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Sperm concentration influences the post-thaw livability of frozen goat semen

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Abstract

In this study, goat semen was cryopreserved in a reduced concentration of 75×10^6, 50×10^6 and 25×10^6 sperm/ml and evaluated based on post-thaw motility and livability. The ejaculated semen samples were collected using an artificial vagina, extended with an egg yolk Tris extender, cryopreserved in straws using glycerol (7%) and egg yolk (5%) as permeating and non-permeating cryoprotectants in LN_2. Post-thaw semen evaluation showed motility scores of 36.6±2.13%, 35.4±2.04%, 27.0±3.0% and 19.2±2.78% for the control, T-A (75×10^6), T-B (50×106) and T-C (25×10^6), respectively. The mean post-thaw percentage livability obtained in the control (60.6±4.39) was higher than T-A (42.86±1.83), T-B (39.36±2.86) and T-C (19.2±5.27). The percentage abnormal sperm in all treatment groups were less than 10%, indicating that the method used for freezing of goat semen in reduced concentration could be useful in artificial insemination and *in vitro* fertilization studies. The reduced sperm motility and livability after thawing merits further studies on the factors influencing the survivability of sperm during cryopreservation in creating a standardized and better freezing protocol for goat semen.

Keywords: Cryopreservation; livability; motility; semen

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Introduction

Commercial goat raising in the Philippines using introduced breeds such as Anglo Nubian, Boer, Saanen, Alpine and Toggenberg is gaining popularity and wide acceptance within the local small ruminant industry. As a result, there has been an emergence or renewed interest in artificial insemination using goat semen either in liquid/extended form or with the use of frozen goat semen. In addition to use processed liquid or extended goat semen for artificial insemination, frozen semen from genetically superior sperm donor can be stored in cryo-conservation *in vitro* for Animal Genetic Resources Cryobanking. One recent development is the optimized goat semen cryopreservation procedure using Tris-citric acid, fructose and raffinose-based semen extender with 5% egg yolk and 7% glycerol (Beltran et al., 2013). In this study, a sperm concentration of frozen semen at 100×10^6 resulted in live birth after artificial insemination of recipient does.

The volume of ejaculated goat semen is fairly small when compared to other livestock species such as

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cattle, buffalo and pigs. Semen volume ranges from 0.5-1.5 ml per ejaculate with a sperm motility of about 70-85% and a sperm concentration 2.5-5.0 × 10^9 sperm/ml. Therefore, the relatively minute volume of sperm ejaculate when processed into frozen semen can produce very few numbers of semen straws if the sperm concentration is set at 100×10^6/ml. It is, therefore, the interest of this study to cryopreserve goat semen in reduced sperm concentration per ml without compromising the number of viable sperm after thawing and possibly increase the production of frozen semen straws.

Materials and Methods

Goat semen collection

Anglo Nubian bucks (n=5, 2-3 yrs old) from the Small Ruminant Centre at Central Luzon State University, Science City of Munoz, Nueva Ecija Philippines used for breeding purposes and trained for semen collection were the sperm donor. Using an artificial vagina, the semen ejaculates were collected in a conical test tube early in the morning and brought to the laboratory for processing.

Evaluation of the semen ejaculate

Upon arrival in the laboratory, the semen was evaluated for its physical characteristics. The volume of the semen was measured against the graduated lines in the conical test tube and recorded accordingly. The pH was determined using Bromthymol blue (BTB) paper or BTB pH strips. The semen colour was assessed visually and scored as either creamy, light creamy and watery. The semen consistency was assessed as thick or thin in consistency after running an aliquot of the semen along the inner side wall of the test tube. Semen ejaculates that were creamy in colour and thick in consistency were considered for processing.

Microscopic evaluation

After the physical characteristics of the ejaculates were observed, microscopic evaluation was performed by taking the sperm concentration, gross and individual motility characteristics. Gross motility was performed by putting an aliquot (10 µl) of the semen in a pre-warmed glass slide and observed for eddies, circular waves and swirls in an inverted Nikon Eclipse Tx10i microscope. Individual motility on one hand was performed next by placing an aliquot of semen and covered with a pre-warmed cover slip. Individual motility was scored based on the motility scoring (Table 1).

