Post-thaw Thermal Resistance Test on Motility and Acrosomal Integrity of Filtered and Non-filtered Frozen Semen of Murrah Buffalo Bulls

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ABSTRACT : Present investigation was conducted to determine the post-thaw sperm motility and acrosomal damage of filtered and non-filtered frozen semen of Murrah buffalo bulls. Twenty semen ejaculates (from four Murrah buffalo bulls collected at weekly interval) were diluted in Tris egg yolk glycerol extender and divided into two parts. One was filtered through sephadex G-100 column and the other portion was kept as such (non-filtered). Both fractions were frozen in liquid nitrogen (-196°C) by the standard method developed in the laboratory. After 24 h of freezing, non-filtered and filtered semen samples were thawed at 37°C for 1 min. These samples were incubated at 37°C in a water both. The different seminal characteristics i.e. percent progressive sperm motility, live and abnormal spermatozoa and spermatozoa with damaged acrosome were assessed at hourly interval till they remained motile. The filtered frozen and thawed semen showed significantly ($p<0.05$) high sperm viability and acrosomal integrity as compared to non-filtered semen.
(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 10 : 1424-1428)

Key Words : Murrah Bull, Sephadex Filtration, Frozen Semen Motility, Acrosomal Integrity

INTRODUCTION

The Murrah buffalo is the most famous breed of domestic Indian buffalo and one of the most efficient milk as well as butter fat producing animals. The main obstacle in maintaining the optimal productivity is the poor freezability of semen from buffalo bulls. The success of artificial insemination technique in buffalo is associated with effective prolongation of fertile life of spermatozoa obtained from genetically superior bulls under *in vitro* storage condition. The variation in temperature during freezing and thawing of semen inevitably reduces the proportion of motile and live spermatozoa and causes ultra structural, biochemical and functional damage. Extensive research work has been carried out both in India and abroad on various aspects of improvement in the freezing technology of buffalo bull semen but still the post-thaw semen quality is not as good as that of bull semen (Dhami, et al., 1995). A high percentage of abnormal and dead spermatozoa in bull (Shannon and Curson, 1972) and buffalo bull semen (Heuer and Tahir, 1982) exert toxic and lytic effect on motile sperm cells in the ejaculate, which leads to lower fertility. Therefore, the removal of non-motile, dead and abnormal spermatozoa from the semen by filtration before freezing, may results in higher post-thaw motility as compared to non-filtered semen.

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Received December 27, 2002; Accepted May 15, 2003

The factors affecting the efficiency of semen filtration through sephadex column have been studied in cattle (Maki-Laurila and Graham, 1968; Graham et al., 1978; Landa et al., 1980; Graham and Graham, 1990; Anzar and Graham, 1995; Anzar et al., 1997), but little information is available on buffalo bull semen (Heuer and Tahir, 1982; Goyal et al., 1996; Panghal and Tuli 1999). The aim of present study was to assess the post-thaw motility and acrosomal integrity of filtered and non-filtered frozen-thawed semen of buffalo bulls at an hourly interval after incubation at 37°C.

MATERIALS AND METHODS

Location of study site

The experiment was carried out at the Chaudhary Charan Singh, Haryana Agricultural University animal farm of college of animal sciences, Hisar which is located at longitude 75°-46E$^{!}$ latitude 29-10N$^{!}$ in a semi-arid tract of the country.

The experimental animals and management

Four healthy Murrah buffalo bulls, 8 to 11 years of age and weighing 650 to 700 kg were used in the study. They were maintained under conventional farm conditions and housed individually in an open bullpen shed. Each bull was fed a daily ration of 2 kg concentrate mixture having (18% CP and 65% TDN). Wheat bhusa (3% CP and 41% TDN) and water were supplied *ad libitum* to these animals through out the investigation. Green fodder was offered to them depending upon its seasonal availability.

Table 1. Seminal characteristics of Murrah buffalo semen (Mean±SE)

Seminal attributes	Bull No.				
	11 (5)	815 (5)	1,082 (5)	1,085 (5)	Over all (20)
Semen vol. (ml)	3.32±0.78[b]	4.29±0.89[a]	2.97±0.95[b]	3.21±0.79[b]	3.45±0.25
Sperm conc. ($\times 10^6$/ml)	728.0±36.52[c]	1,050.0±23.02[a]	738.0±22.00[c]	906.0±38.55[b]	855.5± 33.59
Prog. sperm motility (%)	58.00±2.62[b]	73.00±1.76[a]	41.00±2.29[c]	71.00±2.92[a]	60.75±4.96
Live sperms (%)	71.44±2.14[a]	78.18± 2.63[a]	51.50±3.26[b]	79.80±2.49[a]	70.23±2.79
Abnormal sperms (%)	24.13±1.13[a]	17.50±1.51[b]	22.33±1.81[a]	12.34±1.32[c]	19.08±2.04
Damaged acrosomes (%)	12.86±1.52[a]	16.62±2.01[a]	15.04±1.11[a]	14.52±1.36[a]	14.85±1.10

Figure in parentheses indicate number of semen ejaculates. [a, b, c] Means with different superscript in the same row differ significantly ($p<0.05$).

