes and kept in a water bath at 30°C for evaluation by means of conventional methods and assessed for volume, sperm concentration and motility. Visual motility was assessed microscopically. Sperm concentration was assessed by Neubauer haemocytometer. Ejaculates fulfilling minimum standards of sperm motility 70% and normal sperm morphology 80% were processed for freezing. The ejaculates were pooled in order to have sufficient semen for a replicate and to eliminate the buck effect. The semen was given a holding time for 10 min. at 37 °C in water bath before dilution. To overcome the action of phospholipase A_2, the semen was centrifuged then reconstituted in the basic extender.

C- Semen processing.

One type of extender was used for preserving the goat semen:-

Tris-glucose-glycerol egg yolk (TGGY) was prepared according to Evans and Maxwell (1987) with some modificationas it was composed of Tris-hydroxymethyl-aminomethane (3.786 g), glucose (0.625 g), citric acid monohydrate (2.172 g), glycerol (6.7 ml), fresh chicken egg yolk (10 ml), penicillin (100,000 IU), streptomycin (100 mg) and bidistilled water to 100 ml.

1- Semen assays.

Semen evaluations were performed at three different stages of the cryopreservation protocol: neat semen, after equilibration and after freezing and thawing.

1.1- Motility.

A drop of semen was placed on a pre-warmed (37°C) glass slide and cover slipped**.** Visual motility was assessed microscopically (x 400; Olympus BX20, Japan) at 37 °C with closed circuit television (Graham *et al.,* 1970).

1.2- Sperm morphology and viability.

An Eiosin-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 (1989).

1.3 - Acrosomal integrity examination

Acrosomal staining procedure followed the method of Kovacs and Foote (1992): equal drops of trypane 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 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.

Experiment I:

This experiment was designed to explore the influence of methionine on the conservation and acrosomal integrity of chilled goat semen in TGGY extender. Semen samples were divided and diluted (1:4 according to Evans and Maxwell, 1987) at 30°C with semen extender. Methionine was added to the extended semen at the rate of 0.0, 0.05, 0.10 and 0.15g /100 ml (Khalifa, 2001). After dilution, the extended semen was incubated at 5°C to be examined daily for seven days for sperm motility% (SM%), alive sperm% (AS%) sperm abnormalities% (SA%) using eosin-aniline stain according to Shaffer and Almquist (1948) and acrosomal integrity according to (Kovacs and Foote, 1992) .

Experiment II:

This experiment was designed to investigate influence of methionine (0.0, 0.05, 0.10 and 0.15 g / 100 ml) on the post-thawing motility of goat semen extended in TGGY. So, semen samples were split and diluted 1:4 at 30°C. After few weeks, frozen goat semen was thawed in a water bath at 40°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 (El-Battawy *et al.,* 2003).

Statistical analysis:

Data were analysed using ANOVA test at a confidence limit not less than 95% using SAS program (1988). LSD test was used to evaluate the significant difference between means at P<0.05.

Results

The present results showed that the addition of methionine, to the extended buck semen, increased significantly (P<0.0001) the SM % (Table 1) and AS % (Table 2) while it decreased the SA % (Table 3) as compared with the control treatment.

Meanwhile, the addition of 2.5Mm methionine to the extended semen achieved better results compared with the addition of other concentrations, during the experimental period.

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

Time	**Days**							**Overall mean treatment**	**LSD**
Methionine concentration	**1**	**2**	**3**	**4**	**5**	**6**	**7**		
0 mM (Control)	82.50 ± 6.45	71.25 ± 2.50	63.75 ± 4.79	55.00 ± 5.77	45.00 ± 5.77	32.50 ± 9.57	16.25 ± 4.79	52.321^{D}	**3.038**
1.5mM	91.25 ± 4.79	86.25 ± 6.29	80.00 ± 4.08	76.25 ± 6.29	71.25 ± 6.29	68.75 ± 6.29	63.75 ± 4.79	76.786^{B}	
2.5Mm	91.00 ± 4.79	88.75 ± 4.79	85.00 ± 4.08	81.25 ± 6.29	76.25 ± 6.29	71.25 ± 6.29	68.75 ± 4.79	80.357^{A}	
5 mM	86.25 ± 4.79	81.25 ± 6.29	75.00 ± 4.08	71.25 ± 6.29	66.25 ± 6.29	63.75 ± 7.50	58.75 ± 4.79	52.321^{C}	
Overall mean days	87.813^{g}	81.875^{f}	75.938^{e}	70.938^{d}	64.688^{c}	59.063^{b}	51.875^{a}		
LSD	**4.018**								

Mean ± S.D.; LSD (time x treatment interaction) = 8.042 (P<0.05).

Table 2: Effect of methionine on alive sperm% during incubation of goat semen at 5°C in tris-glucose-glycerol-egg yolk extender (TGGY).

Time / Methionine concentration	1	2	3	4	5	6	7	Overall mean treatment	LSD Treat-ment
				Days					
0 mM (Control)	84.50 ± 6.76	73.25 ± 2.50	66.50 ± 4.36	58.50 ± 5.20	48.50 ± 6.35	35.00 ± 10.10	26.00 ± 4.69	56.036^{D}	**3.16**
1.5Mm	88.50 ± 5.45	83.50 ± 6.76	77.00 ± 4.08	73.50 ± 5.97	70.75 ± 6.45	66.50 ± 7.00	61.75 ± 4.79	74.500^{C}	
2.5 mM	93.75 ± 5.68	90.75 ± 5.85	86.75 ± 4.57	83.50 ± 6.76	78.00 ± 5.83	73.50 ± 5.97	68.75 ± 8.06	82.143^{A}	
5 mM	93.75 ± 5.68	88.00 ± 6.93	81.75 ± 3.69	78.00 ± 5.83	73.50 ± 5.97	71.00 ± 5.83	66.50 ± 4.36	78.929^{B}	
Overall mean days	90.125^{a}	83.875^{b}	78.000^{c}	73.375^{d}	67.688^{e}	61.500^{f}	55.750^{g}		
LSD time				**4.18**					

Mean ± S.D.; LSD (time x treatment interaction) = 8.36 (P<0.05).