Microscopic evaluation was undertaken under medium (40×) and high power (100×) objectives using the inverted microscope (Nikon Eclipse Tx10i). Visual motility was recorded using the imaging software (NIS elements) and the progressive sperm movement of the sample was recorded and assessed by an experienced technical personnel. Semen samples having a motility score of ≥60% were considered for semen processing, while those not fulfilling the criteria were discarded.

Sperm concentration

The sperm concentration was determined after dilution of the sperm sample in an RBC pipette at 1:200. The sperm were counted in the central large area of the Neumbauer haemocytometer which consists of 25 squares and each square consists of 16 smaller squares. In case, where the sperm from 5 of the 25 squares were counted, the following formula was used:

Sperm concentration per ml = n × dilution factor × 50,000

$= n \times 200 \times 50{,}000$

$= n \times 10{,}000{,}000$

$= n \times 10^7$

Preparation of the Tris-Yolk Glycerol (TYG solution)

Table 2 showed the composition of Tris buffer solution prepared by weighing the components accordingly one after the other. Table 3 showed the composition of the Tris buffer solution with egg yolk (5%; v/v) and glycerol (7%; v/v) addition.

Initial dilution of the semen ejaculate

Initial dilution at 1:1 ratio of the semen ejaculate with TYG solution was performed at 37°C. Afterwards, the initially diluted semen ejaculate and the conical tube with TYG solution were placed in a beaker with water at 37°C. The beaker was gradually allowed to cool till 10-15°C. While the gradual cooling process was in progress, the volume of TYG solution to be added was computed to obtain a sperm concentration of 100×10^6/ml.

Experimental treatments

The sperm concentrations/ml used in the study were:

a. Control=100×10^6
b. Treatment A=75×10^6
c. Treatment B=50×10^6
d. Treatment C=25×10^6

Computing for the volume of the TYG solution (Control)

The volume of the TYG solution added into the ejaculate was computed using the following formula:

$$V = \frac{(A)(B)(C)}{D} - C$$

Where:

V = volume of the TYG solution to be added

A = sperm concentration of the ejaculate
B = Percentage motility score
C = Volume of the ejaculate
D = Desired final concentration per ml

Given the following numerical values the volume of TYG solution was computed as follows:
A = 250×10^7
B = 75%
C = 1.2 ml
D = 10×10^7 which is equivalent to 100×10^6

By substitution:

$$V = \frac{(250x10^7)(0.75)(1.5ml)}{10\times10^7} - 1.5 \text{ ml}$$

V = 28.125 – 1.5 ml
V = 26.625 ml

Therefore, the volume of the TYG solution added to the semen ejaculate was 26.625 ml to obtain a desired sperm concentration of 10×10^7/ml which is equivalent to 100×10^6 (Control mixture). During the gradual cooling process the control mixture (100×10^6) was prepared by gradual addition of TYG solution until the temperature reaches 10-15°C.

At the same cooling temperature of 10-15°C, the various sperm treatment concentrations were prepared from the control mixture by dilution with a TYG solution in separate pre-labelled test tubes A, B and C (Table 4). After the experimental treatments were prepared, they were allowed to cool further at 5°C and kept at this temperature for 2 h.

Vapour freezing of the straws and storage in cryotank

The semen straws were filled with the diluted semen of varying sperm concentrations as required, placed 4.0 cm above the level of the LN_2 in a styropore box for 7 min, before plunging into the LN_2 and stored in cryotank until further use.

Post-thaw motility evaluation

The straws were thawed in a water bath at 37°C for 15 sec. Evaluation of post-thaw motility was performed by microscopic visual assessment of the progressive movement under low and high power magnification (40–100×). After a visual evaluation by an experienced semen freezing technician, a ≥30% post thaw motility percentage was considered to have passed and kept for cryostorage until further use. The percentage livability (live and dead) and percentage of normal and abnormal cells was likewise determined by microscopy after staining with eosin-nigrosin of the smeared semen samples. A mixture of 1% eosin and 5% nigrosin in 3% sodium citrate as the standard solution was used to stain the semen smear. At least 500 spermatozoa were counted in different microscopic fields. The number of live and dead sperm were counted and the percentage of live and dead sperm were calculated over the total number of sperm observed multiplied by 100 (Mamuad et al., 2004).

