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Brief Communication

Methionine supplementation improves ram sperm parameters during liquid storage at 5 °C

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ABSTRACT

The aim of this study was to investigate the effects of methionine and lipoic acid on ram sperm parameters during liquid storage (5 °C). Ejaculates collected from five Merino rams were pooled at 37 °C. Each pooled ejaculate was divided into five equal aliquots and diluted (37 °C) with five extenders, one of which was without additives, two of which contained methionine at two different doses, and the other two of which contained lipoic acid at two different doses. Sperm parameters were determined at 0, 24, 48, 72 and 96 h of liquid storage at 5 °C.

The extenders containing 2 and 4 mM of methionine resulted in higher motility percentages, in comparison to the control, up to 96 h of storage. Methionine at doses of 2 and 4 mM led to higher viability and sperm mitochondrial activity percentages, when compared to the controls during 48, 72 and 96 h of liquid storage ($P < 0.05$). The findings of this study showed that methionine was of greater benefit to ram sperm parameters during liquid storage.

In sheep breeding, artificial insemination serves as an important tool for the maintenance of breed improvement. By this way, genetic material from a small number of superior sires can be used for a large number of females [8]. Unfortunately semen transport to distant areas is difficult as semen deteriorates quickly in the short-term [9]. Impaired cell functions caused by oxidative stress, gradually result in decreased motility, morphological integrity and fertilizing capability and in the induction of sperm apoptosis [1]. Alpha-lipoic acid has a high quenching oxygen capacity to react with free radicals and it also plays an important role in mitochondrial dehydrogenase reactions and the protection of mitochondrial functions [2]. It was shown that lipoic acid improved sperm motility in cyclophosphamide-induced rats [13]. Capable of protecting cells from oxidative damage, methionine acts as a precursor amino acid for glutathione and plays a vital role in detoxification [12]. The purpose of this study was to investigate the effects of methionine and lipoic acid, added to a Tris-based extender, on ram sperm parameters during liquid storage up to 96 h at 5 °C.

Semen samples from five mature Merino rams (2 and 3 years of age) were used in this study. These rams, which belonged to the Research and Education Farm of Selcuk University Faculty of Veterinary Medicine, were maintained under uniform feeding, housing and lighting conditions. A total number of 25 ejaculates were collected from the rams using an artificial vagina during the breeding season (autumn to early winter), and the semen was pooled to minimize individual variations. Ejaculates met the following criteria: volume of 0.5–2 ml, minimum sperm concentration of 3×10^9 sperm/ml, motility of 80%. Five pooled ejaculates were included in the study.

A Tris-based extender (Tris 254 mM, citric acid 78 mM, fructose 70 mM, egg yolk 15% (v/v): pH 6.8) was used as the base extender. In this experiment, each pooled ejaculate was divided into five equal aliquots and diluted (37 °C) with the base extender prepared in five different forms, such that one was without additives, two contained methionine at two different doses, and two other contained lipoic acid at two different doses, to a final concentration of approximately 2×10^8 sperm/ml, in a 15-ml plastic centrifuge tube. Diluted semen samples were kept in eppendorf tubes and cooled from 37 to 5 °C in a cold cabinet, and maintained at 5 °C. Sperm parameters were determined during liquid storage at 5 °C.

Motility was assessed using a phase-contrast microscope (200× magnification), with a warm stage maintained at 37 °C. A small volume of stored semen was incubated at 37 °C for 1–2 min, and then examined for motility. Sperm motility estimations were performed by scanning several microscopic fields for each semen sample. The mean of the estimations was recorded as the final motility rate.

