



ISSN: 2456-2912

VET 2017; 2(5): 39-42

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www.veterinarypaper.com

Received: 10-07-2017

Accepted: 11-08-2017

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## Effect of seminal plasma and temperature changes on qualities of frozen-thawed semen extended with egg yolk citrate or skimmed milk extender in Myanmar local bucks

**Hay Mar Kyaw, Zun Zun Wut Hmohn, Su Hlaing Phyu, Chaw Lett Ye Hnin, Than Than Sint, Min Bo and Soe Win Naing**

### Abstract

This research was carried out to observe the effects of seminal plasma and extenders on the motility and viability of local buck spermatozoa at 5 °C and at -20 °C. Three matured bucks were used for this experiment. From each buck, semen was collected two times per week by means of electroejaculator. A total of sixteen ejaculates were collected from each buck. After collecting semen, the effects of seminal plasma removal were analyzed by using two different extenders (Skimmed milk and egg yolk citrate extenders). The removal of seminal plasma gave higher and significant ( $p < 0.01$ ) effect in the motility, and live spermatozoa at 5 °C and at -20 °C. For two different extenders, however, the differences were not observed at 5 °C and at -20 °C on the semen quality of local buck.

**Keywords:** Seminal plasma, egg yolk citrate extender, skimmed milk extender, temperature changes, local buck

### 1. Introduction

Artificial insemination with fresh, chilled or frozen-thawed semen is a basic tool in goat breeding allowing the diffusion of caprine semen of high genetic value (Hidalgo *et al.*, 2007) [16]. The success of an artificial insemination program depends on the appropriate management of semen collection, storage and use (Etches, 1996) [12]. Cryopreservation of semen is a complex process that involves balancing a number of interrelated factors, such as freezing technique, extender, dilution, cooling rates and thawing methods. In case of buck semen cryopreservation, seminal plasma has been blamed as a limiting factor of buck semen freeze ability and fertility.

The deterioration and toxic effect of the seminal plasma were observed when unwashed goat semen was diluted with egg yolk or milk extender. Nowadays, these extenders are widely used for the frozen storage of small ruminant semen (Salamon and Maxwell, 2000) [21]. Notably, it is generally accepted that a substantial number of spermatozoa are damaged during cryopreservation. This damage may decrease sperm motility, viability and fertilization rate. Therefore, the composition of extender, suitable cryoprotectant and optimum freezing and thawing rates are important factors for a better fertilization rate (Curry, 2000; Eiman *et al.*, 2004 and Matsuoka *et al.*, 2006) [9, 11, 22]. In regards with the cryopreservative agents (CPAs), dimethylsulphoxide (DMSO) has been widely used alone or in combination with other cryoprotectants (Singh *et al.*, 1995, Snedeker and Gaunya, 1970) [30, 31]. Regarding to the preparation of extenders, either egg yolk or milk or combination of the two have been used as a basic ingredient. Nowadays, the generally used media for buck semen liquid storage (4-5 °C) are skimmed milk, sodium citrate egg yolk and Tris egg yolk diluents (Leboeuf *et al.*, 2000) [20]. Therefore, this study attempted to investigate the effect of seminal plasma, two different extenders and temperature changes on the semen quality of local buck before freezing and after thawing.

## 2. Materials and Methods

### 2.1 Experimental animals and management

Three bucks of local breed, with the age of ten to twelve months and body weight ranging from thirty-two to thirty-five kilogram were used during successive periods. The bucks, namely A1, A2 and A3, were kept in individual cages in the campus of University of Veterinary Science, Yezin, Nay Pyi Taw. They were supplied with concentrate, green roughage and straw (*ad libitum*). All the animals had free access to fresh water.

### 2.2 Experimental designs

Three bucks (A1, A2 and A3) were used. From each buck, semen was collected twice a week, and a total of sixteen ejaculates were collected. After collecting semen, a small amount of the semen was washed to remove the seminal plasma. Then, both of the washed and unwashed spermatozoa was extended separately in the egg yolk citrate extender and in the skimmed milk extender, and placed at 5 °C for two hours. Motility and live sperm cell percent were assessed. After that, the spermatozoa were placed at -20 °C. Two hours later, the motility and viability were assessed. Experimental period lasted for 8 weeks.

### 2.3 Semen Collection and Evaluation

Semen samples were collected from three mature local bucks. The volume of each ejaculate was measured in a graduated test tube. The sperm concentration of each ejaculate was determined by means of a haemocytometer. Live and dead spermatozoa were assessed using nigrosin-eosin stain (Evan and Maxwell, 1987). The percentage of the motility of spermatozoa in each specimen was evaluated under a phase contrast microscope at  $\times 200$  magnification by placing a small drop of raw semen on a slide covered with a glass cover slip (22 mm  $\times$  22 mm) from three selected representative fields subjectively.

For the assessment of motility and viability of sperm cells, egg yolk citrate extender containing 1.75% DMSO and skimmed milk extender containing 1.75% DMSO were prepared separately in the beakers. Each extender was suspended by a clean and dry pipette. The beakers were labelled and kept in the refrigerator.

