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Effect of Seminal Plasma Removal, Washing Solutions, and Centrifugation Regimes on Boer Goat Semen Cryopreservation

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ABSTRACT

Three experiments were carried out to improve semen quality during cryopreservation process. Total motility, forward motility, acrosome integrity, live spermatozoa, and normal spermatozoa were measured as semen quality. In Experiment 1, the effects of seminal plasma removal were analyzed by using two different extenders (GE and FE). The removal of seminal plasma gave higher and significant ($P<0.05$) effect in the total motility, forward motility, and live spermatozoa after cryopreservation. For two different extenders, however, the differences were not observed on the semen quality. In Experiment 2, three different washing solutions (namely, phosphate buffered saline, normal saline and Tris-based extender) were tested to evaluate the effects of semen quality after cryopreservation. Tris-based extender (TCG) conferred the highest ($P<0.05$) sperm quality values in the total motility, forward motility, and live spermatozoa after cryopreservation. In Experiment 3, the effects of different centrifugation regimes (3000 × g for 3 min, 1600 × g for 10 min, 800 × g for 15 min) were evaluated on Boer semen quality. Semen quality parameters (namely, total motility, forward motility, acrosome integrity, and live spermatozoa) were significantly ($P<0.05$) higher for cryopreserved spermatozoa centrifuged with 3000 × g for 3 min than the others. In conclusion, the removal of seminal plasma, washing solution TCG, and the use short-term centrifugation with a relative high g-force could contribute to the increased Boer semen quality after cryopreservation.

Keywords: Boer goat, centrifugation, cryopreservation, seminal plasma, washing solution

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INTRODUCTION

Semen cryopreservation is essential for the application of reproductive techniques such as AI and IVF which contribute to increase production of goat and genetic selection schemes (Leboeuf *et al*., 2000). However, cryopreservation causes ultrastructural, biochemical, and functional damages on spermatozoa due to the temperature changes resulting in decreased motility and viability. In addition, causes of reduced sperm motility are related to seminal plasma enzymes. Therefore, seminal plasma plays an important role in sperm survival during cryopreservation process (Salamon and Ritar, 1982).

The deterioration and toxic effect of the seminal plasma were observed when goat's semen was diluted with egg yolk or milk extender. Nowadays, these extenders are widely used for the frozen storage of small ruminant semen (Salamon & Maxwell, 2000). The presence of enzymes (bulbourethral secretion glycoprotein-60 and egg yolk coagulating enzyme) in the seminal plasma caused the harmful interactions between seminal plasma and egg yolk or milk (Nunes *et al*., 1982, Leboeuf *et al*., 2000). Meanwhile, bulbourethral secretion glycoprotein-60 (BUSgp60) has a triacylglycerol hydrolase activity which decreases sperm motility and movement quality by disruption of cell membrane (Pellicer-Rubio & Combarnous, 1998). Phospholipase A_2 activity of egg yolk coagulating enzyme (EYCE) catalyse the hydrolysis of egg yolk phosphatidycholine (PC) into fatty acids and lysophophatidycholine (LPC). LPC has toxic effect on buck spermatozoa by acting like a detergent on biomembrane, resulting in a loss of motility, membrane integrity and consequently low fertility rate (Upreti *et al*., 1999).

Therefore, several researchers reported that the removal of seminal plasma had favourable effect on semen freezing and thawing properties in buck (Ritar & Salamon, 1982; Corteel, 1992; Kozdrowski *et al*., 2007). However, Tuli & Holtz (1994), Azeredo *et al*. (2001) and Peterson *et al*. (2007) observed no favourable effect of the removal of seminal plasma in buck semen cryopreservation. Sariozkan *et al*. (2010) also described that a high fertility rate, with or without centrifugation/washing, buck semen could be achieved with the Bioxcell extender. Therefore, the benefits of seminal plasma removal presented in literature are quite variable until now (Purdy, 2006).

