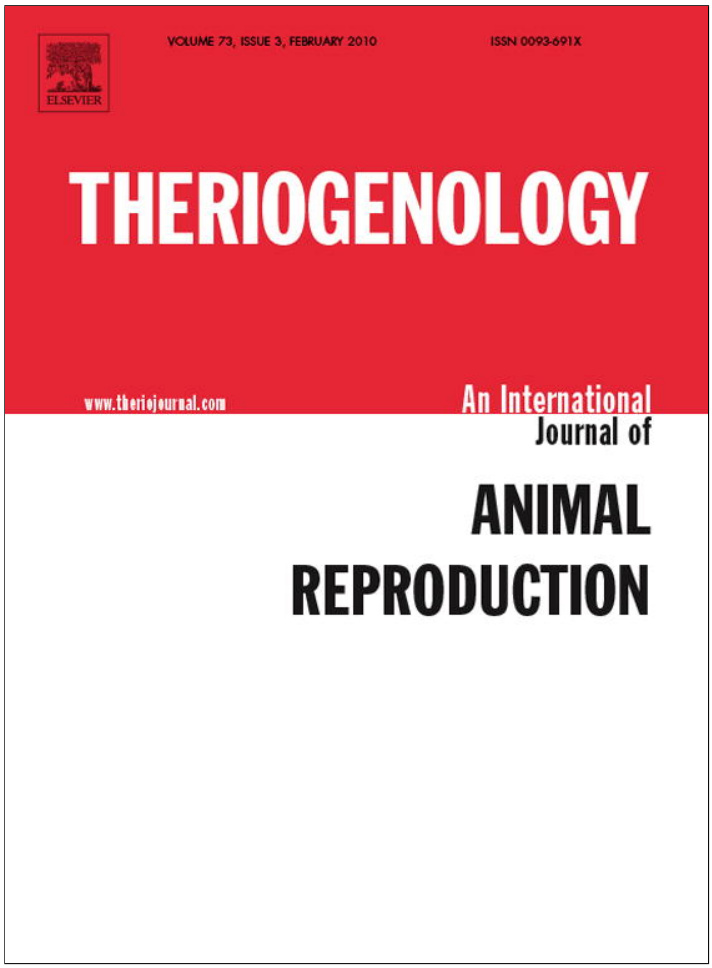

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Theriogenology 73 (2010) 316–323
Theriogenology
www.theriojournal.com

Effects of different extenders and centrifugation/washing on postthaw microscopic-oxidative stress parameters and fertilizing ability of Angora buck sperm

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Received 7 July 2009; received in revised form 4 September 2009; accepted 8 September 2009

