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Effect of sperm number of per dose on quality and fertility of frozen buck semen

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Abstract
We investigated the effect of concentrations of 150, 200 and 300 million sperm /ml containing 37.5, 50 and 75 million sperm respectively in 0.25 ml straws during freezing on post-thaw quality semen pulled from Beetal and Sirohi bucks. The overall mean per cent post-thaw sperm motility, live sperm, intact acrosome and HOST-reacted sperm for straws containing 37.5, 50 and 75 x10^6 sperm/ straw was 56.02 ± 0.47, 57.50 ± 0.41 and 65.57 ± 0.58;67.42 ± 0.62, 70.55 ± 0.55 and 73.45 ± 0.57; 61.12± 0.69, 64.37± 0.66 and 68.25± 0.66; and 59.00 ± 0.62, 62.77± 0.52 and 65.57 ± 0.58 respectively. The post-thaw values of all semen parameters studied in straws with 75 million sperm were significantly (P<0.01) higher than that of 50 and 37.5 x10^6 sperm / straw. The post thaw values with 50 x10^6 sperm/ straw was significantly (P<0.01) higher than that with 37.5 x10^6 sperm /straw for sperm motility, intact acrosome and HOST-reacted sperm but not for live sperm. The fertility rate recorded using the three different sperm concentrations separately in Beetal and Sirohi goats revealed that it was the highest with 75 x10^6 sperm /straw in both the breeds. It was revealed from the present study that the post thaw quality and fertility of goat semen for 75 x10^6 sperm / straw was superior to that for 50 x10^6 and 37.5 x10^6 sperm / straw.

Keywords: freezing, fertility, goat semen, post thaw quality, sperm number

Introduction
It is essential that a semen sample should be diluted properly so that there are sufficient numbers of sperm and sufficient diluent to accommodate the cells in an insemination straw, so that the high fertility rate can be achieved with the lowest number of sperm per dose in a single insemination. Historically, semen samples in farm animals have been diluted by either diluting semen with specific volumes of diluents or by diluting semen to specific sperm concentration. Dilution rates of 1:1–1:23 (v/v; semen to diluent) have been used successfully (Evans and Maxwell, 1987; Ritar *et al*., 1990 a,b) [9, 19, 20]. Perhaps a better way of diluting semen, for comparison purposes, is based on the sperm concentration. Reports of successfully freezing of semen with reasonable fertility have been obtained with varying insemination doses of 80 to 500×10^6 sperm/ml in goat (Corteel, 1974; Ritar *et al*., 1990 a; Ritar *et al*., 1990 b; Karatzas *et al*., 1997) [7, 19, 20, 10]. Goat semen is usually frozen allowing higher sperm concentration per ml of extended semen. While there is no clear cut industry standard, goat semen is commonly frozen only after 4-5 folds (v/v) extension with cryodiluent (leaving approximately 800-1000 million spermatozoa per ml) or to a specific concentration (Purdy, 2006) [16]. This is most likely for the sake of convenience and to avoid negative effect associated with high dilution rates of goat semen (Purdy, 2006) [16]. However, there is a lack of information on the rate to which semen can be diluted during pre-freezing without the reduction in their post-thaw survival of spermatozoa and fertility. Hence the present study was taken up.

Materials and Methods
Two Sirohi and two Beetal adult bucks maintained at Goat Research Station, Assam Agricultural University, Burnihat were used in the study. The animals were maintained under uniform feeding and managemental practices. Semen was collected from each buck once/twice a week with the help of a standard artificial vagina using a restrained doe as a mount. Immediately after collection, each ejaculate was evaluated for volume, mass activity (Zemjanis, 1970) [22] (based on the numerical scale 0–4) and initial sperm motility. Only ejaculates having volume 0.8 ml or more, mass activity 3+ or more and initial sperm motility 70 % or more were used for the study.

A total of 40 pooled ejaculates, 20 each from Beetal and Sirohi breeds were used. The sperm concentration was determined with the help of a Neubauer counting chamber. Each pooled ejaculate was split into three parts and extended in warm tris extender containing 150, 200 and 300 x10^6 sperm/ml respectively so as to maintain the final number of sperm at 37.5, 50 and 75 x10^6 respectively per 0.25 ml straws. The extended semen was cooled gradually to 5˚C @ 1˚C / 3 minutes, equilibrated for 4 hours at 5˚C and frozen in liquid nitrogen using French mini straws (0.25 ml) following standard method. After 24 h of storage in liquid nitrogen, the frozen semen was thawed in warm (37°C) water for 30 sec. The semen was evaluated for sperm motility, live sperm (Blom 1977) [6], live intact acrosome (Watson 1975) [21] and hypo osmotic swelling test (HOST) - reacted sperm (Revel and Mrode 1994) [18] after equilibration and after freezing on thawing. Statistical analyses of the data were performed with one way ANOVA using Statistical Analysis Systems (enterprise Guide 4.2 version) and Duncan's Multiple Range Test (DMRT) was used to compare the differences between mean values. Fertility rate using three different concentrations was recorded on artificial insemination separately for Beetal and Sirohi bucks.

