The effect of bovine serum albumin and fetal calf serum on sperm quality, DNA fragmentation and lipid peroxidation of the liquid stored rabbit semen

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ABSTRACT

The aim of the present study was to determine the effects of the bovine serum albumin (BSA) and fetal calf serum (FCS) on sperm quality, DNA fragmentation and lipid peroxidation of liquid stored rabbit semen stored up to 72 h at 5 °C. Ejaculates were collected from five New Zealand male rabbits by artificial vagina and pooled at 37 °C following evaluation. Each pooled ejaculate was split into three equal experimental groups and diluted to a final concentration of approximately 40×10^6 sperm/ml (single step dilution), in an Eppendorf tube, with the Tris based extender containing BSA (5 mg/ml), FCS (10%) or no additive (control) at 37 °C, cooled to 5 °C and stored for up to 72 h. The extender supplemented with BSA and FCS did not improve the percentages of motility and acrosomal abnormality during 48 h compared to the control. The additives BSA and FCS had a significant effect in the maintaining of plasma membrane integrity between 48 and 72 h storage period, compared to the control ($P < 0.01$). The supplementation of BSA and FCS had a protective effect on motility ($P < 0.05$), plasma membrane integrity ($P < 0.01$) and acrosomal integrity ($P < 0.01$) at 72 h compared to the control. The supplementations with BSA and FCS led to a reduction in DNA damage of rabbit sperm at 48 and 72 h during storage period, compared to the control ($P < 0.001$). Although supplementation of BSA and FCS caused significant ($P < 0.01$) decreases in malondialdehyde (MDA) level at 48 h and 72 h, they significantly ($P < 0.01$) increased the glutathione peroxidase (GPx) antioxidant activity up to 72 h when compared to the control group. In conclusion, BSA and FCS supplementation to liquid stored rabbit semen provide a protection for spermatozoa against cool storage-induced DNA damage and plasma membrane integrity by their antioxidative properties.

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Introduction

Semen cryopreservation contributes to the expansion of reproductive techniques, such as artificial insemination (AI) and in vitro fertilization. AI with cryopreserved semen is essential in breeding and selection schedules contributing to increase production of domestic species. Liquid storage is an alternative semen preservation techniques which allows semen to storage at refrigerated temperature for a short period [6,9,25,46,62]. However, semen cryopreservation procedures including cooling, freezing and thawing induces sperm cell damages derived from the formation of intracellular ice crystals, cold chock and osmotic injury resulting in loss of motility, viability, and biochemical changes and finally lead to reduces to fertilizing capability [27,47]. It has been reported

that rabbit sperm has low water permeability and high activation energy when compared to sperm from other species [15]. The use of cryopreserved semen in AI is a practical and useful tool for cattle breeding [62]. Despite the usability of AI in the cattle breeding, rabbit AI with cryopreserved semen has generally been used only in research and laboratory procedures [19,33].

Cryopreservation leads to deleterious effects on rabbit sperm as in other species. Freeze–thawing process causes a decrease in the percentage of motility, lifespan of the spermatozoa and oxidative stress which finally leading to a reduction in the fertilizing ability [2,13,34,63]. Therefore, AI is usually performed by use of freshly diluted or cooled semen for short periods of time (2–3 days) in rabbit because of the lower fertility with the use of frozen-thawed sperm [17,30,33,44,56].

Oxidative stress is known to be one of the major causes and exhibits adverse effect on the physiology of sperm through the induction peroxidation of sperm plasma membrane. The

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detrimental effect of oxidative stress to sperm is known to originate from the generation of reactive oxygen species (ROS) [1,2, 48]. Normally, ROS is physiologically generated by sperm [16]. However, excess ROS has been generated when the balance is broken between ROS generation and scavenging activity [1]. Excessive ROS generation has been associated with decreased motility, impaired sperm plasma membrane, morphology and DNA integrity which in turn decrease the capability to fuse with the oocyte, thereby resulting in infertility [40,48]. Sperm DNA is a valuable tool for evaluating the detrimental effect originating from cooling period or cryopreservation processes. Sperm DNA damage has been reported in equine spermatozoa [29] and rabbit spermatozoa [49] as a result of cooled storage. Besides, the sperm DNA damage such as single and double DNA strand fractures has been demostrated in human spermatozoa originating from ROS [59].