Abstract

The main objective of the current study was to evaluate the effects of extender type and centrifugation/washing prior to cryopreservation on the postthaw sperm parameters, lipid peroxidation, and superoxide dismutase activity of Angora buck (Capra hircus ancryrensis) sperm. Ejaculates collected from three Angora bucks were used in this study. Two consecutive ejaculates from each buck were pooled and split into equal parts in four Falcon tubes. Two tubes were diluted at 37 °C and then centrifuged to remove semen plasma. After centrifugation, two sediment parts were diluted with a Tris-based extender and commercial Bioxcell extender, respectively. The remaining two parts, which were not centrifuged/washed, were diluted with the above-mentioned extenders, respectively. Diluted samples were cooled to 5 °C and frozen in 0.25-mL French straws to be stored in liquid nitrogen. Frozen straws were thawed individually at 37 °C for 20 sec in a water bath for evaluation. The semen part with centrifugation/washing in the Bioxcell extender (BC) demonstrated a higher rate of subjective motility (58.1 ± 3.0%) compared with that of groups with (TC) or without (T) centrifugation/washing in the Tris-based extender ($P < 0.01$). Angora buck sperm frozen with (BC) or without (B) centrifugation/washing in the Bioxcell extender demonstrated higher percentages of motility (60.6 ± 2.7% and 54.3 ± 4.8%, respectively) compared with that of groups T and TC. The postthaw progressive motility rate (22.3 ± 2.7%) was significantly greater for semen parts diluted in B compared with that of other groups. BC gave rise to a lower value of average path velocity (90.0 ± 5.2 μm/sec) compared with that of other groups ($P < 0.01$). For straight linear velocity and linearity index, the highest values (103.2 ± 4.7 μm/sec, 47.5 ± 1.6% and 94.8 ± 3.0 μm/sec, 44.8 ± 1.1%, respectively) were obtained from B and TC ($P < 0.001$). For sperm acrosome and total abnormalities, TC gave the highest values (11.2 ± 0.6% and 26.6 ± 1.5%, respectively, $P < 0.01$). In the group frozen in BC, the percentage of membrane integrity assessed by hypo-osmotic swelling test was higher (61.2 ± 2.2%) than that of the other groups ($P < 0.001$). With respect to fertility results based on 35-d pregnancy rates, BC gave a higher rate (76.5%) than that of TC (27.8%, $P < 0.05$). Malondialdehyde formation was found to be lower (1.64 ± 0.26 nmol/L) in BC than in the other groups after the freeze-thawing process ($P < 0.001$). In the semen part frozen in BC, superoxide dismutase activity was higher (0.18 ± 0.02 U/mg protein) compared with that of the other groups ($P < 0.05$). Further studies are required to obtain more precise results for the characterization of oxidative stress parameters and fertilizing ability in cryopreserved buck spermatozoa.

Keywords: Angora buck; Laparoscopic insemination; Microscopic-oxidative stress parameters; Semen freezing

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0093-691X/$ – see front matter.
doi:10.1016/j.theriogenology.2009.09.015

1. Introduction

Cryopreservation and artificial insemination (AI) techniques play an important role in goat breeding, especially to provide satisfactory fertility rates after the long-term storage of semen from quality bucks [1]. Some of the problems encountered with the use of AI in small ruminants include decrease in sperm parameters (e.g., motility, membrane integrity, and fertilizing potential) and alteration in membrane responses to physiologic stimuli of the sperm [2]. Laparoscopic insemination is preferred to be used in the breeding of small ruminants due to the particular cervix anatomy of these animals, which renders AI less efficient [3]. Laparoscopic insemination was reported to improve the conception rate in comparison with cervical insemination when frozen semen was used [4–7].

It is reported that seminal plasma has a detrimental effect on the viability of buck spermatozoa in the case of egg yolk in the extender during cryopreservation. Seminal plasma contains egg yolk coagulating enzyme (EYCE), which hydrolyzes egg yolk lecithin into fatty acids, and lysolecithin, which is highly detrimental to buck spermatozoa. This hydrolysis leads to a subsequent loss of motility, membrane integrity, fertilizing capability, and metabolic changes of buck semen during cryopreservation [8–10]. Many researches show that the washing of semen is necessary to increase motility, membrane integrity, and fertility after freeze-thawing [11–13], whereas others indicate that postthaw results were achieved without washing prior to cryopreservation [4,14,15].

Furthermore, all the processes of cryopreservation, including cooling, freezing, and thawing, also create oxidative stress on the sperm membrane, resulting in the reduction of parameters [16–19]. It is well known that mammalian spermatozoa contain high concentrations of polyunsaturated fatty acids, and therefore they are highly susceptible to lipid peroxidation (LPO), which damages lipids, proteins, and DNA, and thus ultimately leads to sperm death [20,21]. In recent years, many researches have focused on different cryopreservation systems with or without the removal of seminal plasma as well as on the use of extenders with antioxidants to reduce the harmful effects of LPO [22,23].

The improvement of buck semen cryopreservation technologies requires in-depth knowledge of the properties of current extenders. Tris-based extenders with egg yolk have been widely used for the freezing of buck spermatozoa [24]. However, there is a lack of research evaluating the effect of centrifugation/washing on sperm (motility, morphology, functional integrity, and fertilizing ability) and oxidative stress parameters of Angora buck spermatozoa cryopreserved in a commercial Bioxcell extender.