Semen collection and evaluation

Semen from 4 Murrah buffalo bulls was collected weekly using artificial vagina (42 to 43°C) and examined for its quality characteristics viz., volume, colour, consistency and sperm concentration. Live, dead and abnormal spermatozoa were estimated using eosin-nigrosine stain (Campbell et al., 1960). Giemsa stain (Watson and Martin, 1972) was used for determining the damaged acrosomes. In all, twenty semen ejaculates (5 /bull) were collected during the experimental period.

Preparation of sephadex column

Slurry of sephadex G-100, 3.3% w/v (Sigma chemical Co. USA) was prepared by allowing the sephadex to swell in Tris-citric acid buffer for overnight at room temperature (22 to 25°C). The filtration column of one cm height was prepared in a 5 ml disposable plastic syringe (external dia. 1 cm). A small amount of glass wool fibre (Sigma chemical Co. USA) was compressed at the bottom of syringe to support the sephadex slurry and also to prevent its loss. Under gentle but constant stirring sephadex slurry was layered over the glass wool. Tris-citric acid buffer was added twice in each syringe and allowed to drain by gravity and to pack the sephadex column. These columns were prepared just before filtration and kept in a vertical position at room temperature.

Extension and filtration of semen

Twenty semen ejaculates obtained from 4 Murrah buffalo bulls were processed. Tris egg yolk glycerol extender (Goyal, 1993) was used for semen extension. Each extended semen sample was divided into two parts, one part was kept as control (non-filtered) and second part of extended semen was filtered through the sephadex G-100 column of 1 cm height. Two ml Tris egg yolk glycerol extender was added into the column to complete the process of semen elusion. In the control semen, 2 ml extender was also added. German mini straws filled with filtered and non-filtered semen separately were equilibrated at 5°C for 3 h and then frozen in the vapours of LN_2. These straws were stored in the LN_2 at -196°C for 24 h before thawing and evaluation.

Thawing and evaluation of semen

After 24 h of freezing and storage, sufficient number of straws from control (non-filtered) as well as from filtered semen of each ejaculate were taken and thawed at 37°C for 1 min. The use of digital thermometer and stopwatch was made to have accuracy in thawing procedure. Straws were wiped dry and the contents of straws were incubated at 37°C. Various seminal parameters i.e. percent progressive sperm motility, live sperms, abnormal spermatozoa and spermatozoa with damaged acrosome were recorded at the different hours of incubation till the spermatozoa showed movement (motility).

Statistical analysis

The means and standard errors, multivariate analysis of variance, Duncan's multiple range test for means and linear product moment of biological parameters were calculated using SPSS/PC student software (Norusis, 1988).

RESULTS

Table 1 presents the mean values of semen volume, sperm concentration, percent progressive motile sperms, live sperms, abnormal sperms and damaged acrosomes. The data showed significant difference ($p<0.05$) in all the above parameters except percent damaged acrosomes in the semen among bulls.

The (mean±SE) percent progressive sperm motility, live spermatozoa, abnormal spermatozoa and damaged acrosomes in frozen-thawed semen incubated at 37°C for different hours have been presented in Table 2. The data revealed significant ($p<0.05$) increase in percent progressive motility in filtered frozen-thawed semen up to 1 h of incubation than non-filtered frozen-thawed semen. At 2 h and 3 h of incubation, the percent progressive motility of filtered frozen semen was higher than non-filtered frozen semen but did not reach to the level of significance. A significant ($p<0.05$) decline in percent live sperms at different hours of incubation was observed in filtered as well as in non-filtered frozen-thawed semen. The filtered frozen semen had significantly ($p<0.05$) more number of live spermatozoa at every stage of incubation than non-filtered frozen-thawed semen. Significantly ($p<0.05$) higher

Table 2. Quality based thermal resistance of filtered and non-filtered frozen-thawed Murrah buffalo semen incubated at 37°C

Treatments	Incubation hours (h)			
	0	1	2	3
Percent progressive sperm motility				
Filtered	43.25±2.06^{Aa}	30.25±2.31^{Ab}	15.25±1.83^{Ac}	6.75±0.59^{Ad}
Non-filtered	30.25±2.09^{Ba}	20.0±1.92^{Bb}	11.0±1.30^{Bc}	4.67±0.28^{Ad}
Percent live sperms				
Filtered	59.11±2.51^{Aa}	47.47±2.75^{Ab}	33.68±2.01^{Ac}	25.61±1.25^{Ad}
Non-filtered	46.89±2.37^{Ba}	36.55±1.73^{Bb}	28.33±1.61^{Bc}	20.66±1.39^{Bd}
Percent abnormal sperms				
Filtered	15.39±0.52^{Bab}	14.87± 0.59^{Bb}	17.16± 0.62^{Ba}	17.00± 0.81^{Ba}
Non-filterd	24.41±1.50^{Ab}	27.03±2.71^{Aab}	29.29±2.33^{Aa}	31.24±2.29^{Aa}
Percent damaged acrosome				
Filtered	39.16±1.19^{Ba}	37.56±1.63^{Ba}	38.59±1.22^{Ba}	39.88±1.81^{Ba}
Non-filtered	44.13±2.63^{Aa}	45.59±1.59^{Aa}	46.30±1.16^{Aa}	48.14±2.47^{Aa}

$^{a, b, c}$ Means with different superscript in the same row differ significantly ($p<0.05$).