Removal of seminal plasma is a time consuming process that can damage cells if it is performed improperly; however, if it is done correctly, it can be beneficial (Purdy, 2006). Therefore, some previous research have used a variety of washing solutions and concentration regimes to find out the proper method for the improvement of cryopreserved semen quality. The centrifugation regimes and washing solutions used in buck semen cryopreservation were 800 x g for 15 min with Tris citric acid buffer (Tuli & Holtz, 1994), 600 × g for 10 min with Krebs-Ringer phosphate plus sodium citrate (Azeredo *et al*., 2001), 1200 × g for 15 min with Tris citric acid glucose (TCG) buffer (Peterson *et al*., 2007), 1500 × g for 10 min with TCG (Kozdrowski *et al*., 2007), and 1000 × g for 10 min with Ringer's latate (Sariozkan *et al*., 2010). In other species, several researchers have studied the effects of centrifugations regimes. Among other, Carvajal *et al*. (2004) observed the influence of different centrifugation regimes (400, 800, 1600, and 2400 × g) and reported that the use of short-term centrifugation with a relative high g-force (2400 × g for 3 min) caused a positive effect on Boar sperm cryosurvival. Webb & Dean (2009) described that post thaw motility of frozen stallion sperm was not different between centrifugation treatments (700 × g for 15 min, 600 × g for 12 min, and 400 × g for 7 min). Nonetheless, studies evaluating the effects of washing solutions and centrifugation regime on Boer goat semen cryopreservation are still very limited. Moreover, the results presented in the literature are quite variable. Hence, to overcome this variability, the researchers should endeavour to reach a consensus that generally addresses accepted practices for the effects of seminal plasma and centrifugation in Boer goat semen cryopreservation. Therefore, these experiments were carried out to analyze the effects of seminal

plasma removal using two different extenders, three different washing solutions and different centrifugation regimes on the characteristics of Boer goat semen before freezing and after thawing.

MATERIALS AND METHODS

Animals, Semen Collection and Evaluation

Semen samples from five mature Boer goats (2 and 4 yr of age) were used in this study. Ejaculates were collected twice a week from the Boer goats with the aid of an artificial vagina. Immediately after collection, the ejaculates were immersed in warm water bath at 37°C and semen assessment was performed within approximately 20 min. Only ejaculates between 1 and 2 ml in volume, spermatozoa with >70% progressive motility, and a concentration of higher than 2.5 x 10^9 spermatozoa per straw were used for the freezing process. A total of 30 ejaculates were used in this study.

The volume of each ejaculate was measured in a graduated test tube and consistency was subjectively scored (0 to 3). The mass activity of the semen was measured on a score of 0 to 5. The sperm concentration of each ejaculate was determined by means of a haemocytometer. Live and dead spermatozoa, as well as morphologically normal spermatozoa percentages were assessed using nigrosin-eosin stain (Evan & Maxwell, 1987). The percentage of acrosome integrity (with normal apical ridge) was determined by evaluating sperm smears which were stained with nigrosin-eosin and examined under phase contrast microscope at x1000 magnification under oil immersion objective with bright light (Yildiz *et al*., 2000). Spermatozoa (200 cells per slide) in duplicate for each treatment were assessed as well. The percentage of the motility of spermatozoa in each specimen was evaluated under a phase contrast microscope at ×200 magnification by placing a 5 µl drop of diluted semen on a slide covered with a glass cover slip (22 mm × 22 mm) from three selected representative fields subjectively. The mean of the three successive estimations was recorded as the final motility score. S perm motility was assessed by modifying the category of the WHO laboratory manual (WHO, 2002).

Extenders

Two types of semen extenders were used to analyse the effects of seminal plasma removal on Boer goat semen cryopreservation (Foote, 1970; Liu *et al*., 1998).

Glucose based extender (GE) - the cooling extender consisted of 250 mM Tris, 88.5 mM citric acid, 70 mM glucose, 18% egg yolk (v/v) and antibiotics (5000 IU penicillin, 5 mg streptomycin, 10 mg neomycin per ml). The freezing extenders were composed of 250 mM Tris, 88.5 mM citric acid, 18% egg yolk (v/v) and 8% glycerol (v/v).