Statistical analysis

The experimental treatments were replicated five times in a Complete Randomized Design. Analysis of variance was performed to evaluate the effect of different sperm concentrations on sperm characteristics such as post-thaw motility percentage, live sperm and abnormal sperm. The differences among treatment means using Tukey's HSD was set at 5% ($P<0.05$) level of significance.

Results and Discussion

The fresh semen characteristics used in this study were semen volume (ranges from 0.5–2.0 ml), milky or creamy white in colour without any sign of coagulation or colour abnormalities that may indicate contamination or injury, thick consistency, sperm motility score of 70-80%, mean sperm concentration of $104.4\pm18.23\times10^7$ sperm/ml and a mean pH of 6.56 (Table 5). The differences in the sperm concentration were not affected by the volume of the ejaculates. The smallest volume of ejaculate at 0.5 ml had a sperm concentration of 103.4×10^7 while the highest volume of ejaculate at 2 ml had a sperm count of 95.2×10^7. The highest sperm concentration obtained was 171.2×10^7whereas the lowest sperm concentration was 60.4×10^7. Overall, the semen used have a sperm concentration of 6×10^6 - 1.7×10^9 sperm per/ml. and therefore were considered as a potential source for semen processing.

Thereafter, the semen samples were cryopreserved and stored in the LN_2 tank for at least a week before thawing. The post-thaw motility rate obtained were 36.6%, 35.4%, 27.0% and 19.2% for the Control, T-A, T-B and T-C, respectively. The percentage livability observed in the control was 60.6%, whereas in T-A, T-B and T-C were 42.8%, 39.3% and 18.7%, respectively. Overall, the post-thaw motility rate of the sperm decreases as the sperm concentration decreases reflecting the observed decreasing percentage livability as the sperm concentration decreases.

In this study, the mean semen volume obtained was 1.04 ml, which was higher compared to the semen volume reported in using similar goat breed (Anglo-Nubian; 0.6±0.08 ml, Bondoc et al., 2007) and in South African indigenous goat (0.7±0.08 ml; Ramukhithi et al., 2011). In Boer goat, a semen volume of more than 1.5 ml per ejaculate has been reported (Tuli and Holtz, 1995; Sundararaman et al., 2007). In some cases, however, even using similar goat breed had some

Table 1: Scoring system for the motility of sperm cells*

Motility (%)	Grade	Characteristics
91 – 100	Excellent motility	90% or more of the spermatozoa are very rigorous in motion. Swirls caused by the movement of the sperm are extremely rapid and constantly going forward progressively.
76 – 90	Very good motility	Approximately 75-90% of the spermatozoa is in vigorous rapid motion. Waves and eddies form rapidly but lesser as in excellent motility.
60 – 75	Good motility	About 60-75% of the spermatozoa is in motion. Motion is vigorous but waves and eddies formed moved slowly across the field of vision.
40 – 59	Fair motility	From 40-55% of the sperm is in motion. The movements are largely vigorous or eddies are formed.
< 40	Poor motility	Less than 40% of the sperm is in motion. The motion is not progressive but mostly
0	Zero motility	weak and oscillary. No recognizable movements of the sperm.

*(Mamuad et al., 2004).

Table 2: Composition of Tris-buffer solution

Chemicals/Reagents	mM	g/100 ml
Tris hydroxymethylamino methane	320.0	3.875
Sodium citrate	10.0	2.125
Fructose	3.0	0.625
Raffinose	8.0	0.535
Distilled water	-	100 ml

Table 3: Composition of Tris-buffer solution with egg yolk and glycerol

Chemicals/ Reagents	100 ml preparation	Final concentration in Tris-buffer solution
Tri-buffer solution	88.0 ml	88.0% (v/v)
Glycerol	7.0 ml	7.0% (v/v)
Egg yolk	5.0 ml	5.0% (v/v)
Antibiotic (10mg/ml stock)	100 µl	

Table 4: Dilution of the control mixture with TYG solution to obtain the experimental treatments