Results

The results obtained are furnished in Table 1 presenting the effect of number of sperm per straw on quality on different parameters investigated. Analyses of a variance of means of different parameters along with their critical differences were worked out.

Table 1: Per cent of sperm motility, live sperm, intact acrosome and HOST- reacted sperm (mean*± SE) after equilibration (AE) and after freezing (AF) of buck semen in tris extender containing 37.5, 50 and 75 x 10^6 sperm/straw

Sperm number (x 10^6)	Sperm motility (%)		Live sperm (%)		Intact Acrosome (%)		HOST (%)	
	AE	AF	AE	AF	AE	AF	AE	AF
37.5	70.30^{c}± 0.32	56.02^{c} ± 0.47	79.60^{b} ± 0.55	67.42^{b} ± 0.62	74.50^{c} ± 0.86	61.12^{c} ± 0.69	65.97^{c}± 0.72	59.00^{c} ± 0.62
50	72.07^{b} ± 0.33	57.50^{b} ± 0.41	80.87^{b} ± 0.49	70.55^{b} ± 0.55	77.30^{b}± 0.71	64.37^{b} ± 0.66	68.97^{b}± 0.61	62.77^{b}± 0.52
75	73.30^{a}± 0.38	65.57^{a}± 0.58	83.75^{a}± 0.44	73.45^{a} ± 0.57	79.97^{a}± 0.70	68.25^{a} ± 0.66	72.55^{a}± 0.65	65.57^{a} ± 0.58

*40 observations; Means bearing different superscripts in a column under each parameter differ significantly (P< 0.05).

The fertility rate for 37.5, 50 and 75 x10^6 sperm/straw was recorded to be 40.00, 60.00 and 70.00 per cent in Beetal, and 33.33, 33.33 and 66.66 per cent in Sirohi buck frozen semen respectively. Fertility rate was found to be the highest at a concentration of 75 x10^6 sperm / straw in both Beetal and Sirohi bucks (70.00 and 66.66% respectively).

Discussion

The present values could not be compared for all the sperm concentrations used during freezing due to the paucity of available literature. However, very low post-thaw mean sperm motility (14.2 ± 2.2 %) was recorded in Angora buck semen (Daskin *et al.*, 2011) [8] when extended in Bioxcell extender to contain 200 million sperm per ml as compared to the present value of 57.50 ± 0.41 per cent in Beetal and Sirohi buck semen extended in Tris extender with 50 million sperm in 0.25 straw ml that amounts to 200 million spermatozoa per ml. The mean post-thaw sperm motility of 65.57 ± 0.58 per cent in the present study when extended with a sperm concentration of 300 million/ml. (*i.e.*, 75 million per 0.25 ml straw) also compared unfavorably with the findings of Daskin *et al.* (2011) [8] who obtained 20.0 ± 2.9 per cent post-thaw motile sperm allowing 400 million sperm/ml after dilution in the Bioxcell extender before freezing. The present findings on incidence of post-thaw intact acrosome and spermatozoa with plasma membrane integrity were also at wide variance with that recorded by Daskin *et al.* (2011) [8].

In the present study the mean post-thaw sperm motility, live sperm, intact acrosome and HOST-reacted sperm increased significantly (P<0.05) from 56.02 ± 0.47 to 65.57 ± 0.58 per cent, 67.42 ± 0.62 to 73.45 ± 0.57 per cent, 61.12 ± 0.69 to 68.25 ± 0.66 per cent and 59.00 ± 0.62 to 65.57 ± 0.58 per cent respectively with rise in pre-freeze sperm concentration from 150 to 300 million sperm per ml (corresponding to 37.5 to 75 million sperm per 0.25 ml straws). The finding of the present study gained support from the report of Daskin *et al.* (2011) [8] who recorded increase in the percentage of mean post-thaw sperm motility and spermatozoa with plasma membrane integrity from 14.00 ± 2.20 to 38.00 ± 4.80, and 14.00 ± 3.40 to 39.00 ± 4.10 respectively with increase in sperm concentration (*i.e.*, lowering in dilution rate) from 200 to 800 million sperm per ml in Angora buck semen extended in Bioxcell extender. Rekha *et al.* (2016) [17] recorded the percentage of mean post-thaw sperm motility to be 44.50 ± 0.60 and 36.60 ± 0.60 when extended in Triladyl and Tris-fructose-egg-yolk extenders respectively to contain 200 million spermatozoa per ml before freezing in ram semen. They found that the mean per cent post-thaw sperm motility increased significantly to 62.00 ± 0.60 and 48.00 ± 0.30 when pre-freeze level of sperm concentration was increased to 400 million sperm per ml in the aforementioned extenders respectively. The mean percentage of viable sperm was also recorded to increase significantly from 46.60 ± 0.60 to 64.80 ± 0.60 and from 38.10 ± 0.60 to 49.30 ± 0.30 with increase in pre-freeze sperm concentration from 200 to 400 million per ml. in Triladyl and tris fructose egg yolk glycerol extender respectively.

