OPTIMIZED EXTENDERS FOR CRYOPRESERVATION OF BUCK SEMEN FOR ARTIFICIAL INSEMINATION

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ABSTRACT

The study was conducted to evaluate the efficiency of different non-permeating cryoprotectants for freezing buck semen. Semen samples were collected from three healthy bucks and were evaluated for volume, color, consistency, pH, motility and concentration. Samples with 60% and above motility were allocated to the following treatments of Tris-Citric Acid-Fructose –Raffinose-Glycerol-based extenders: T1 – 5% egg yolk; T2 – 10% egg yolk; T3 – 15% egg yolk; T4 – 20% egg yolk; T5 – 2.5% goat serum; T6 – 5% goat serum; T7 – 10% goat serum; T8 – 10mg Bovine Serum Albumin (BSA); T9 – 30 mg BSA; and T10 – 50 mg BSA. Post-thawing evaluation revealed that extenders with 5% egg yolk (T1), 2.5% goat serum (T5), and 30 mg BSA (T9) yielded high rates of sperm motility and acrosome integrity. In terms of the proportion of live sperm at post thawing, 5% egg yolk was found significantly higher than 2.5% goat serum and 30 mg BSA. Moreover, cost analysis revealed that supplementation with 5% egg yolk was cheaper than with goat serum and BSA. Artificial insemination (AI) using frozen-thawed semen with 5% egg yolk resulted in 70.50% birthrate. This work demonstrates the successful production of kids from frozen buck semen enhancing the potential of AI technology for goat production.

Keywords: artificial insemination, buck semen, cryopreservation, ultrasonography

INTRODUCTION

Artificial insemination (AI) is a vital technique used in animal production to improve livestock productivity. However, one critical factor that affects the success of AI is the efficient cryopreservation of spermatozoa from quality animals. Semen quality and its relationship to fertility are said to be a major concern in AI; hence, accurate measurement of semen quality and fertilizing potential is of great importance. To meet the requirements for AI, many extenders have been used like egg yolk-phosphate, skim milk and coconut juice. Cryopreservation diluents supply the sperm cells with sources of energy, protect the cells from temperature-related

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damage and maintain a suitable environment for the spermatozoa to survive temporarily. Logically, each of the different components present in the media must be investigated separately to maximize the post-thaw sperm viability and fertility. In general, goat sperm cryopreservation medium includes a non-permeating cryoprotectant (milk or egg yolk), a permeating cryoprotectant (glycerol, ethylene glycol or dimethyl sulfoxide), a buffer (Tris), one or more sugars (glucose, lactose, fructose, raffinose or trehalose), salt (sodium citrate, citric acid) and antibiotics (penicillin or streptomycin) (Evans and Maxwell, 1987).

Limited studies done on cryopreservation of buck semen showed its differences from those of other domestic species. As such, there is a need to determine an efficient extender for the cryopreservation of buck semen for AI. Hence, this study was developed to address the kind of non-permeating components of the extender in order to come up with an efficient extender for the cryopreservation of buck semen in terms of semen quality at post-thawing and fertilizing ability following artificial insemination.

MATERIALS AND METHODS

Study 1. Comparison of egg yolk, goat serum and bovine serum albumin as extender supplements for buck semen

Semen was collected from three 1.5 to 3 years old apparently healthy bucks (Anglo-Nubian, Boer and Saanen) two times a week. Semen collection was done early in the morning using an artificial vagina filled with warm water at 42-44ºC. Collected semen samples were initially evaluated for volume, color, consistency, pH, percent motility and concentration. Semen samples with 60% and above motility were selected for processing. Sperm concentration was determined using a red blood cell (RBC) dilution pipette and a hemocytometer counting chamber (Neubauer, USA). The concentration of spermatozoa per milliliter of semen was calculated by multiplying the total number of spermatozoa by 10×10^7.

The buffer solution was composed of 320 mM (3.875 g) Tris (hydroxymethyl) amino methane (Sigma Chemical Co., St. Louis, MO, USA), 10 mM (2.125 g) Citric Acid Monohydrate (Sigma Chemical Co., St. Louis, MO, USA), 3 mM (0.625 g) Fructose (Sigma Chemical Co., St. Louis, MO, USA) and 8 mM (0.535 g) Raffinose (Sigma Chemical Co., St. Louis, MO, USA), dissolved in 100 ml triple distilled water. In the preparation of the extenders, the volume of the Tris buffer solution in each treatment was calculated and placed in plastic tubes. The different volumes of egg yolk, goat serum and BSA required for each treatment were added in the tube. Glycerol was added last.