In the current study, the effects of the extender type (Tris or Bioxcell) and pretreatment processes such as centrifugation/washing, prior to cryopreservation, on motility, morphology, functional integrity, fertilizing ability, LPO, and superoxide dismutase (SOD) activity of Angora buck sperm were investigated during the freeze-thawing process.

2. Materials and methods

2.1. Chemicals

The Bioxcell extender was purchased from IMV Technologies, L'Aigle, France (Şark Kemikal Ltd., Ankara, Turkey), and other chemicals used in this study were obtained from Sigma-Aldrich, St Louis, Mo, USA.

2.2. Animals and semen collection

Semen samples from three mature Angora bucks (Capra hircus ancryrensis) (3 and 4 yr of age), of superior genetic merit and fertility capacity, were used in this study. The Angora bucks, belonging to the Lalahan Livestock Central Research Institute, were maintained under uniform nutritional conditions. Ejaculates were collected twice a week from the Angora bucks with the aid of an artificial vagina during the breeding season (autumn to early winter). Immediately after collection, the ejaculates were immersed in a warm water bath at 33 °C until their assessment in the laboratory. Semen assessment was performed within approximately 20 min.

2.3. Semen extending, freezing and thawing

The groups established in this study were as follows:

B: Experimental group that was frozen in a Bioxcell extender without the centrifugation/washing of semen.
BC: Experimental group that was frozen in a Bioxcell extender with the centrifugation/washing of semen.
T: Experimental group that was frozen in a Tris-based extender without the centrifugation/washing of semen.
TC: Experimental group that was frozen in a Tris-based extender with the centrifugation/washing of semen.

The volume of ejaculates was measured in a conical tube graduated at 0.1-mL intervals. The sperm concentration was determined by means of a hemocytometer [25], and sperm motility was estimated using phase-contrast microscopy (×400). Only ejaculates between 1 and 2 mL in volume, spermatozoa with >80% progressive motility, and a concentration higher than 2.5×10^9 spermatozoa/mL were used for the freezing process. A total of 60 ejaculates were used in this study. Two consecutive ejaculates from each buck were pooled and split into equal parts in four Falcon tubes. Two tubes were diluted at 37 °C in a 1:5 ratio in Ringer's lactate and then centrifuged for 10 min at $1000 \times g$ at room temperature to remove seminal plasma. After centrifugation, two sediment parts were diluted in a Tris-based extender (Tris 254 mM, citric acid 78 mM, fructose 70 mM, egg yolk 15% [vol/vol], glycerol 6% [vol/vol], pH 6.8) and commercial Bioxcell extender, respectively. The remaining two parts were diluted in the above-mentioned extenders, respectively. Diluted semen was packaged in 0.25-mL French straws (100×10^6 spermatozoa per straw), sealed with polyvinyl alcohol powder, and equilibrated at 5 °C for 3 h. After equilibration, the straws were frozen in liquid nitrogen vapor, 4 cm above the liquid nitrogen, for 15 min and plunged into liquid nitrogen for storage. After being stored for a week, the frozen straws were thawed individually at 37 °C for 20 sec in a water bath for semen evaluation.