Into each test tube, sperm and extender were added at the ratio of 1:4 (sperm: extender). Into the test tube 1, washed sperm and egg yolk citrate extender were put. Into the test tube 2, unwashed sperm and egg yolk citrate extender were put. Into the test tube 3, washed sperm and skimmed milk extender were put. Into the test tube 4, unwashed sperm and skimmed milk extender were put. The test tubes were gently shaken and placed at 5 °C for two hours. After equilibration time, the cooled samples were rewarmed by placing the test tubes in the water bath at 37 °C for two seconds. Then semen samples were assessed for motility and live spermatozoa. After that, the test tubes were moved to the freezer (-20 °C). Two hours later, the samples were thawed for a few seconds in a water bath at 37 °C and assessed for motility and live spermatozoa.

### 2.4 Statistical Analysis

The data were analyzed using the SPSS software system (Version 20, SPSS). The values were expressed as the mean  $\pm$  the standard error of the mean (S.E.M) and the level of statistical significance was considered as  $p < 0.05$ .

## 3. Results

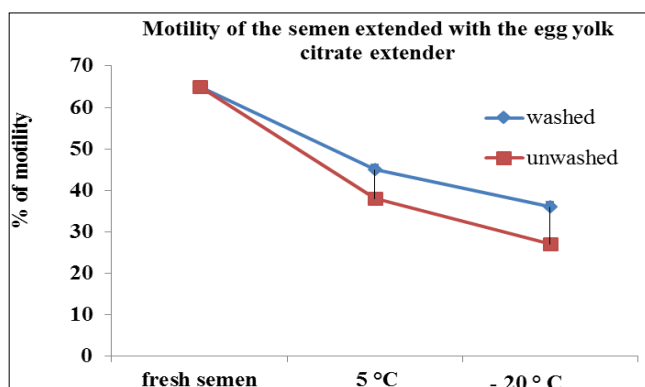
The removal of seminal plasma had a significant influence on semen quality before freezing and after thawing (Table 1 and 2). Washing of spermatozoa provided significantly ( $p < 0.01$ ) higher effect on the percentage of motility and live spermatozoa before freezing. After thawing, washed spermatozoa were found to have significant ( $p < 0.01$ ) effects on the percentage of motility and live spermatozoa. No significant differences ( $p < 0.05$ ) were observed between the two different extenders in all the semen quality of local bucks.

**Table 1:** Percentage of motility of the spermatozoa and live spermatozoa in the semen extended with the skimmed milk extender and egg yolk citrate extender placed at 5 °C (Mean  $\pm$  SEM).

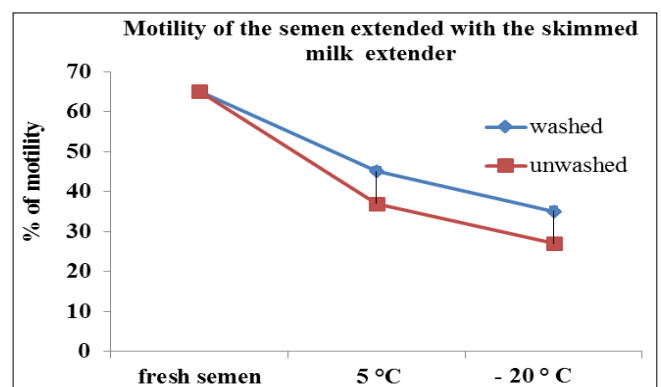
	Washed semen		Unwashed semen		p value
	Skimmed milk extender	Egg yolk citrate extender	Skimmed milk extender	Egg yolk citrate extender	
Motility	45 $\pm$ 1 <sup>a</sup>	47 $\pm$ 1 <sup>a</sup>	37 $\pm$ 1 <sup>b</sup>	38 $\pm$ 1 <sup>b</sup>	<0.01
Live sperm	52 $\pm$ 1 <sup>a</sup>	54 $\pm$ 1 <sup>a</sup>	34 $\pm$ 3 <sup>b</sup>	44 $\pm$ 1 <sup>b</sup>	<0.01

**Table 2:** Percentage of motility of the spermatozoa and live spermatozoa in the semen extended with the skimmed milk extender and egg yolk citrate extender placed at -20 °C (Mean  $\pm$  SEM).

	Washed semen		Unwashed semen		p value
	Skimmed milk extender	Egg yolk citrate extender	Skimmed milk extender	Egg yolk citrate extender	
Motility	35 $\pm$ 1 <sup>a</sup>	36 $\pm$ 2 <sup>a</sup>	27 $\pm$ 1 <sup>b</sup>	29 $\pm$ 1 <sup>b</sup>	< 0.01
Live sperm	40 $\pm$ 1 <sup>a</sup>	44 $\pm$ 2 <sup>a</sup>	31 $\pm$ 1 <sup>b</sup>	32 $\pm$ 1 <sup>b</sup>	< 0.01