Rabbit semen has been effectively stored for short periods of time without serious loss of its motility, viability and fertilizing capability especially when using diluents based on tris buffer extenders [30,44,49]. Maintaining sperm quality characteristics during liquid storage of rabbit semen for several days after semen collection is highly important for AI programs of rabbit breeding [45]. Bovine serum albumin (BSA) is known to improve sperm motility, plasma membrane integrity and acrosome against temperature shock during the freezee–thawing process in ram semen [60]. It may also help sperm in surviving in the reproductive tract of the cow prior to fertilization [14]. Besides, it was reported that BSA gives best fertility rates and increases the activity of catalase antioxidant activity following the freezee–thawing process in bull semen [52]. Fetal calf serum (FCS) is a constituent of most media used for the culture of animal cells. FCS has a variety of proteins that maintain cultured cells in a medium [22]. FCS has been widely used in the culture of cumulus–oocyte complexes, since it is believed that FCS stabilizes the expanding cumulus extracellular matrix [54]. The influential use of cooled semen for AI is affiliated with the power of the semen extender to provide an appropriate environment for rabbit semen during liquid storage. Some studies have been conducted to evaluate the effect of BSA on the semen characteristics in rams [60] and bulls [14,51] during cryopreservation. It was demonstrated that a simple culture medium containing BSA may be sufficient to develop in vitro from zygote to the blastocyst stage but further development and hatching pig blastocysts needs the presence of FCS [32,37,39,43].

AI may be performed with cooled semen stored for short periods of time, <24 h after semen collection, because the use of AI doses older than 24–48 h may decrease the fertility [44]. Fertility losses with the use of cooled stored rabbit semen older than 24–48 h may be the consequence of deterioration in semen quality observed over storage time [30,44]. Besides, there is no information is available on the individual effects of BSA and FCS on DNA damage and oxidative stress parameters as well as spermatological parameters in rabbit semen during liquid storage. On the basis of the lack of available information as well as with the aim of prevention of fertility losses formed with cooled stored rabbit semen; the present study was therefore conducted to determine the effects of BSA and FCS on semen characteristics including motility, acrosomal abnormality, plasma membrane integrity, DNA fragmentation, and also malondialdehyde (MDA) level and glutathione peroxidase (GPx) endogenous antioxidant enzyme activity of cooled rabbit semen during liquid storage.

Materials and methods

Chemicals

BSA (A9647), FCS (F2442) and other chemicals were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA).

Animals, semen collection and processing

Five sexually mature New Zealand white male rabbits were used as semen donors. They were obtained from Experimental and Clinical Research Center of Erciyes University, Kayseri, Turkey. Males were housed in individual cages under standard laboratory conditions (12 h/12 h light/dark cycle, 22–24 °C temperature and 55–60% relative humidity). A commercial pellet diet (Optima Food Co., Bolu, Turkey) and fresh drinking water were given ad libitum.

Semen was collected two times a week with an artificial vagina from male rabbits. After semen collection, any gel plug was removed. Only ejaculates having good wave motion ($\geqslant 3$ on a 0–5scale), $\geqslant 300 \times 10^6$ spermatozoa per ml and $\geqslant 75\%$ motility, were used in this study. Collected ejaculates from each rabbit were pooled in order to eliminate individual variations. Six pooled ejaculates were included in the study. A Tris-based extender (313.8 mM Tris, 103.1 mM citric acid and 33.3 mM glucose) was used as the base extender. Each pooled ejaculate was split into three equal experimental groups and diluted to a final concentration of approximately 40×10^6 sperm/ml (single step dilution), in an Eppendorf tube, with the base extender containing BSA (5 mg/ ml), FCS (10%) or no additive (control) at 37 °C. 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 motility, acrosomal abnormality, plasma membrane integrity (HOST), DNA damage, MDA level, GPx antioxidant enzyme activity were determined at 5 °C for periods of 0, 24, 48 and 72 h during liquid storage of rabbit semen.

Evaluation of semen characteristics (motility, plasma membrane integrity, acrosomal abnormality)

Motility was assessed at 37 °C under light microscope at 100×. Sperm motility estimations were performed in several microscopic fields for each semen sample. The mean of the estimations was recorded as the final motility score and expressed as percentage.

The hypoosmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm plasma membrane. HOST relies on the resistance of the membrane to loss of permeability barriers under stress condition of stretching in a hyposmotic medium [12,41]. Sperm cells with resistant membranes exhibited a swelling around the tail such that the flagella become curled and the membrane maintained a swollen 'bubble' around the curled flagellum. The assay was performed by mixing 30 μl of semen with a 300 μl 100 mOsm/kg hypoosmotic solution (9 g fructose plus 4.9 g sodium citrate per liter of distilled water). This mixture was incubated (37 °C) for 1 h, where 0.2 ml of the mixture was placed on a microscope slide and mounted with a cover slip and immediately evaluated (magnification × 400) under phase-contrast microscope. A total of 200 spermatozoa were counted in at least five different microscopic fields. The percentages of sperm with swollen and curled tails were then recorded.

For the assessment of acrosomal abnormality, at least three drops of each sample were added to 1 ml of Hancock solution. Hancock solution: *sodium saline solution;* 9.01 g NaCl and 500 ml of double-distilled water. *Buffer solution*: (1) 21.682 g $Na_2HPO_4.2H_2O$ and 500 ml of double-distilled water; (2) 22.254 g KH_2PO_4 and 500 ml of double-distilled water. Amounts of 200 ml of (1) and 80 ml of (2) were mixed to obtain 280 ml of buffer solution. The final Hancock solution was mixed as follows: 62.5 ml formalin (37%) + 150 ml sodium saline solution + 150 ml buffer solution, and 500 ml of double-distilled water. One drop of this mixture was put on a slide and covered with a cover slip. The percentages of acrosomal abnormality were determined by counting a total of 400 spermatozoa under phase-contrast microscope (magnification × 1000 and oil immersion) [52].

Assessment of sperm DNA damage

Diluted semen samples were centrifuged at 300g for 10 min at 4 °C. Seminal plasma was removed and remaining sperm cells were washed with (Ca^{2+} and Mg^{2+} free) PBS to yield a concentration of 1×10^5 spermatozoa/ml [7]. Sperm DNA damage was investigated using the single cell gel electrophoresis (comet) assay that was generally performed at high alkaline conditions. Firstly, each microscope slide was pre-coated with a layer of 1% normal melting point agarose in PBS and thoroughly dried at room temperature. Next, 100 µl of 0.7% low melting point agarose at 37 °C was mixed with 10 µl of the cell suspension and dropped on top of the first layer. Slides were allowed to solidify for 5 min at 4 °C in a moist box. The cover slips were removed and the slides were immersed in freshly prepared cold lysis buffer containing 2.5 M NaCl, 100 mM Na_2–EDTA, 10 mM Tris, 1% Triton X-100,10% DMSO and 40 mM dithiothereitol (pH 10) for 1 h at 4 °C. Then the slides were incubated overnight at 37 °C in 100 µg/ml proteinase K and added to the lysis buffer. The slides were removed from the lysis buffer, drained and placed in a horizontal electrophoresis unit filled with fresh alkaline electrophoresis solution, containing 300 mM NaOH and 1 mM EDTA, (pH 13), for 20 min to allow the DNA to unwind. Electrophoresis was performed for 20 min at 8 °C at 12 V and was adjusted to 250 mA. Subsequently, the slides were washed with a neutralizing solution of 0.4 M Tris, pH (7.5), in order to remove alkali and detergents. After neutralization the slides were stained with 50 µl of 2 µl/ml ethidium bromide and covered with a cover slip. All steps were performed under dim light to prevent further DNA damage [21,55]. The images of 50 randomly chosen nuclei were analyzed by Comet Assay Software Project (CASP-1.2.2, Windows 2010) [49]. Observations were made at a magnification of 400× using a fluorescent microscope (Olympus, BX51, Japan). Damage was detected by a tail of fragmented DNA that migrated from the sperm head, causing a 'comet' pattern, whereas whole sperm heads, without a comet, were not considered damaged.

Biochemical measurements

GPx activities and MDA levels were measured in the samples on a UV–VIS Recording Spectrophotometer (UV-2100S, Shimadzu Co., Kyoto, Japan).