Fructose-based extender (FE) - the cooling extender consisted of 312.53 mM Tris, 110 mM citric acids, 55 mM fructose, 20% egg yolk (v/v) and antibiotics (5000 IU penicillin, 5 mg streptomycin, 10 mg neomycin per ml). The freezing extender was composted of 312.53 mM tris, 110 mM citric acid, 55 mM fructose, 20% egg yolk (v/v) and 6.8% glycerol (v/v).

Glucose based extender (GE) was used to evaluate the effects of washing solutions and centrifugation regimes. All the chemicals were reagent grade and were purchased from Sigma-Aldrich, St. Louis, MO.

Experimental Designs

A total of three experiments were conducted in this study. The first experiment was replicated three times from each of the four Boer goats. Following the initial evaluation, the ejaculates were equally divided into two parts. One part was diluted with normal saline at the ratio of (1:1 v/v) and subjected to centrifuge at 1500 x g for 5 min for the removal of seminal plasma. The washed spermatozoa was split again into two equal aliquots and immediately extended with two different extenders. The other part of the ejaculate was also divided into two aliquots, but without the removal of seminal plasma and diluted with the two extenders (GE and FE), respectively.

In Experiment 2, the study was replicated two times from each of five Boer goats. Immediately after the initial evaluation, the ejaculated semen was equally divided into three aliquots and diluted with each of the three different washing solutions (phosphate-buffered saline [PBS], normal saline [NS], Tris-based extender [TCG], cooling extender of GE without egg yolk at the ratio of (1:1 v/v) (O'Meara *et al*., 2007; Evan & Maxwell, 1987; Kozdrowski *et al*., 2007). Meanwhile, the seminal plasma was removed by centrifugation at 1500 × g for 5 min.

Experiment 3 was replicated two times from each of the four Boer goats. Following the initial evaluation, the semen was mixed with cooling extender of GE without egg yolk at the ratio of (1:1 v/v) and equally divided into three parts. Diluted spermatozoa were centrifuged by using three different centrifugation regimes (3000 × g for 3 min, 1600 × g for 10 min, and 800 × g for 15 min) and all of supernatants were discarded.

Cooling, Freezing and Thawing of Spermatozoa

After the removal of seminal plasma, the semen was diluted with cooling extender. Semen dilution procedure was carried out by using two step dilution methods. The diluted semen was incubated at room temperature (26°C) for 10 min. The extended semen were subsequently placed in a cooling chamber at 5°C and maintained for 2.5 hr. The extended semen was then diluted in the freezing extender to obtain the final concentration of 150×10^6 spermatozoa per straw and kept for 30 min. After that, the sperm suspension was loaded into 0.25 ml straws. The total cooling time lasted for about three hours. The straws were horizontally placed on an aluminium rack and frozen in liquid nitrogen vapour, about 5 cm above the surface of liquid nitrogen for 7 min, and then immersed into the liquid nitrogen for storage. After 2 days, the thawing procedure was carried out in a water bath (37°C) for 30 sec. Immediately after thawing, the total motility, forward motility, acrosome integrity, percentage of live spermatozoa, and normal spermatozoa were evaluated.

Statistical Analysis

The data were analysed using the SPSS software system (Version 12.0, SPSS, Chicago, IL). The sperm quality parameters in Experiment 1 were analysed using a 2-way factorial analysis of variance. The values were expressed as the mean ± the standard error of the mean (S.E.M) and the level of statistical significance was considered as $P<0.05$. In Experiments 2 and 3, the means were analyzed using a one-way analysis of variance, followed by the Tukey's post-hoc test to determine the significant differences in all the semen quality parameters between the groups.