2.4. Semen evaluation

2.4.1. Analysis of microscopic sperm parameters

Subjective motility was assessed using a phase-contrast microscope (×100), with a warm stage maintained at 37 °C. A wet mount was made using a 5-μL drop of semen placed directly on a microscope slide and covered by a coverslip. Sperm motility estimations were performed in three different microscopic fields for each semen sample by the same researcher. The mean of the three successive estimations was recorded as the final motility score. Besides estimating subjective sperm motility, a computer–assisted sperm motility analysis (CASA; IVOS version 12; Hamilton-Thorne Biosciences, Beverly MA, USA) was also used to analyze sperm motion characteristics. CASA was set up as follows: phase contrast; frame rate 60 Hz; minimum contrast 70; low and high static size gates 0.6 to 4.32; low and high intensity gates 0.20 to 1.92; low and high elongation gates 7 to 91; default cell size 10 pixels; default cell intensity 80. Thawed semen was diluted (5 μL semen + 95 μL extender) in a Tris-based extender (without egg yolk and glycerol) and evaluated immediately after dilution. A 4-μL sample of diluted semen was put onto a prewarmed chamber slide (20 μm; Leja 4; Leja Products Luzernestraat B.V., Holland), and sperm motility characteristics were determined with a ×10 objective at 37 °C. The following motility values were recorded: motility (%), progressive motility (%), VAP (average path velocity, μm/sec), VSL (straight linear velocity, μm/sec), VCL (curvilinear velocity, μm/sec), ALH (amplitude of lateral head displacement, μm), LIN (linearity index; LIN = [VSL/VCL] × 100). For each evaluation, 10 microscopic fields were analyzed to include at least 300 cells.

For the assessment of sperm abnormalities, at least three drops of each sample were added to Eppendorf tubes containing 1 mL Hancock solution (62.5 mL formalin [37%], 150 mL sodium saline solution, 150 mL buffer solution, and 500 mL double-distilled water) [26]. One drop of this mixture was put on a slide and covered with a coverslip. The percentages of total sperm abnormalities (acrosome and other abnormalities) were determined by counting a total of 200 spermatozoa under phase-contrast microscopy (×1000, oil immersion).

The hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane based on curled and swollen tails. This was performed by incubating 30 μL of semen with 300 μL of a 100 mOsm hypo-osmotic solution (9 g fructose + 4.9 g sodium citrate per liter of distilled water) at 37 °C for 60 min. After incubation, 0.2 mL of the mixture was spread with a coverslip on a warm slide. Two hundred spermatozoa were evaluated (×1000) under bright-field microscopy. Sperm with swollen or coiled tails were recorded [27,28].

In fertility trials, estrous was detected once a day at 0800 h (morning) in the natural breeding season by introducing bucks fitted with an apron. Females in natural estrus were removed from the herd. After detection, a total of 69 does were inseminated intrauterine by laparoscopy with frozen samples pertaining to four experimental groups, each comprising the semen of one Angora buck. The dose of each straw was 0.25 mL and contained 100×10^6 spermatozoa for an insemination. Pregnancy was diagnosed by rectal ultrasound scanning at Day 35 after AI.

2.4.2. Biochemical assays

Biochemical assays were performed on sperm samples immediately after thawing following centrifugation and washing. An aliquot (500 μL) of semen from

each sample was centrifuged at 800 × *g* for 10 min, and the sperm pellet was separated and washed by resuspending in PBS and recentrifuging (three times). After the last centrifugation, Triton X-100 (0.1%) was added to the sperm pellet. The supernatant was used for enzymatic measurements. LPO and SOD activities in spermatozoa were determined immediately from these samples. LPO and SOD activities were calculated for 2×10^8 sperm cells [29,30].

2.4.2.1. MDA concentrations. Concentrations of malondialdehyde (MDA), as indices of LPO in sperm samples, were measured using the thiobarbituric acid reaction and according to the method described by Yoshioka et al. [31]. The 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. The MDA concentrations were expressed as nmol/L.

2.4.2.2. SOD activity. Superoxide dismutase activity was analyzed with the technique of Sun and Zigman [32]. A volume of 0.4 mL sample, 0.1 mL 0.9% NaCl, and 0.4 mL chloroform was centrifuged for 10 min at 2000 × *g*. The reaction mixture contained 20 mL 3 mmol/L xanthine, 10 mL 0.6 mmol/L ethylenediamine tetraacetic acid (EDTA), 10 mL 0.15 mmol/L nitroblue tetrazolium (NBT), 6 mL 400 mmol/L sodium carbonate, and 3 mL 1 g/L Bovine serum albumin (BSA) for 20 tests. An aliquot (50 μL) of xanthine oxidase (20 μmol/2 mL) was added to 2.45 mL of the reaction mixture, and this was incubated at 25 °C for 20 min. Subsequently, the reaction was stopped by adding 1 mL 0.8 mmol/L $CuCl_2$ to the test. The absorbance was recorded at 560 nm by a spectrophotometer. SOD activity was expressed as U/g protein.