GPx activity was measured as described by Pleban [38]. Briefly, a reaction mixture containing 1 mmol/l Na_2-EDTA, 2 mmol/l reduced glutathione, 0.2 mmol/l NADPH, 4 mmol/l sodium azide and 1000U glutathione reductase in 50 mmol/l TRIS buffer (pH 7.6) was prepared. 20 µl of samples and 980 µl of the reaction mixture were mixed and incubated for 5 min at 37 °C. The reaction was initiated by adding 8.8 mmol/l hydrogen peroxide and the decrease of absorbance recorded at 340 nm for 3 min. GPx activity is expressed in U/ml.

MDA level, a marker of lipid peroxidation, was estimated by measurement of thiobarbituric acid reactive substances (TBARS) in samples by the method described by Richard [42]. After the reaction of MDA with thiobarbituric acid, the reaction product was followed spectrophotometrically at 532 nm, using tetrametoxypropane as a standard. The results are expressed as nmol/ml.

Statistical analysis

The results of sperm DNA damage were expressed as the Mean ± S.E.M ($\bar{X} \pm S_x$). Means were analyzed by General Linear Models, repeated measures followed by Tukey's post hoc test to determine significant differences in all the parameters between groups using the SPSS (Version 15, SPSS, Chicago, IL). For spermatological parameters including motility, plasma membrane integrity and acrosomal abnormality a Kruskal–Wallis test was conducted to evaluate differences among control and treated groups. Follow-up tests were conducted to evaluate pairwise differences between the three groups, controlling for Type I error across tests by using the Bonferroni approach.

Results

1.1. Sperm characteristics
The effects of BSA and FCS on sperm motility, acrosomal and plasma membrane integrity (HOST) of New Zealand rabbit semen during different storage times at 5 °C are presented in Table 1. BSA and FCS supplementation to rabbit semen did not improve the percentages of motility and acrosomal abnormality during 48 h when compared to the control. However, both BSA and FCS addition significantly decreased the percentage of spermatozoa with damaged membrane in diluted samples examined at 48 h in comparison to the control. The supplementation of BSA and FCS had a protective effect on motility, plasma membrane integrity and acrosomal structure at 72 h compared to the control.

Sperm DNA damage

The effect of additives on the DNA damage of liquid stored rabbit semen over time is shown in Table 2. It was determined that used additives and storage time were statistically significant in sperm DNA damage of rabbit semen during liquid storage. Total values refer to an increase in sperm DNA damage for up to storage time and used additives (BSA and FCS) which provide a decrease in sperm DNA damage at 48–72 h of liquid storage. The supplementation of BSA and FCS led to a reduction in DNA damage of rabbit semen at 48 and 72 h during storage period, compared to the control.

Biochemical parameters

MDA level and GPx antioxidant activity in semen samples of rabbits, containing BSA and FCS for different storage periods at 5 °C are given in Table 3. The level of MDA in control group was higher statistically when compared to experimental groups supplemented with BSA and FCS at 48 h and 72 h of liquid storage period at 5 °C. The additives BSA and FCS had a protective role in maintaining the GPx antioxidant activity compared to the control group during the liquid storage period for up to 72 h.

Discussion

Liquid storage of semen is a necessary technique for experimental or genetic resource bank purposes in rabbits [33]. Since frozen-thawed semen has low fertility in rabbits, AI is applied with liquid stored semen or fresh semen [17,30,33,44]. In the present study, we investigated the effects of the additives BSA and FCS on sperm motility, acrosomal abnormality, plasma membrane integrity, DNA fragmentation, lipid peroxidation and GPx activity at 5 °C during the liquid storage of rabbit semen.

Many studies have been conducted to determine the optimal conditions for preserving the fertilizing potential of rabbit semen during liquid storage [4,30,44]. Alternatively, different semen preserving protocols, extenders and additives for rabbit sperm have been developed to prolong motility and viability resulting in higher fertility results. Tris-buffer extenders are widely used in many studies that have been found the extender to be beneficial in maintaining sperm characteristics [35,44,45].