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Sperm viability was determined by means of staining with the Sperm Viability Kit (SYBR-14/PI Molecular Probe: L 7011 Invitrogen, Carlsbad, CA). The staining protocol of the kit was modified from a study of Garner and Johnson [4]. A working solution of SYBR-14 was diluted 1:10 with DMSO (Applichem A3006) and then divided into equal aliquots (30 μl) after being filtered through a 0.22-μm Millipore Millex-GV filter, and stored at −20 °C. Propidium iodide (PI) was dissolved in distilled water at a concentration of 2 mg/ml, divided into equal aliquots (30 μl) after being filtered through a 0.22-μl Millipore Millex-GV filter, and stored at −20 °C. A thawed straw was diluted 1:3 with Tris stock solution without glycerol and egg yolk, and then 30 μl of diluted semen was mixed with 6 μl of SYBR-14 and 2.5 μl of PI. The sample was mixed gently, incubated at 37 °C in the dark for 20 min, and added 10 μl of Hancock solution for sperm fixation. A wet mount was made using a 2.5 μl-drop of sample placed directly on a microscope slide and covered by a cover slip. At least 200 spermatozoa were examined at 400× magnification under a fluorescence microscope to assess sperm viability. Sperm displaying green–red or red colorisation were considered to be dead, while those displaying green colorisation were considered to be viable.

Sperm mitochondrial activity was assessed with a staining protocol modified from Garner et al. [5] using JC-1/PI. A stock solution of 5,5′, 6,6′-Tetrachloro-1,1′, 3,3′tetraethyl-benzimidazolylcarbocyanineiodide (1.53 mM) (T3168 JC-1, Invitrogen, Carlsbad, CA) was prepared in DMSO solution, divided into equal aliquots (100 μl) after being filtered, and stored at −20 °C. A thawed straw was diluted 1:3 with Tris stock solution without glycerol and egg yolk, and then 300 μl of diluted semen was mixed with 2.5 μl of JC-1 and 2.5 μl of PI. The sample was mixed gently, incubated at 37 °C in the dark for 20 min, and added 10 μl of Hancock solution for sperm fixation. A wet mount was made using a 2.5 μl-drop of sample placed directly on a microscope slide and covered by a cover slip. At least 200 sperm cells were examined at 400× magnification under a fluorescence microscope to assess activity. A high level of yellow/orange fluorescence associated with the sperm midpiece (where mitochondria are located) indicated high mitochondrial activity.

In result, the extenders supplemented with 2 and 4 mM of methionine resulted in higher motility percentages, in comparison to the control group, up to 96 h of storage ($P < 0.05$) (Table 1). Methionine at doses of 2 and 4 mM led to higher viability and sperm mitochondrial activity percentages, when compared to the controls during 48, 72 and 96 h of liquid storage ($P < 0.05$) (Tables 2 and 3).

This study investigated the effects of the antioxidants methionine and lipoic acid on sperm motility, viability and mitochondrial activity at 5 °C during the liquid storage of ram semen. The sperm plasma membrane is rich in polyunsaturated fatty acids and is therefore sensitive to peroxidative damage with consequent loss of sperm parameters during aerobic incubation [6]. The inclusion of antioxidants into the semen extender prior to storage avoids such damage [10]. In the present study, an improvement in sperm motility was achieved when methionine was added at all doses. The addition of 2 and 4 mM of methionine increased sperm motility up to 96 h of liquid storage. This is in contrast to the findings of Coyan et al. [3], who reported no improvement in ram sperm motility at 72 h. Mitochondria are located in the sperm midpiece and provide accessible energy to the tail filaments, thus facilitating efficient propulsion for the sperm both to reach the oocyte and to penetrate its zona pellucida [11]. We can suppose that methionine at doses of 2 and 4 mM displayed a protective effect on the viability and high mitochondrial activity of ram semen during 48, 72 and 96 h of liquid storage.

Table 1
Mean (S.E.) motility (%) of Merino ram sperm supplemented with different doses of lipoic acid and methionine for different storage times at 5 °C.