2.5. Statistical analysis

The experiment was repeated 10 times. The results were expressed as mean ± SEM. Means were analyzed using a repeated measurement model of ANOVA, followed by Duncan's post hoc test to determine significant differences for all parameters between all groups using the SPSS/PC software package (version 12.0; SPSS Inc., Chicago, IL, USA). Differences with values of $P < 0.05$ were considered statistically significant. Pregnancy rates were evaluated using the chi-square test.

3. Results

3.1. Microscopic sperm parameters (percentages of motility, acrosome, total abnormalities, HOST, and in vivo fertility)

The influence of a Bioxcell or Tris extender with or without centrifugation/washing on standard semen parameters after the freeze-thawing process is shown in Table 1. Group BC resulted in a better rate of subjective motility (58.1 ± 3.0%) compared with that of groups T and TC ($P < 0.01$). Angora buck sperm frozen in B and BC demonstrated higher percentages of motility (60.6 ± 2.7% and 54.3 ± 4.8%, respectively) compared with that of groups T and TC. Postthaw progressive motility rate (22.3 ± 2.7%) was significantly greater for semen parts diluted in B compared

Table 1
Mean ± SEM sperm parameters in frozen-thawed buck semen.

Groups	BC	B	TC	T	P
Subjective motility (%)	58.1 ± 3.0^{c}	53.5 ± 3.8^{bc}	40.9 ± 1.8^{a}	45.0 ± 3.1^{ab}	**
CASA motility (%)	60.6 ± 2.7^{c}	54.3 ± 4.8^{c}	31.5 ± 2.7^{a}	43.8 ± 3.5^{b}	***
Progressive motility (%)	10.1 ± 1.4^{a}	22.3 ± 2.7^{b}	7.2 ± 1.1^{a}	7.0 ± 1.2^{a}	***
VAP (μm/sec)	90.0 ± 5.2^{a}	117.5 ± 4.2^{c}	109.0 ± 2.5^{bc}	102.1 ± 4.4^{b}	**
VSL (μm/sec)	52.8 ± 5.7^{a}	103.2 ± 4.7^{b}	94.8 ± 3.0^{b}	65.9 ± 6.1^{a}	***
VCL (μm/sec)	183.0 ± 10.1	199.6 ± 11.5	211.4 ± 5.1	208.9 ± 7.4	–
LIN (%)	32.0 ± 2.5^{a}	47.5 ± 1.6^{b}	44.8 ± 1.1^{b}	32.0 ± 2.0^{a}	***
ALH (μm)	8.7 ± 0.3	7.3 ± 0.2	8.1 ± 0.3	8.2 ± 0.7	–
Acrosome abnormality (%)	6.8 ± 1.0^{a}	7.1 ± 0.8^{b}	11.2 ± 0.6^{c}	9.3 ± 0.6^{bc}	**
Total abnormalities (%)	16.7 ± 1.0^{a}	17.7 ± 0.8^{a}	26.6 ± 1.5^{b}	19.4 ± 0.8^{a}	***
HOST (%)	61.2 ± 2.2^{c}	52.7 ± 2.2^{b}	30.6 ± 1.0^{a}	49.3 ± 0.6^{b}	***
Pregnancy rate (%)	76.5^{b} (13/17)	52.9^{ab} (9/17)	27.8^{a} (5/18)	47.1^{ab} (8/17)	**

[a–c]Different superscripts within the same row demonstrate significant differences among groups (**$P < 0.01$; ***$P < 0.001$).
–, no significant difference ($P > 0.05$).