The extender suplemented with BSA and FCS did not improve the percentages of motility and of acrosomal abnormality during 48 h for liquid storage of rabbit semen, compared to the control. The additives BSA and FCS had a significant effect in maintaining

of plasma membrane integrity at 48 h of storage period, compared to the control. Our results also demonstrated a protective effect on acrosomal integrity at 72 h with the addition of BSA and FCS to the extender, compared to the control. Furthermore, the percentages of intact plasma membrane and motility were better than controls at 72 h in the presence of BSA and FCS in this study. These results are in agreement with the previous results in ram [31], whale [23], turkey [8], and buffalo [18] semen. These studies showed that BSA significantly improved semen characteristics. BSA is found in reproductive tract fluids, and helps to preserve sperm motility and acrosomal integrity during the semen freezing process in goat [64]. Embryos from swine can be developed in vitro from the zygote to the blastocyst stage in a culture medium. However, further development and hatching of cultured blastocysts requires the presence of serum [32,43]. Sera seem to provide numerous amino acids, known and unknown growth factors and other macromolecules for the hatching of mammalian blastocysts [20,24,32]. BSA may also provide beneficial factors such as energy substrates or scavenger ions and small molecules [10]. Based on current results, it appeared that BSA and FCS had protective effect on semen characteristics providing beneficial factors.

Sperm DNA integrity is an important indicator on the transmission of genetic information to future generations. Spermatozoa are susceptible to a number of endogenous and exogenous factors. They possess limited defensive cytoplasm [53], therefore significant DNA damage may occur arising from lipid peroxidation [61]. The addition of the semen extender with various additives shows protective role against DNA damages during long-term sperm storage process [11,57,58]. In this study, the supplementation of BSA and FCS provided significant reduction in DNA damage of rabbit semen at 48 and 72 h during storage period, compared to the control. This finding is in agreement with the results of the studies performed on bovine [57] and goat [26,58] sperm in which an improvement was observed in DNA damage in the presence of various additives, following the freeze–thawing process.

Spermatozoa are very susceptible to lipid peroxidation since the spermatozoa have the high concentration of long chain polyunsatured fatty acid within the phospholipids [3]. Phospholipids in the sperm plasma membrane undergo peroxidation, which results in the formation of ROS and lipid hydroperoxides [5]. Spermatozoa have antioxidant capacity as a defense functioning mechanism against the lipid peroxidation of semen [28]. However, the antioxidant capacity in spermatozoa is insufficient in preventing lipid peroxidation [36]. In that regard, many studies showed that supplementations of antioxidant to semen extender prior to storage that enhance functional properties of spermatozoa [14,50,65]. In this study, MDA level and GPx activity were detected in rabbit semen for different liquid storage period at the presence of BSA and FBS. It was found that the level of MDA in control group was higher statistically when compared to experimental groups supplemented with BSA and FCS at 48 h and 72 h of liquid storage period at 5 °C. The additives BSA and FCS had a protective role in maintaining the GPx antioxidant activity compared to the control group during the liquid storage period for up to 72 h. The current results agree with a study previously conducted in the bull [51] with respect to the endogenous antioxidant activity in a group with BSA which study was demostrated that BSA (5 mg/ml) protected sperm morphology and fertilizing potential of bull spermatozoa, with a significant antioxidant property as a result of increased activity of catalase against cryodamage after the freeze–thawing process.

Conclusions

Our study showed that the addition of BSA and FCS to extender improved the quality of liquid stored rabbit semen. Compared to

Table 1
Mean ± SEM values of sperm parameters in liquid stored rabbit semen supplemented with BSA and FCS at 5 °C for 72 h.

Groups	Time (h)											
	0			24			48			72		
	Motility (%)	Acrosomal abnormality (%)	HOST (%)	Motility (%)	Acrosomal abnormality (%)	HOST (%)	Motility (%)	Acrosomal abnormality (%)	HOST (%)	Motility (%)	Acrosomal abnormality (%)	HOST (%)
n	6			6			6			6		
BSA	85.00 ± 1.83	3.33 ± 0.42	68.33 ± 1.05	77.50 ± 1.12	4.67 ± 0.21	65.67 ± 1.58	76.67 ± 1.05	8.83 ± 0.60	64.00 ± 0.63[a]	75.83 ± 0.83[a]	9.83 ± 0.48[a]	61.50 ± 1.69[a]
FCS	85.83 ± 1.54	3.17 ± 0.31	68.17 ± 1.33	75.83 ± 2.01	5.50 ± 0.43	66.67 ± 1.28	75.00 ± 1.83	9.00 ± 0.26	63.00 ± 0.58[a]	70.00 ± 1.83[b]	11.17 ± 0.48[a,b]	62.00 ± 0.93[a]
Control	85.83 ± 1.54	3.67 ± 0.33	69.00 ± 1.75	75.83 ± 2.01	5.33 ± 0.21	65.00 ± 1.34	75.00 ± 1.83	9.50 ± 0.43	60.17 ± 0.40[b]	70.00 ± 1.83[b]	13.00 ± 0.58[b]	56.67 ± 1.05[b]
Statistical significance (Kruskal WallisTest)	NS	NS	NS	NS	NS	NS	NS	NS	P < 0.01	P < 0.05	P < 0.01	P < 0.01