Semen samples were divided and distributed into the following treatments of extenders: T1 – 5% egg yolk; T2 – 10% egg yolk; T3 – 15% egg yolk; T4 – 20% egg yolk; T5 – 2.5% goat serum; T6 – 5% goat serum; T7 – 10% goat serum; T8 – 10 mg BSA; T9 – 30 mg BSA; and T10 – 50 mg BSA.

Prior to initial dilution, the semen and the extenders were allowed to warm separately in a water bath at 28ºC for equilibration. After dispensing the semen in plastic tubes of different treatments, the extenders were added to the semen at 2:1

ratio (two volumes of extender for every given volume of semen). After the initial dilution, the tubes containing the extended semen were transferred to a water bath pre-cooled at 15-20ºC. The extended semen samples were finally transferred to a refrigerator maintained at 5ºC for further cooling.

The remaining volumes of the extenders were added after the initially diluted semen reached a temperature of 5-7ºC inside the refrigerator. The total volume of extender to be added to the semen was calculated using the formula as:

$$\text{Total volume of extender} = \frac{\text{Semen volume} \times \%\text{Motility} \times \text{Sperm concentration}}{\text{Desired sperm concentration (100,000,000/ml)}}$$

Cooled semen from the refrigerator was poured in the bubbler dish. Semen was loaded by filling two to three straws at a time by sucking the semen from a bubbler dish. Semen straws were sealed with the use of colored polyvinyl powder. The sealed straws were submerged in a styrofoam box with 5ºC pre-cooled water to allow complete sealing, then dried, and arranged horizontally in a metal tray in preparation for freezing.

The freezing of buck semen was done using the simple and commercially available styrofoam box**.** The metal tray containing the semen straws was transferred at about 4 cm above the level of the liquid nitrogen (LN_2) inside a styrofoam box. The tray was maintained in LN_2 vapor for at least 7 min. The semen straws were directly plunged in LN_2, placed in a goblet and then stored in LN_2 tank until post- thawing evaluation or use.

Frozen semen straw was individually removed from the LN_2 tank, exposed to air for about 10 sec and was thawed in a water bath set at 37ºC for 15-20 sec. The post-thaw motility of cryopreserved semen was rated following the criteria of scoring system before freezing. Sperm morphology, was evaluated using Hancock's solution under a phase contrast microscope. Sperm cells with abnormal morphology were counted and expressed as mean percentage of sperm head, neck or tail abnormality.

Evaluation of percent acrosome integrity was done after staining samples with Giemsa stain following the method described by Mamuad *et al*. (2004). Briefly, a drop of 2.9% sodium citrate (0.01 ml) was placed near the center of a slide and semen sample was added and gently mixed with the use of a small platinum loop. Three semen smears per treatment were prepared and dried immediately before flooding with a freshly prepared Giemsa stain. After staining, the slides were rinsed with double distilled water and then dried before examination. Two hundred spermatozoa were examined and the mean percentage of reacted acrosome as a result of the treatment was determined.

Percentage of live and dead spermatozoa was determined by differential staining with Eosin-Negrosin stain. Three smears from each treatment were prepared and two hundred or more spermatozoa were examined from each smear per slide. Dead sperms absorb eosin stain and, thus, appeared pink or purple in color while viable sperm cells do not take the stain, thus, retaining their white color. The percentages of live sperms were calculated over the total number of sperm observed x 100 (Mamuad *et al*., 2004).

Study 2. Utilization of cryopreserved buck semen for artificial insemination

Does in natural estrus at the Small Ruminant Center, Central Luzon State University were artificially inseminated using frozen-thawed buck semen cryopreserved with 5% egg yolk. The does were inseminated twice; once in the afternoon of the first day of estrus and in the morning of the second day of estrus.

Determination of pregnancy was initially done based on the record of non-return to estrus of animals inseminated after the first and second estrous cycles (21 and 42 days after insemination). Confirmation by ultrasonography was performed at day 56-60 of pregnancy using a portable ultrasound machine (Vetko Plus, Noveko, Canada) equipped with 5.0 MHz transducer. Images during trans-rectal ultrasonography were taken. Water bag appeared as black images. The hind legs and bones of fetus appeared solid white. AI efficiency was finally assessed by live births of kids.