Groups	0 h	24 h	48 h	72 h	96 h
Lipoic acid 2 mM	80 ± 3.2^{ab}	65 ± 5.0^{a}	55 ± 6.3^{a}	38 ± 3.4^{a}	18 ± 5.4^{ab}
Lipoic acid 4 mM	75 ± 3.2^{a}	68 ± 3.0^{a}	54 ± 4.0^{a}	29 ± 6.8^{a}	15 ± 5.0^{a}
Methionine 2 mM	83 ± 1.2^{bc}	81 ± 1.0^{b}	72 ± 2.5^{b}	65 ± 3.5^{b}	61 ± 4.3^{c}
Methionine 4 mM	89 ± 1.0^{c}	84 ± 1.0^{b}	79 ± 1.9^{b}	73 ± 2.0^{b}	68 ± 2.5^{c}
Control	76 ± 1.9^{ab}	67 ± 5.6^{a}	54 ± 6.6^{a}	44 ± 7.0^{a}	31 ± 4.3^{b}
p	*	*	*	*	*

-: No significant difference.
a, b, c: Different superscripts within the same column demonstrate significant differences
(*$P < 0.05$).

Table 2
Mean (S.E.) viability (%) of Merino ram sperm supplemented with different doses of lipoic acid and methionine for different storage times at 5 °C.

Groups	0 h	24 h	48 h	72 h	96 h
Lipoic acid 2 mM	67.18 ± 3.2^{b}	61.78 ± 2.9^{ab}	57.92 ± 3.5^{ab}	53.96 ± 3.3^{a}	51.86 ± 3.0^{b}
Lipoic acid 4 mM	60.22 ± 3.9^{a}	57.36 ± 3.7^{a}	53.16 ± 3.1^{a}	49.62 ± 2.9^{a}	47.36 ± 2.7^{b}
Methionine 2 mM	71.24 ± 1.3^{b}	67.66 ± 1.5^{bc}	65.66 ± 1.6^{b}	63.26 ± 1.6^{b}	61.60 ± 1.4^{c}
Methionine 4 mM	73.10 ± 0.4^{b}	71.02 ± 0.8^{c}	65.98 ± 1.5^{b}	63.54 ± 1.4^{b}	59.74 ± 1.1^{c}
Control	70.98 ± 1.5^{b}	$61 \pm 42 \pm 2.0^{ab}$	55.02 ± 3.1^{a}	47.30 ± 3.6^{a}	34.28 ± 3.8^{a}
p	*	*	*	*	*

-: No significant difference.
a, b, c: Different superscripts within the same column demonstrate significant differences
(*$P < 0.05$).

Table 3
Mean (S.E.) high mitochondrial activity (%) of Merino ram sperm supplemented with different doses of lipoic acid and methionine for different storage times at 5 °C.

Groups	0 h	24 h	48 h	72 h	96 h
Lipoic acid 2 mM	9.13 ± 1.7^{a}	8.19 ± 1.7^{a}	6.42 ± 1.2^{a}	4.06 ± 1.4^{a}	2.53 ± 1.0^{a}
Lipoic acid 4 mM	7.62 ± 2.2^{a}	6.49 ± 1.8^{ab}	5.65 ± 1.5^{a}	4.64 ± 1.5^{a}	3.57 ± 1.5^{a}
Methionine 2 mM	30.30 ± 5.4^{bc}	24.05 ± 3.3^{c}	23.42 ± 2.8^{b}	18.59 ± 2.9^{b}	14.47 ± 2.4^{b}
Methionine 4 mM	35.08 ± 7.6^{c}	26.85 ± 2.1^{c}	26.12 ± 2.2^{b}	24.86 ± 2.2^{c}	21.76 ± 2.2^{c}
Control	17.48 ± 3.1^{ab}	14.80 ± 3.3^{c}	12.30 ± 2.3^{a}	8.22 ± 1.5^{a}	6.52 ± 1.4^{a}
p	*	*	*	*	*

-: No significant difference.
a, b, c: Different superscripts within the same column demonstrate significant differences
(*$P < 0.05$).

Although lipoic acid provided an improvement in post-thawed goat sperm motility and mitochondrial function [7], it did not result in any improvement in sperm motility or mitochondrial activity in the present study. In conclusion, based on the current findings, methionine (2 and 4 mM) provided improved sperm parameters during the extended liquid storage of ram sperm.

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