RESULTS

The removal of seminal plasma had a significant influence on semen quality parameters before freezing and after thawing (Table 1). Washing of spermatozoa provided significantly ($P<0.05$) higher effect on progressive motility and live spermatozoa before freezing. After thawing, washed spermatozoa were found to have significant ($P<0.05$) effects on the total motility, forward motility, acrosome integrity and the percentage of live spermatozoa. However, the percentages of normal spermatozoa were not affected by washing spermatozoa. No significant differences ($P>0.05$) were observed between the two different extenders in all the semen quality parameters, but GE conferred a better effect in Boer goat semen cryopreservation.

Meanwhile, different washing solutions (PBS, NS, and TCG) had significant ($P<0.05$) effects on the semen quality parameters (Table 2). In particular, TCG washing solution conferred the highest sperm quality values in progressive motility and live spermatozoa before freezing. Acrosome integrity and normal spermatozoa, on the contrary, were not significantly ($P>0.05$) affected by different washing solutions before freezing, but higher values were found in TCG. After thawing, washing solution TCG was found to have the highest significant effect ($P<0.05$) on

TABLE 1
The effects of seminal plasma removal using two extenders on the semen characteristics of Boer goat

Before freezing	Progressive motility %	Intact acrosome %	Live spermatozoa %	Normal spermatozoa %
SP removed (FE)	74.58 ± 1.86	81.17 ± 1.25	76.58 ± 1.52	95.50 ± 0.53
SP removed (GE)	77.50 ± 1.69	80.50 ± 1.81	75.67 ± 2.06	95.83 ± 0.52
SP retained (FE)	69.58 ± 2.42	77.83 ± 1.66	71.83 ± 1.80	96.33 ± 0.58
Sp retained (GE)	70.83 ± 2.03	78.67 ± 1.47	72.08 ± 1.90	96.92 ± 0.69

After thawing	Total motility %	Forward motility %	Intact acrosome %	Live spermatozoa %	Normal spermatozoa %
SP removed (FE)	61.67 ± 2.41 ab	52.08 ± 2.08 ac	66.67 ± 1.92 ab	61.08 ± 2.20 ab	88.42 ± 1.22
SP removed (GE)	64.17 ± 2.59 b	55.83 ± 2.37 a	70.25 ± 1.95 b	64.08 ± 2.12 b	89.25 ± 1.57
SP retained (FE)	55.83 ± 2.45 a	43.33 ± 2.33 b	63.92 ± 1.65 a	57.08 ± 2.05 a	90.50 ± 1.35
Sp retained (GE)	58.33 ± 2.49 a	47.08 ± 2.08 bc	64.50 ± 2.31 a	58.33 ± 2.19 a	91.08 ± 1.21

Means within column with different alphabetical superscripts (a,b,c) are significantly different at least as P<0.05 (by LSD). Mean ± SEM of the semen of three ejaculates obtained from four individuals are shown. SP=seminal plasma, FE=fructose based extender, GE=glucose based extender

TABLE 2
The effects of three different washing solutions on Boer goat semen characteristics

Before freezing	Progressive motility %	Intact acrosome %	Live spermatozoa %	Normal spermatozoa %
PBS	79.50 ± 1.17^{a}	85.30 ± 1.32	78.70 ± 1.45^{a}	93.40 ± 0.81
NS	76.50 ± 1.50^{b}	84.20 ± 1.16	78.90 ± 1.27^{b}	91.40 ± 0.60
TCG	84.50 ± 0.89^{c}	87.50 ± 1.43	83.20 ± 1.45^{c}	95.60 ± 0.58

After thawing	Total motility %	Forward motility %	Intact acrosome %	Live spermatozoa %	Normal spermatozoa %
PBS	54.50 ± 2.29^{a}	47.50 ± 1.11^{a}	67.70 ± 0.78^{a}	61.40 ± 0.95^{a}	88.70 ± 1.51
NS	51.50 ± 2.89^{b}	44.50 ± 1.74^{b}	66.10 ± 1.19^{a}	59.60 ± 1.31^{a}	87.90 ± 1.50
TCG	61.50 ± 2.24^{c}	54.50 ± 0.89^{c}	73.90 ± 0.66^{b}	68.50 ± 0.86^{b}	89.90 ± 0.89

Mean ± SEM within each column, means with different alphabetical superscripts (a,b,c) are significantly different at least as P<0.05 (ANOVA – Post hoc test). PBS = Phosphate Buffer Solution, NS = Normal Saline, TCG = Tris citric acid glucose extender

all the semen quality parameters, except for the normal spermatozoa.