Sperm concentration (sperm/ml)	Control treatment mixture volume	Dilute with TYG solution	Volume
Control 100×10^6	10.0 ml	-	10ml
Treatment A (T-A) 75×10^6	7.5 ml	2.5 ml	10 ml
Treatment (T-B) 50×10^6	5.0 ml	5.0 ml	10 ml
Treatment (T-C) 25×10^6	2.5 ml	7.5 ml	10 ml

Table 5: Fresh semen characteristics*

Goat No.	Mean sperm volume (ml)	Mean sperm motility (%)	Mean sperm concentration ($n \times 10^7$)	pH
1	1.0	70	91.6	6.6
2	2.0	80	95.2	6.6
3	0.5	70	103.4	6.4
4	0.9	80	171.2	6.6
5	0.8	80	60.4	6.6
Mean	1.04	76	104.4	6.56

*Mean values were taken from 5 replicates of each buck used for semen collection.

differences in semen volume during ejaculation as influenced by age and feeding system (Dombo, 2002; Almeida et al., 2007; Daramola et al., 2007). Semen volume was also affected by both the physiological status and genetic effects of the bucks (Webb et al., 2004; David et al., 2007). Also, breed differences contribute to variability in semen volume of buck (Choe et al., 2006) as in the case of Korean native goats with semen volume of 2.1±1.0 ml.

The sperm cell motility of 70% and above obtained in this study was acceptable as it was within the range considered of high quality (Yamashiro et al., 2006) compared to the sperm cell motility of 62.5% as earlier reported using Saanen goats (Nur et al., 2005). Also, the sperm cell concentration of 104.4×10^7/ml observed in Anglo-Nubian goat used was higher than the sperm cell concentrations (116.7×10^6–663.6×10^6/ml) reported in South African indigenous goat and Gorno Altai (Dombo, 2002; Ramukhithi et al., 2011) but lower compared to sperm concentration of 3×10^9/ml (Tuli and Holtz, 1995; Sundararaman et al., 2007) to 4.7×10^9/ml (Hidalgo et al., 2006) in Florida goats. Some factors considered contributory to the differences observed in this study and other related studies are, the breed used, nutrition, system used for collection and evaluation, age of buck and time of the year (breeding vs non-breeding season) (Gacitua and Arav, 2005; Jimenez et al., 2005; Bester, 2006; Daramola et al., 2006; Zarazaga et al., 2009).

The normal pH of mammalian semen ranges from 7.2–7.8 (Prins, 1999), and was reported to be most favourable for sperm cell motility (Molinia et al., 1994; Purdy, 2006). In this study, an acidic pH of 6.56 was obtained following collection through the use of an artificial vagina early in the morning. Similarly, a pH of 6.4 was observed in South African indigenous goat when using an electro-ejaculator (Ramukhithi et al., 2011). The use of electro-ejaculator stimulates the buck to release acidic urine, which contaminates the semen, thus reducing the semen pH (Jimenez et al., 2008; Moreno et al., 2009). In this study, the reasons for having an acidic semen remained unclear.