**Fig 1:** Motility of spermatozoa extended in the egg yolk citrate extender at different temperatures.



**Fig 2:** Motility of spermatozoa extended in the skimmed milk extender at different temperatures.

#### 4. Discussion

Removal of seminal plasma from semen has a beneficial effect on the freezing ability of Boer goat semen (Leboeuf *et al.*, 2000; Gacitua and Arva, 2005; Kozdrowski *et al.*, 2007, Ustuner *et al.*, 2009 and Naing *et al.*, 2011) [20, 14, 19, 33, 25]. Leboeuf *et al.* (2003) [20] stated that a specific problem in the preservation of goat semen was the detrimental effect of seminal plasma on the subsequent viability of spermatozoa *in vitro*. In this experiment, the removal of the seminal plasma has a beneficial effect on the semen quality of Myanmar bucks, at 5 °C and -20 °C (Table 1 and 2). Seminal plasma had a negative effect on Myanmar goat sperm viability diluted in 20% egg-yolk-citrate extender and kept at ambient temperature (Than Than Sint, 2004). Phillips and Lardy (1940) [26] described that when bovine spermatozoa were stored at 5 °C, they remained viable and capable of fertilizing ova for 3-4 days. Beer-Ljubic *et al.* (2009) [4], reported that 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. Washing of semen prior to freezing has also been advised but generally it is time consuming and can affect the sperm viability (Gacitua and Arav 2005) [14]. A detrimental effect of seminal plasma on the maintenance of motility of cooled equine spermatozoa; however, the mechanism for the adverse effect of seminal plasma during cooled storage remains undetermined.

In this experiment, the percentage of motile and live spermatozoa in the washed semen extended with the egg yolk citrate and skimmed milk extenders were higher than those in the unwashed semen extended with the egg yolk citrate and skimmed milk extenders at 5 °C and -20 °C. Phillips and Lardy (1940) [26] and De Leeuw *et al.* (1993) [10], reported that egg yolk extender could prevent sperm cell damage during cooling and freezing. Egg yolk had lipoprotein and lecithin which could maintain and protect the integrity of spermatozoa (Blackshow, 1954) [5]. Higher percentage of motility at -20 °C was occurred when the seminal plasma was present than when it was removed (Ritar and Salamon, 1983; Chauhan and Anand, 1990; Tuli and Holtz, 1994 and Azerado *et al.*, 2001) [27, 7, 32, 3].

Plasma removal and subsequent addition of the extender containing egg yolk had deleterious effects to sperm (Azerêdo *et al.*, 2001, Gil *et al.*, 2002 and Viana *et al.*, 2006) [3, 15, 34]. Goat semen contains coagulating enzymes which convert the lecithin in the egg yolk to lysolecithin and this substance seemed to be lethal to sperms (Leboeuf *et al.*, 2000) [21]. The addition of egg yolk based extenders to buck semen, give rise to coagulation of egg yolk and sperm died owing the action of an enzyme secreted by the bulbourethral gland named egg yolk coagulation enzyme. The enzyme hydrolyses the lecithin of the egg yolk to fatty acids and lysolecithin which are toxic to spermatozoa and cause coagulation of the storage medium (Roy 1957; Iritani *et al.*, 1961, 1964; Aamdal *et al.* 1965, Iritani and Nishikawa, 1972) [28, 18, 2]. When egg yolk was included in the diluted buck seminal plasma, hydrolysis of lecithin led to further deterioration of the environment. As a consequence, the spermatozoa did not survive beyond two hours (Iritani and Nishikawa 1961). Egg yolk show negative effect on frozen goat semen due to the presence of phospholipase (Iritani and Nishikawa, 1972) [18]. Low density lipoprotein is better than whole egg yolk to preserve sperm motility after freezing (Moussa *et al.*, 2002; Amirat *et al.*, 2004) [24, 1].

Leboeuf *et al.* (2003) [20] found motility parameters of goat sperm, diluted in skimmed milk extenders and stored at 4 °C, could be maintained with success until Day 4. Seminal plasma has an adverse effect on the survival of goat spermatozoa stored frozen in milk-based extenders (Corteel 1980; Memon *et al.*, 1985) [8]. However, a glycoprotein component of bulbourethral gland secretion contains lipase activity that is detrimental to sperm motility when stored in skimmed milk-based extenders (Carver *et al.*, 2002) [6]. Bulbourethral secreting BUSgp60 has a triacylglycerol hydrolase activity that causes deterioration of buck spermatozoa in skimmed milk extender. BUSgp60 causes a decrease in the percentage of motile spermatozoa and the death of buck spermatozoa in bucks (Leboeuf and Salamon, 2000) [21].

#### 5. Conclusion

In this experiment, it is found that removal of the seminal plasma had beneficial effect on the semen quality at 5 °C and -20 °C. There was no difference between the beneficial effects of egg yolk citrate extender and skimmed milk extender on the percentages of motility and live spermatozoa at 5 °C and -20 °C. Further researches are needed to deeply understand the beneficial effects of removal of seminal plasma and the effects of egg yolk citrate extender and skimmed milk extender on the percentages of motility and live spermatozoa kept at 5 °C for longer (more than two hours) duration in Myanmar local bucks.

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