Centrifugation regimes had a significant influence on the semen quality of Boer goat, before freezing and after thawing (Tables 3). Before freezing, centrifugation regime C1 (3000 × g for 3 min) showed significantly (P<0.05) higher effect in progressive motility than the others. However, there were no significant differences in intact acrosome, live spermatozoa and normal spermatozoa between C1 (1600 × g for 10 min) and C2 (800 × g for 15 min) before freezing. After thawing, the semen quality parameters (total motility, forward motility, acrosome integrity, and live spermatozoa) were significantly (P<0.05) higher for frozen-thawed spermatozoa centrifuged with C1 than for the others. The differences between the spermatozoa centrifuged with C2 and C3 were not significant for the semen quality parameters forward motility, intact acrosome, live spermatozoa and normal spermatozoa. Nonetheless, C2 gave a higher significant (P<0.05) effect in the total motility and better sperm quality values than C3 in the Boer goat semen cryopreservation.

TABLE 3
The effects of different centrifugation regimes on the semen characteristics of Boer goat

Before freezing	Progressive motility %	Intact acrosome %	Live spermatozoa %	Normal spermatozoa %
C1	73.75 ± 1.83[a]	79.50 ± 1.46	73.88 ± 2.40[a]	91.25 ± 1.64
C2	66.88 ± 2.30[b]	76.63 ± 1.73	70.75 ± 2.19[a]	92.63 ± 1.31
C3	62.50 ± 3.13[b]	75.13 ± 1.98	68.13 ± 1.96[b]	94.13 ± 0.93

After thawing	Total motility %	Forward motility %	Intact acrosome %	Live spermatozoa %	Normal spermatozoa %
C1	60.62 ± 2.74[a]	50.63 ± 2.74[a]	68.38 ± 2.73[a]	63.38 ± 2.69[a]	86.75 ± 2.05
C2	53.12 ± 2.30[b]	44.37 ± 2.74[b]	63.88 ± 2.64[b]	56.50 ± 2.69[b]	87.75 ± 1.89
C3	48.75 ± 1.83[c]	43.75 ± 2.27[b]	61.25 ± 0.66[b]	55.25 ± 2.43[b]	89.38 ± 1.75

Mean ± SEM within each column, means with different alphabetical superscripts (a,b,c) are significantly different at least as $P<0.05$ (ANOVA – Post hoc test). C1 = 3000 x g for 3 min, C2 = 1600 × g for 10 min, C3 = 800 × g for 15 min

DISCUSSION

The removal of seminal plasma increased Boer semen quality in cryopreservation process. This is in agreement with the findings of Ritar & Salamon (1982), Machado & Simplicio (1992), Love *et al*. (2005) and Kozdrowski *et al*. (2007) who reported a beneficial effect of removing seminal plasma on the freezeability of semen. This, however, contradicts with the findings of Tuli & Holtz (1994), Gil *et al*. (2000), Azeredo *et al*. (2001) and Peterson *et al*. (2007) who reported that the removal of seminal plasma decreased motility in frozen-thawed spermatozoa. Furthermore, Angora buck sperm frozen, with or without centrifugation/ washing in the Bioxcell extender, demonstrated higher percentages of subjective motility (58.1 ± 3.0% and 53.5 ± 3.8%, respectively) compared to that of the groups Tris extender (40.9 ± 1.8% with centrifugation and 45.0 ± 3.1% without centrifugation) (Sariozkan *et al*., 2010). Some of these contraindications may be attributed to several factors, such as season, different processing procedures (namely, washing solution, centrifugation regimes, cooling and freezing rate), and the concentration of seminal plasma remaining after centrifugation. Moreover, species, breeds and individual variation are also critical factors because the compositions of seminal plasma and sperm membrane vary greatly between species and individuals. Among other, seasonal variation is one of the most important considerations (Leboeuf *et al*., 2000).