Statistical analysis

Data in Study 1 were analyzed using Randomized Complete Block Design. Treatment means were compared using Duncan's Multiple Range Test and adapting the SAS 9.0 Statistical program. In Study 2, descriptive analysis was used to determine the pregnancy rate after insemination.

RESULTS AND DISCUSSION

Study 1. Comparison of egg yolk, goat serum and bovine serum albumin as extender supplements for buck semen

The volume of semen per collection ranged from 0.6 to 1.0 ml from the three bucks with a mean volume of 0.78 ml (Table 1). All the collected semen samples were relatively uniform and appeared creamy opaque. The result is in agreement with the findings of a previous work (Hafez, 1993). All the semen samples have thick consistency indicating a high sperm concentration. The pH of the semen samples ranged from 6.5 to 7.0 with a mean pH of 6.85 (Table 2).

Sperm motility during initial dilution with different extenders was noted to be 90% in most treatments (T1, T2, T4, T6, T7, T8, T9 and T10) and were comparable

Table 1. Volume of semen collected from three bucks used in the study.

Source of Semen	Number of collections										Ave vol (ml)
	1	2	3	4	5	6	7	8	9	10	
Anglo-Nubian	0.8	0.8	1.0	1.0	1.0	1.0	0.6	0.6	0.8	0.6	0.82
Boer	1.0	0.8	0.8	0.6	0.8	0.6	0.6	1.0	1.0	1.0	0.82
Saanen	0.7	0.7	0.8	0.8	0.8	0.8	0.7	0.7	0.6	0.6	0.72
Mean Volume of Semen											0.80

Table 2. Basicity or alkalinity (pH) of initially diluted semen with different extenders.

Source of semen	Number of evaluations										Ave pH
	1	2	3	4	5	6	7	8	9	10	
Anglo-nubian	7.0	6.5	7.0	7.0	7.0	7.0	6.5	6.5	7.0	7.0	6.85
Boer	6.5	7.0	7.0	7.0	7.0	7.0	6.5	6.5	6.5	7.0	6.80
Saanen	7.0	7.0	7.0	7.0	7.0	6.5	6.5	7.0	7.0	7.0	6.90
									Mean pH		6.85

with each other. In contrast, percent motility of semen treated with 15 ml egg yolk (T3) and 0.25 ml goat serum (T5) had comparably lower mean sperm percent motility of 86.66% (Table 3). Analysis of variance disclosed highly significant differences between treatment means. The percent motility data derived in this study were found to be within the range of 76-90%. This indicated a good semen quality based on the standard of sperm motility of Mamuad *et al*. (2004).

Data on sperm concentration showed means that ranged from 130 x 10^7 to 349 x 10^7/ml (Table 3). Statistical analysis revealed highly significant differences

Table 3. Percent motility and sperm concentration of goat semen before cryopreservation using different extenders.

Extender	Motility (%)	Sperm conc x 10^7
	Fresh	Sperm/ejaculate
T1	90.00^a	349^a
T2	90.00^a	244^{ab}
T3	86.66^b	137^b
T4	90.00^a	164^{ab}
T5	86.66^b	164^{ab}
T6	90.00^a	296^{ab}
T7	90.00^a	130^b
T8	90.00^a	153^b
T9	90.00^a	299^{ab}
T10	90.00^a	231^{ab}

Means with the same superscripts are not different from each other (P<0.01).

(P<0.01) in sperm concentration with the different treatments. The highest concentration of sperm per milliliter was observed in samples treated with 5% egg yolk (T1). Sperm concentration was lower in samples treated with 10% egg yolk (T2), 20% egg yolk (T4), 2.5% goat serum (T5), 5% goat serum (T6), 30 mg BSA (T9) and 50 mg BSA (T10). The least sperm concentration was found in semen samples treated with 15% egg yolk (T3), 10% goat serum (T7) and 10 mg BSA (T8). In a report made by Hafez (1993), ram and buck had 0.8-1.2 ml of ejaculate volume or 1.6-3.5 billion sperm per ejaculate.