Table 3: Effect of methionine on sperm abnormalities % during incubation of goat semen at 5°C in tris-glucose-glycerol-egg yolk extender (TGGY).

Time / Methionine concentration	1	2	3	4	5	6	7	Overall mean treatment	LSD
				Days					
0 mM (Control)	10.50 ± 1.29	12.75 ± 0.50	12.75 ± 0.50	14.00 ± 1.15	15.50 ± 0.58	18.25 ± 2.22	22.50 ± 1.91	15.179^{A}	**0.573**
1.5 mM	8.75 ± 0.96	10.00 ± 0.82	11.00 ± 0.82	11.75 ± 1.26	12.75 ± 1.26	13.50 ± 1.00	14.25 ± 0.96	11.714^{B}	
2.5 mM	6.75 ± 0.96	7.50 ± 0.58	9.00 ± 0.82	10.00 ± 1.41	10.75 ± 0.96	11.75 ± 1.26	12.50 ± 1.29	9.750^{D}	
5 mM	7.50 ± 0.58	8.75 ± 0.96	10.00 ± 0.82	11.00 ± 0.82	11.75 ± 0.50	12.50 ± 1.00	13.25 ± 0.96	10.679^{C}	
Overall mean days	8.375^{g}	9.750^{f}	10.688^{e}	11.688^{d}	12.688^{c}	14.000^{b}	15.625^{a}		
LSD				**0.7584**					

Mean ± S.D.; LSD (time x treatment interaction) = 1.158 (P<0.05).

Data shows also that the acrosomal integrity in case of methionine (0.1 g/dl) was increased significantly (P<0.0001) than the control group (Table 4).

Table 4: Effect of methionine on acrosomal integrity.

Day / Treatment	1	2	3	4	7	Overall mean	LSD
Control	79.60 ± 0.51	74.00 ± 0.71	72.00 ± 0.45	64.40 ± 0.75	52.00 ± 0.71	68.40C	**0.9088**
Methionine 2.5 mM	81.60 ± 0.51	76.40 ± 0.93	73.00 ± 0.71	68.00 ± 0.89	67.40 ± 0.93	73.28B	
Overall mean	81.267A	76.067B	72.733C	67.933D	63.067E		
LSD			**1.1732**				

P<0.0001; LSD (day x treatment interaction) = 2.0317, (P<0.05).

Post-thawing motility % of cryopreserved goat semen added to its extender a concentration of 2.5 Mm methionine was the best motility (43.75%) and it was highly significant (P<0.0088) in comparison with other methionine concentrations and control (38.75, 37.5 and 32.50% respectively) as shown in table 5.

Table 5: Effect of methionine on post-thawing motility % of cryopreserved goat semen.

Control	Methionine			F-cal
	1.5 mM	2.5 mM	5 mM	
32.50c ± 3.23	38.75abc ± 2.39	43.75ab ± 1.25	37.50bc ± 3.23	3.91**

** P<0.0088, LSD = 6.3004

Discussion

The current investigation declared that the addition of methionine to the extended buck semen, improved significantly the SM%, AS% and post thaw motility while it reduced the SA% when compared to the control results. The present results are in agreement with Nizza *et al*. (2000) and Singh *et al*. (2000) 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 in compatible with Smirnov *et al*. (1978) and Khalifa (2001) who recorded that in vitro supplementation of ram and buffalo semen extenders with methionine resulted in pronounced enhancement in postthaw sperm motility and plasma membrane integrity. On the contrary, Scholkamy (1996) reported a negative relationship between the concentration of methionine in buffalo seminal plasma and sperm motility.

The beneficial impact of methionine on semen preservation and freezability may be attributed to one and / or the following physiological mechanisms:

I- Its ability to maintain a high level of alpha-tocopherol in seminal plasma and spermatozoa (Kaludin and Dimitrova, 1986). Alpha tocopherol was proved to be a very potent antioxidant that could inhibit lipid peroxidation in sperm membrane (Cerolini *et al.,* 2000).

II- Its tendency to stabilize the integrity of sperm plasma membrane and acrosomal membrane by keeping the sulphydryl groups of the membrane in a reduced state (Lin and Keefe, 2000).

Moreover, several hypotheses have been advanced to explain the role of amino acids in cryoprotection against freezing-thaw damage including:

1- Acting as energy substitutes through undergoing glucogenesis and then converted into glucose which become available as energy substrate (Leng, 1970).
2- Protection of calcium uptake (Lalonde *et al.,* 1991).
3- Protection of certain enzymes (Kruuv *et al.,* 1988).
4- Protection of phosphofructokinase, lactate dehydrogenase and alcohol dehydrogenase (Heinz *et al.,* 1990).

In conclusion the promising results of the amino acids on chilling and cryopreservation of goat semen help in advancement of the goat semen preservation and reaching the best extender additive to be used in large scale.

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