Findings of the present study indicated that the post-thaw quality of spermatozoa improved significantly with higher sperm concentration allowed to before freezing as compared to lower concentration. This could be due to lower dilution rate of semen allowing higher sperm concentration per ml before freezing resulting in higher availability of seminal plasma per spermatozoa as compared to higher dilution that rendered the spermatozoa with lower concentration of seminal plasma. It was suggested that the amount of seminal plasma surrounding each spermatozoa in an ejaculate varied among different concentrations (Kommisrud *et al.*, 2002) [12]. The 'dilution effect' that cause loss of sperm motility and viability when semen is diluted to high levels (Mann, 1964) [13] could be avoided in the present study by lower dilution of semen leaving higher sperm concentration at the time of processing of semen for preservation. The 'dilution effect' was ascribed to a reduction in the concentration of protective factors in male reproductive tract secretions. Seminal plasma contains a mixture of secretions from the testis, epididymis and accessory glands. Beneficial proteins from these secretions could support sperm function, sperm motility and viability

(Bergeron and Manjunath, 2006) [5]. Thus at higher dilution that resulted in lower number of 37.5 million sperm per ml in the present study, the beneficial effects of seminal plasma proteins was diminished with lower availability of the plasma around spermatozoa that exerted detrimental effects on spermatozoa as evidenced by recording of significantly (P<0.05) lower percentages of sperm motility, live sperm, sperm with intact acrosome and HOST-reacted sperm with sperm number of 37.5 million per straw i.e., 150 million sperm/ml. However, a threshold of high sperm concentration appears to exist beyond which beneficial effect of the lower dilution rate of semen before freezing using an extender could not be obtained. The post-thaw characteristics of ram spermatozoa were reported to be significantly affected by elevation of pre-freezing sperm concentration from 500 to 800 million/ml (Alessandro *et al.,* 2001) [2], 200 to 400-800 million/ml (Akcay *et al.,* 2012) [1], 200 to 400-1600 million/ml (Alvaraz *et al.,* 2012) [3] and 400 to 600 million/ml (Rekha *et al.,* 2016) [17]. This was attributed to lower ratio of available nutrients of the extender and cryoprotective agents at higher sperm concentration. (Akcay *et al.*, 2012) [1]. It was probable that lowering in amount of cryoprotectant per sperm cell lowered the percentage of unfrozen water channels which could be detrimental to post-thaw seminal characteristics. (Nascimento *et al.*, 2008) [15]. The decline in seminal attributes could also be ascribed to increase in extracellular oxidative stress due to increase in endogenous free radical production with rise in sperm concentration per volume of extender. The decline in post-thaw semen characteristics was conspicuous with increased pre-freeze sperm concentration of 400 million spermatozoa or more in ram semen.

The obtained highest fertility rate on artificial insemination of does could be due to significantly higher post-thaw sperm quality with the highest sperm number used in the study. It might also be attributed to the higher seminal plasma concentration per spermatozoa at the lowest dilution rate used. Alessandro *et al.* (2001) [2] observed that in ewes the rate of lambing increased from 64.3 per cent to 65.6 per cent with the increment in dose of spermatozoa from 20 $x10^6$ to 40 $x10^6$ per ewe that corresponded to increase in pre-freeze spermatozoa concentration from 100 $x10^6$ to 200 $x10^6$ per ml extending the semen in milk-lactose egg yolk based extender. The non-return rate was found to be the lowest with 37.5 $x10^6$ sperm/straw *i.e.,* with the highest dilution of semen in the present study leaving a lower proportion of seminal plasma per spermatozoa. Beneficial proteins from the seminal plasma were reported to prevent spontaneous capacitation or acrosome exocytosis that rendered the spermatozoa infertile (Ballester *et al.*, 2007) [4]. Substances from seminal plasma protected spermatozoa from premature aging during storage (Kasimanickam *et al.*, 2006) [11]. The reduction in seminal plasma concentration following dilution could lead to induction in capacitation and acrosome reaction. Maxwell and Johnson (1999) [14] proposed that although the acrosome reaction was vital for fertilization, it must occur in the direct proximity of the oocyte. The lower concentration of seminal plasma might cause acrosome reaction of spermatozoa before it was used for insemination and the reduction in the number of cells with intact acrosome might eventually decrease fertility in the does when inseminated with semen having higher dilution. This might explain the obtained lower fertility rate in does in the present investigation that were inseminated with frozen semen that contained 37.5 million sperm per straw.

Conclusion

It could be concluded from the present study that per cent post thaw sperm motility, live sperm, intact acrosome and HOST-reacted sperm and fertility rate was superior for 75 $x10^6$ sperm/straw as compared to 50 $x10^6$ and 37.5 $x10^6$ sperm /straw.

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