HOST: Hypo-osmotic swelling test.
NS: Non-significant.
[a] Different superscripts within the same column demonstrate significant differences.
[b] Different superscripts within the same column demonstrate significant differences.

Table 2

Mean ± SEM values of DNA damage of rabbit semen supplemented with BSA and FCS at 5 °C for 72 h ("n" refers to 50 randomly chosen sperm nuclei for DNA analysis in each of the six pooled ejaculates).

Groups	n	Time (h)					Statistical significance (GLM-repeated measures)		
		0	24	48	72	Total			
BSA	50	1.83 ± 0.09	2.17 ± 0.12	2.16 ± 0.09	2.23 ± 0.14	2.10 ± 0.06[a]	Groups	F: 42.55	P < 0.001
FCS	50	1.98 ± 0.12	1.89 ± 0.09	1.99 ± 0.09	2.44 ± 0.10	2.08 ± 0.06[a]	Time	F: 48.75	P < 0.001
Control	50	1.53 ± 0.09	1.94 ± 0.11	3.65 ± 0.17	3.76 ± 0.14	2.72 ± 0.06[b]	Groups-Time	F: 24.19	P < 0.001
Total	150	1.78 ± 0.64[A]	2.00 ± 0.62[A]	2.60 ± 0.72[B]	2.81 ± 0.76[C]				

[A] Different superscripts within the same row demonstrate significant differences.
[B] Different superscripts within the same row demonstrate significant differences.
[C] Different superscripts within the same row demonstrate significant differences.
[a] Different superscripts within the same column demonstrate significant differences.
[b] Different superscripts within the same column demonstrate significant differences.

Table 3

Mean ± SEM values of malondialdehyde (MDA) level and glutathione peroxidase (GPx) activity of rabbit semen supplemented with BSA and FCS at 5 °C for 72 h.

Groups	Time (h)							
	0		24		48		72	
	MDA (nmol/ml)	GPx (U/ml)	MDA (nmol/ml)	GPx (U/ml)	MDA (nmol/ml)	GPx (U/ml)	MDA (nmol/ml)	GPx (U/ml)
n	5	5	5	5	5	5	5	5
BSA	0.02 ± 0.001[a]	9.15 ± 0.001[a]	0.02 ± 0.001[a]	10.51 ± 0.06[a]	0.05 ± 0.001[a]	13.74 ± 0.01[a]	0.03 ± 0.001[a]	2.74 ± 0.01[a]
FCS	0.53 ± 0.01[b]	1.37 ± 0.01[b]	0.50 ± 0.04[b]	4.91 ± 0.02[b]	0.09 ± 0.001[b]	12.85 ± 0.02[b]	0.08 ± 0.001[b]	1.92 ± 0.01[b]
Control	0.08 ± 0.001[c]	1.98 ± 0.01[c]	0.07 ± 0.001[a]	2.86 ± 0.03[c]	0.11 ± 0.001[c]	3.71 ± 0.01[c]	0.70 ± 0.001[c]	1.34 ± 0.01[c]
Statistical significance (Kruskal Wallis Test)	P < 0.01	P < 0.01	P < 0.01	P < 0.01	P < 0.01	P < 0.01	P < 0.01	P < 0.01

[a] Different superscripts within the same column demonstrate significant differences.
[b] Different superscripts within the same column demonstrate significant differences.
[c] Different superscripts within the same column demonstrate significant differences.

the controls, the additives provided a protective effect on the motility, morphological integrity, plasma membrane integrity and DNA integrity following liquid storage up to 72 h at 5 °C. Additionally, it was found that the additives BSA and FCS had a protective role in maintaining the GPx antioxidant activity and decreasing the level of MDA compared to the control group during the liquid storage period for up to 72 h.

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