Table 2
Mean ± SEM malondialdehyde and superoxide dismutase activities in frozen-thawed buck semen.

Groups	BC	B	TC	T	P
MDA (nmol/L)	1.64 ± 0.26^{a}	2.64 ± 0.18^{b}	4.39 ± 0.06^{c}	2.82 ± 0.28^{b}	***
SOD (U/mg protein)	0.18 ± 0.02^{b}	0.11 ± 0.02^{a}	0.13 ± 0.01^{a}	0.12 ± 0.01^{a}	*

[a–c]Different superscripts within the same row demonstrate significant differences among groups (*$P < 0.05$; ***$P < 0.001$).

with that of the other groups. BC gave rise to a lower value of VAP (90.0 ± 5.2 μm/sec) compared with that of the other groups ($P < 0.01$). For VSL and LIN, the highest values (103.2 ± 4.7 μm/sec, 47.5 ± 1.6% and 94.8 ± 3.0 μm/sec, 44.8 ± 1.1%, respectively) were obtained from B and TC ($P < 0.001$). For sperm acrosome and total abnormalities, TC gave the highest values (11.2 ± 0.6% and 26.6 ± 1.5%, respectively, $P < 0.01$). In the group frozen in BC, the percentage of membrane integrity assessed by HOST was higher (61.2 ± 2.2%) than that of the other groups ($P < 0.001$). With respect to fertility results based on 35-d pregnancy rates, BC gave a higher rate (76.5%) than that of TC (27.8%, $P < 0.05$).

3.2. Analysis of oxidative stress parameters

As shown in Table 2, MDA formation was found to be lower (1.64 ± 0.26 nmol/L) in BC than in the other groups after the freeze-thawing process ($P < 0.001$). In semen parts frozen in BC, SOD activity was significantly increased (0.18 ± 0.02 U/mg protein) compared with that of the other groups ($P < 0.05$).

4. Discussion

When cells are frozen, they are subjected to various stresses such as cold shock and oxidative stress that arise through ice crystallization and LPO due to membrane changes. Cryopreservation disrupts the transbilayer-phospholipid asymmetry in the plasma membrane of mammalian sperm. Ultrastructural damage of the plasma membrane increases the susceptibility to LPO when high production of reactive oxygen species (ROS) occurs during the freeze-thawing process—predisposing mammalian sperm to gross morphologic damage and decreased motility and fertilizing capability. This was stated for bull [33–35], ram [15,36], buck [23], boar [37,38], and human [39] sperm. However, in vivo physiologic concentrations of ROS are involved in providing membrane fluidity and maintaining the fertilizing ability and acrosome reaction of sperm [20,40]. Endogenous antioxidant mechanisms are, therefore, important in maintaining postthaw sperm parameters, namely, motility, membrane integrity, and fertilizing ability, particularly in the absence of seminal plasma, which is known as the prime source of antioxidant protection [41–45]. Among endogenous antioxidant mechanisms, SOD, which is a well-known enzymatic biological antioxidant, scavenges superoxide and peroxide and thus controls oxidative stress in mammalian sperm [5,46]. It also removes (O_2^-) generated by NADPH-oxidase in living cells [36,47]. In this study, Bioxcell showed an antioxidative property, resulting in higher antioxidant enzyme activity such as SOD. This finding was similar to the results of a study performed by Stradaioli et al. in bovine species [48].

Buck semen is centrifuged/washed to remove seminal plasma during the freeze-thawing process. Centrifugation/washing is considered as an essential process particularly when egg yolk is used in the freezing extender. The fact that EYCE located in seminal plasma interacts with lecithin of egg yolk, generating substances harmful for spermatozoa (e.g., isolecithin), necessitates the centrifugation/washing of semen prior to cryopreservation. However, some authors showed that the freezing of semen diluted in an extender containing egg yolk without centrifugation/washing was not detrimental to postthaw sperm parameters [23,49,50].