Sperm cryopreserved in 5% egg yolk (T1) recorded the highest post thaw motility of 59.58% (Table 4). This was followed by semen with 2.5% goat serum and 30 mg BSA added to the extender. Treatment with 10% egg yolk (T2), 15% egg yolk (T3) and 20% egg yolk (T4) had low mean percent motility of semen. Treatments with 5% goat serum (T6), 10% goat serum (T7), 10 mg BSA (T8) and 50 mg BSA (T10) had the lowest mean percent motility. These observations conformed with the findings of Ritar and Salamon (1982) who recommended the use of lower level of egg yolk (1.5%) because a higher concentration can decrease the post-thaw viability of buck semen samples that were not washed of seminal plasma prior to cryopreservation. This was in contrast, however, with the findings of Tuli and Holtz (1992) who used 16.8% egg yolk.

Table 4. Semen quality after cryopreservation using different extenders.

Treatments	% motility	% acrosome integrity	% live sperm
T1	59.58^{a}	99.27	72.60^{a}
T2	49.58^{b}	98.74	62.37^{bc}
T3	52.50^{b}	99.52	61.61^{bc}
T4	49.16^{b}	98.73	57.97^{cd}
T5	57.50^{a}	98.09	65.63^{a}
T6	32.50^{c}	98.56	56.25^{cd}
T7	32.50^{c}	97.81	54.25^{d}
T8	32.91^{c}	98.76	58.20^{cd}
T9	57.50^{a}	99.07	64.27^{b}
T10	30.83^{c}	99.22	56.38^{cd}

Means with the same superscripts are not different from each other (P<0.01).

Goat serum and BSA were not normally incorporated in extenders of semen but results of this study confirmed the findings of Yamashiro (2006) who used BSA to freeze goat spermatozoa to enhance freezability. BSA can reduce contact between the seminal plasma components and spermatozoa, and minimize the various kinds of damage due to the exposure to the seminal plasma. Meanwhile, reports on harmful interactions between seminal plasma and egg yolk had been previously reported. Roy (1957) observed that addition of buck semen to egg yolk

media without removing the seminal fluid caused the egg yolk to coagulate and induced death of sperm. Egg yolk coagulation was claimed to be mediated by an enzyme with bulbourethral origin, and this was named as egg yolk-coagulating enzyme (EYCE) (Nunes *et al*., 1982). The EYCE was identified as phospholipase A and that the higher level of egg yolk has higher levels of EYCE according to Iritani and Nishikawa (1961 and 1963). EYCE acts as a catalyst that hydrolyzes egg yolk lecithin into fatty acids and lysolecithin (Iritani and Nishikawa, 1961). This hydrolysis causes 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 (Amdal *et al*., 1965). Moreover, the inclusion of 20% egg yolk in extender in the earlier study of Ritar and Salamon (1982) recorded significant number of tail abnormality in semen. This abnormality was associated with the coagulative properties of this extender which inhibited sperm from making progressive movements. The results of the studies of Roy (1957) and Ritar and Salamon (1982) in using egg at a level greater than 1.5% indicated induced coagulation of the egg yolk in the extender.

All of the treatments had high acrosome integrity scores ranging from 97.81-99.27% (Table 4). Treatment with 15% egg yolk (T3) had the highest acrosome integrity. This was followed by extender with 5% egg yolk with 99.27% (T1), with 50 mg BSA (T10) at 99.22%, and with 30 mg BSA at 99.07% (T9). However, statistical analysis showed no significant differences among the treatment means.

Monitoring for acrosome integrity is important in the cryopreservation of semen as it plays an important role in fertilization. As the apical ridge of the acrosome of bull or boar sperm deteriorates with aging or injury in the cell, the acrosin enzyme may be lost (Plachot *et al*., 1984).

The results of the present study showed that sperms cryopreserved with 5% egg yolk (T1) had the highest percent of live sperm with 72.60% (Table 4). It was noted further that the higher the concentration of egg yolk incorporated in the extender (T4) the lower is the percentage of live sperm in the samples. Statistical analysis revealed highly significant differences ($P<0.01$) in the percentage of live sperm among the semen samples from the different treatments used (Table 4). The treatment with 5% egg yolk (T1) and 2.5% goat serum (T5) demonstrated the highest percentage of live sperm in cryopreserved goat semen. Comparable but of lower percentages of live sperm were noted in treatments with 30 mg BSA (T9), 10% egg yolk (T2) and 15% egg yolk (T3). Results showed that the percentage of live sperm was congruent with the results of the percent post-thaw motility. Sperm cryopreserved in 5% egg yolk (T1) and 2.5% goat serum (T5) showed consistently high percentage of post-thaw motility and live sperm. This conformed with the findings of Tuli and Holtz (1992) who determined which buffer was the most compatible with buck semen in a cryopreservation medium. Their experiments used an egg yolk (16%)–fructose diluents that were variable. There were significantly greater progressive motility (44%) and percentage of live cells (49%) observed in the semen samples.