Moreover, sperm sample with concentrations ranging from 8–50×10^7 sperm/ml could be frozen successfully with reasonable fertility (Purdy, 2006). In this study, the mean sperm concentration obtained was 104.4±18.23×10^7/ml, thus regarded as useful for cryopreservation studies, although our main interest was on the cryopreservation of buck semen with reduced sperm concentration. The results showed a decrease in post-thaw motility and livability as the sperm concentration decreased. These findings are in agreement with Kozdrowski et al.. (2007) reporting more or less than 30% sperm cell motility, but lower than the sperm cell motility of 50% recommended in goat (Biswas et al., 2002). Some factors that may have influenced the post-thaw motility and livability of buck semen used include, a) *pH* – acidic semen usually results from sperm cell apoptosis that occur during cryopreservation due to toxic substances secreted during death of other sperm cells (Bester, 2006; Uysal and Bucak, 2009), b) *the presence of egg yolk in the cryopreservation diluents* – if neat semen (as in the case of this study) is added to egg yolk media, the egg yolk coagulate and the sperm may die due to an enzyme of bulbourethral origin named egg yolk-coagulating enzyme (EYCE). This acts as a catalyst that hydrolyzes egg yolk lecithin into fatty acids and lysolecithin causing the sperm membranes to be more fusogenic thereby inducing the acrosome reaction (Upreti et al., 1999) and chromatin decondensation (Sawyer and Brown, 1995), which is toxic to the sperm, c) *sperm dilution/concentration*– to achieve a high fertility rate using the lowest number of sperm for insemination, it is important that the semen sample be diluted properly so that sufficient numbers of sperm and diluents are available to accommodate the cells in an insemination straw. Dilution rates of 1:1–1:23 (v/v; semen to diluent) have been used successfully (Ritar et al., 1990 a&b). It is suggested that a better way of diluting semen should be based on sperm concentration for comparison purposes, d) *straw size and freezing height above the LN_2*- when using 0.5 ml straws, it should be frozen 4 cm above LN_2 for 5 min before plunging into LN_2, while 0.25 ml straws should be placed 16 cm above the LN_2 for 2 min, lowered to 4 cm for 3 min before plunging into the LN_2 for storage (Chemineau et al., 1991) though 4-5 cm above LN_2 for 4-5 min also had acceptable results (Gravance et al., 1997; Leboeuf et al., 2000). In this study, the straws were held at 4 cm in a styropore for 7 min, before plunging into the LN_2, e) *diluents pH and concentration of buffering additives* – oxygen uptake of goat sperm is maximal between pH 7.2–7.5 and sperm cell motility is optimal between 7.0–7.2, indicating that goat sperm survival in vitro should be 7.2 (Fukuhara and Nishikawa, 1973). That, increasing the medium pH from 7–8 stimulates about 50% of cauda epididymal sperm to become motile, indicating a sensitivity of the sperm to pH fluctuation (Jaiswal and Majumder, 1998). Also, increasing intracellular pH activates downstream or parallel pathways that activate protein kinase A, an indicator of capacitation in mammalian sperm cells (Visconti and Kopf, 1998). In this study, the diluents pH was mistakenly not taken, a critical information that might have contributed on the observed reduced sperm motility post-thawing. On the other hand, post-thaw motility was reported greater with the Tris buffer at concentrations ranging from 350–450 mM combined with 21, 42 or 62 mM glucose (33%) or fructose (33%). The addition of lactose (29%) or raffinose (17%) resulted in lower percentages of motile cells (Salamon and Ritar, 1982). Similarly, in this study, a combination of fructose (3 mM) and raffinose 8 (mM) resulted in a reduced sperm motility post thawing. It was suggested that for successful cryopreservation of buck semen, the sugar concentrations should range from 0–62.4 mM when combined with Tris, that monosaccharides offer greater cryoprotective effects than disaccharides (Molinia et al., 1994), f) choice of sugar(s) in the cryopreservation diluents-goat sperm readily utilizes fructose, glucose, lactose and other sugars for respiration, osmotic balance and cryprotection. Of these, fructose has the greatest molar concentration in neat goat semen and is the primary substrate for glycolysis in goat seminal plasma making it the most logical sugar of choice to include in the medium (Pellicer-Rubio et al., 1997; Aboagla and Terada, 2003). Likewise, glucose is an excellent substrate in goat sperm metabolism and essential for providing energy for the sperm cells to function in a normal physiological manner (Fukuhara and Nishikawa, 1973; Corteel, 1974) and, g) *choice of cryoprotectants*- the use of penetrating cryoprotectant (glycerol at 6%) alone resulted in high percentage motility (35%) post thawing, but when combined with other cryoprotectants (eg., dimethyl sulfoxide at 5.9%) resulted in 45% progressive motility (Kundu et al., 2001). In this study, the inclusion of non-penetrating cryprotectants in the cryopreservation medium (e.g., egg yolk at 5%) together with glycerol at 7% afforded a progressive motility and liveability of 35.4±2.04% and 42.86±1.83%, respectively when using 75×10^6 sperm/ml concentration.

Conclusion

The volume of information provided in this study could be used for the improvement of cryopreservation procedures in goat semen. That, it is possible to cryopreserve goat semen in a reduced concentration of 50-75 × 10^6 sperm/ml and used for artificial insemination, *in vitro* fertilization studies and for eventual embryo production, given the fact that it can result in almost 40% sperm livability post-thawing.

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