In this study, BC gave a stronger cryoprotective effect on postthaw spermatologic quality such as subjective and CASA motility, morphology, functional integrity, and fertilizing ability compared with that of B. However, this difference was not statistically important, except for the value of functional integrity assessed by HOST. With respect to oxidative stress parameters, BC showed higher antioxidative properties than B, TC, and T resulting in a decrease in MDA formation ($P < 0.01$) and an increase in SOD enzyme activity ($P < 0.05$). Additionally, the MDA level of T was lower than that of TC. In the current study, it was demonstrated that TC led to the formation of higher levels of MDA compared with that of the other groups. This is in contradiction with Carnaval et al. [51], who reported that MDA production was not affected by centrifugation in boar semen after the freeze-thawing process.

Contrary to Bioxcell groups, T caused stronger cryoprotection in spermatologic values (motility, morphology, functional integrity, and fertilizing ability) compared with that of TC after the freeze-thawing process. Among values of total abnormalities and HOST, these differences were not statistically important. In this study, we did not find any important difference in pregnancy rates in either washed or nonwashed spermatozoa prior to cryopreservation, similar to the results obtained in a previous study [4]. Some researchers [11,13,52] showed that centrifugation/washing provided better postthaw sperm survival, in contrast with our current results suggesting that centrifugation/washing was detrimental to postthaw sperm parameters. In the current study, whereas centrifugation/washing in a Bioxcell extender led to more positive effects on functional integrity and oxidative stress parameters, no centrifugation/washing in a Tris-based extender showed important superiority in the protection of total morphology and functional integrity and decrease in MDA formation compared with that of TC. Changes in the extenders and centrifugation/washing process may explain contradictory results in sperm.

Regarding the centrifugation/washing process, BC was superior in maintaining sperm motility, morphology, functional integrity, fertilizing ability, and eliminating MDA formation compared with TC. In groups of no centrifugation/washing process, B was more effective in elevating CASA and progressive motility compared with T. The egg yolk in the Tris-based extender increased the viscosity of the extender, which might also have complicated motility and fertilization.

Generally, Bioxcell groups gave higher results of postthaw parameters, excluding sperm motion characteristics, than those of groups with Tris containing egg yolk. Only differences in CASA motilities were statistically important. Therefore, it can be implied that egg yolk contains some substances harmful to sperm quality parameters. Additives that may be used as external cryoprotectants in extenders for buck semen alternatively to egg yolk include soybean lecithin, which is also present in a commercial Bioxcell extender having less viscous fluid. This study agrees with a study where Bioxcell is recommended as a freezing extender for ram semen freezing [53]. We can say that a high fertility rate with or without centrifugation/washing buck semen can be achieved with the Bioxcell extender. This is in agreement with results obtained by Gil et al. [54] indicating that the light viscous form of Bioxcell may make AI easier when frozen-thawed semen is used.

In conclusion, antioxidative activity was significantly higher with the BC extender, leading to a significant reduction of LPO, compared with that of other groups. In addition, even though the fertility rate of BC was 76.5%, groups B and T gave rise to fertility rates of 52.9% and 47.1%, respectively. But these differences were not statistically important. The fertility rate of group TC was lower than that of other groups. Thus, BC may be recommended for use as a freezing extender.

It may be stated that the centrifugation and washing of Angora buck semen to remove the seminal plasma prior to cryopreservation is time consuming and useless when egg yolk extenders are used, but not for Bioxcell. This process deteriorated sperm motility, morphology, functional integrity, and fertilizing ability and led to a high release of MDA in TC. The use of Bioxcell with or without the centrifugation/washing of semen prior to cryopreservation may be recommended to facilitate semen cryopreservation systems in the goat breeding industry.

Acknowledgments

This study was supported by the Republic of Turkey, Ministry of Agriculture and Rural Affairs, General Directorate of Agricultural Research (GDAR) Project number: 08.08.04/02.

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