Results of the present study showed a significantly higher percentage of live sperm with 5% egg yolk (72.60%), 2.5% goat serum (65.63%) and 30 mg BSA (64.27%). These values were similar to the result of the 16% egg yolk in extender that had 49% of live sperm (Tuli and Holtz, 1992). The lower concentration of egg

yolk in the present study was found to be better than with higher concentrations which could be due to the lower level of egg yolk coagulating enzyme in the extender as claimed by Iritani and Nishikawa (1961).

Table 5 shows that the highest percentage of abnormal head morphology was observed in the treatment with 10% goat serum (T7). However, there were no significant differences among the different extenders. As to the middle piece abnormality**,** a high percentage of sperm with abnormal neck morphology was noted in goat semen treated with 10 mg BSA (T8). Similarly, no significant differences were observed among the different treatments. In terms of tail abnormality, results revealed no significant differences among treatments. In terms of the total sperm abnormality scores, the lowest sperm abnormality was observed in the sperm cryopreserved with 5% egg yolk (T1) at 6.49%, while the highest was with sperm cryopreserved in 10 mg BSA (T8) with 12.65%. Generally, it was observed that semen samples in this study contained some abnormally formed spermatozoa. Earlier studies (Jager *et al*., 1985), however, stated that abnormalities in the sperm morphology were usually not associated with lower fertility rates, but only if the proportions of abnormal sperm exceed about 20% (Purdy, 2006).

Table 5. Percent abnormality of goat sperm treated with different extenders.

Treatment	Types of abnormality (%)			
	Head	Mid-piece	Tail	Total
T1	0.98	2.39	3.12	6.49
T2	1.51	2.78	3.45	7.74
T3	0.89	3.45	5.12	9.46
T4	2.01	3.42	5.46	10.89
T5	2.06	2.48	2.67	7.21
T6	1.53	4.06	5.93	11.52
T7	2.27	3.95	3.71	9.93
T8	0.85	4.08	7.72	12.65
T9	0.96	2.79	5.40	9.15
T10	1.24	2.89	7.07	9.96

Study 2. Utilization of cryopreserved goat semen for artificial insemination

In view of the overall superiority of semen cryopreserved with extender with 5% egg yolk, the frozen-thawed semen was used to inseminate does in natural estrus. Out of the 23 does inseminated in the first batch, 16 were confirmed pregnant, while 5 out of 7 does were pregnant in the second batch, resulting in an overall pregnancy rate of 70.50% (Table 6). Confirmation of pregnancy was made through the birth of kids after 5 months (150 days) of pregnancy. This considerably high AI efficiency rate could be attributed to the superior quality semen used in the present work.

Table 6. Pregnancy rate (%) following insemination of cryopreserved goat semen with 5% egg yolk extender.

AI batches	No. of does inseminated	No. of pregnant does	Pregnancy rate (%)
1st	23	16	69.56
2nd	7*	5	71.43
Total	30	21	70.50

*Does from the 1st batch which returned to estrus after 21 days.

CONCLUSION

The results of the present study revealed that Tris-Citric Acid-Raffinose-Glycerol extender supplemented with 5% egg yolk, 2.5% goat serum or 30 mg BSA were suitable for the cryopreservation of buck semen in terms of sperm motility, percentage of intact acrosome and percent abnormalities at post thawing. The proportion of live sperm in 5% egg yolk extender, however, was higher compared with those of 2.5% goat serum and 30 mg BSA. It is, therefore, concluded that extender with 5% egg yolk is the most efficient extender for the buck semen cryopreservation in the present study. Furthermore, insemination of does with frozen -thawed semen extended with 5% egg yolk resulted in 70.50% pregnancy and birth rate. In sum, the present study demonstrated the successful development of a simple and economical cryopreservation protocol for buck semen using extender with lower concentration of egg yolk and involving the use of a simple freezing device for wider application. The results expand the potential of AI as a tool for genetic improvement of goat species in the country.

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