Seminal plasma cholesterol concentration, which was most probably related to the use of extracellular lipids for the protection of sperm membrane integrity vary between seasons (Beer-Ljubic *et al*., 2009). Furthermore, seasonal differences influence the type of proteins found in seminal plasma. The absence of critical proteins, such as 20-kDa (Perez-Pe *et al*., 2001), 25-kDa (Lessard *et al*., 2000), 26-kDa (Gerena *et al*., 1998) caused lower recovering effect on sperm viability during non-breeding season. These proteins are found only during the breeding season. Major proteins of bovine seminal plasma bind to the low-density lipoprotein fraction of hen's egg yolk leading to adhesion of these proteins to sperm membrane and prevent cryodamage (Manjunath *et al*., 2002). However, there was a negative correlation between one of the protein bands (16 kDa) and semen freezability due to the impaired linking of egg yolk lipoproteins to sperm membrane (Brandon *et al*., 1999; Zahn *et al*., 2006). Meanwhile, vesicular gland solutions, which only occur in the breeding season, partially inhibit the negative effect of BUSgp-60 bulbourethral gland secretion (Nunes *et al*., 1982). EYCE hydrolyses egg yolk phosphatidylcholine (PC) and produces lysophosphatidylcholine (LPC) which has toxic effects by acting on biomembranes of spermatozoa as a detergent. The formation and

strength of detergent properties are temperature dependent (Peterson *et al*., 2007). Therefore, the amount of compositions formed in the seminal plasma and the strength of detrimental effect to the spermatozoa during cryopreservation process is dependent on the type of weather or type of season.

Meanwhile, washing solution TCG increased semen quality during cryopreservation process. It seems that the addition of glucose into the washing solution causes better ability to support energy utilisation for goat sperm cryosurvival. This result is supported by Waite *et al*. (2008) who found that the centrifugation media INRA 96 containing proteins gave more superior effect in equine sperm motility than HELL centrifugation media. Cochran *et al*. (1984) have reported that the effect of centrifugation on equine spermatozoa motility is influenced by the type of extender used prior to or following centrifugation. Therefore, this experiment further confirms that washing solution could influence sperm quality parameters after cryopreservation.

Centrifugation at 3000 × g for 3 min markedly improved the quality of Boer semen cryopreservation. Hence, the use of high g-force and short-term centrifugation is clearly recommended in the centrifugation step of Boer goat sperm. Carvajal *et al*. (2004) confirmed the current results by reporting that the use of short-term centrifugation with a relative high g-force (2400 x g for 3 min) showed significantly higher on boar sperm cryosurvival and oocyte penetration ability. Moreover, Shekarriz *et al*. (1995) have also reported that the time of centrifugation is more critical than g-force for inducing human sperm damage in the preparation of sperm for assisted reproductive techniques. Although centrifugation causes potential damage to the spermatozoa, the use of high g-force centrifugation could enhance to remove the ejaculate contaminants, such as abnormal and dead spermatozoa. Dead spermatozoa produced reactive oxygen species (ROS) which have harmful effect on spermatozoa during cryopreservation (Upreti *et al*., 1999). Therefore, the use of high g-force centrifugation could lead to increase in the percentage of high quality spermatozoa. Based on the criteria adopted in these studies, washing solution and centrifugation regimes could be stated to have significantly influenced the buck semen cryosurvival. Washing solution TCG and high g-force centrifugation with short duration caused better improvement in Boer goat semen quality after cryopreservation.

In conclusion, this study has indicated that the practical and beneficial effects can be obtained by removing seminal plasma through centrifugation (3000 × g for 3 min) with TCG washing solution in Boer goat semen cryopreservation protocol. However, these results are based only on motility characteristics, and therefore, further fertility trials are required as the ultimate test of improved cryopreservation.

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