$^{A, B}$ Means with different superscript in the same column for individual parameter differ significantly ($p<0.05$).

numbers of percent live sperms up to 1 h of incubation were obtained in filtered than in non-filtered frozen-thawed semen. Onwards one to three hours the filtered semen had more live sperms than non-filtered frozen-thawed semen but did not show significant difference ($p>0.05$). As incubation hours advances, a steady increase in sperm abnormalities was recorded in filtered as well as in non-filtered frozen-thawed semen. The filtered semen had significantly ($p<0.05$) lower percentage of sperm abnormalities at each hour of incubation as compared to non-filtered frozen-thawed semen. From Table 2 it is clear that filtered semen had significantly ($p<0.05$) less number of percent damaged acrosomes at each hour of incubation than non-filtered frozen-thawed semen. From zero to three hour of incubation the non-significant ($p>0.05$) difference in percent damaged acrosomes was observed between filtered and non-filtered frozen-thawed semen.

DISCUSSION

The variation in percent progressive sperm motility of semen observed in the present study was because of environmental and nutritional status of the bulls. The higher values of dead and abnormal sperms in the semen might be due to age of bulls and time interval between collection of semen and preparation of smears can further add to this variation. The variation in sperm concentration among bulls may be due to method of semen collection, frequency of semen collection and the pre-ejaculatory stimulus. The seminal characteristics obtained in the present study were within the range of values reported by other workers (Raizada, 1979; Dhami, et al., 1992; Goyal, 1993; Panghal, 1996; Younis et al., 1998).

The results of present study indicate that filtered semen had good quality spermatozoa as compared to non-filtered frozen-thawed buffalo bull semen. The increase in percent progressive sperm motility and live spermatozoa and decrease in percent abnormal sperms and damaged acrosome was noticed in filtered semen than in non-filtered frozen-thawed semen. The improvement in semen quality was due to retention of non-motile spermatozoa in the column, which may be due to interaction between sephadex beads, and abnormal spermatozoa and cells with damaged acrosomal membrane. The findings of Heuer and Tahir (1982), Kumar (1989), Chauhan et al. (1993), Spreckels (1994), Goyal et al. (1996), Panghal and Tuli (1999), Kumar et al. (1999) and Maurya et al. (2003) also supports our results. It has been proposed that sephadex traps stallion spermatozoa with capacitation like changes, as suggested by the results obtained with uterine incubated spermatozoa and with sperm cells incubated under *in vitro* capacitation media (Samper and Crabo, 1993). Results of the present investigation revealed that sephadex gel plays a significant role in retaining the dead, abnormal, non-motile and sperm with damaged acrosomes in the column. The motility of spermatozoa was increased after filtration, so it is regarded as being of major importance for the viability of cells. Based on this assumption, visual assessment of motility is the most commonly used criteria for semen quality. Many researchers have reported promising relationship between fertility and progressive sperm motility (Linford, et al., 1976). Our result also shows increase in intactness of acrosome in filtered frozen thawed semen than non-filtered frozen-thawed semen. In this context Saacke (1970), reported that the intactness of acrosome has a significant relationship with fertility. Physical barrier constituted by glass wool and sephadex can affectively obstruct the passing of low quality spermatozoa (i.e. cell with low motility or with a morphological abnormal spermatozoa). Roberts (1972) reported that motile spermatozoa kept in the column orient them selves downwards due to gravitational force while dead spermatozoa float at the top since they have lost the power of orientation. Filtration of semen through sephadex column appears to be a physiochemical

reaction and sephadex particles provide a barrier that allowed immotile/dead spermatozoa to agglomerate (Graham et al., 1978). Sperm motility and sperm membrane characteristics appear to be the factors influencing the trapping of bull spermatozoa in the sephadex column. Heuer and Tahir (1982) reported a positive correlation between sephadex filtration and sperm motility. In this context Graham and Crabo (1982) also found that sephadex allows only to pass normal motile spermatozoa through column. The column affectively retains the acrosome-damaged spermatozoa and allows the normal acrosome to the filtrate there by shown increase in percent normal acrosomes spermatozoa in the filtrate samples. The intact acrosomes carry hydrolytic enzymes necessary for oocyte penetration and therefore have strong relationship with fertility.

The quality as well as the viability of buffalo bull spermatozoa deteriorate as a consequence of freezing and thawing (Rao et al., 1993; Goyal et al., 1996 and Panghal and Tuli, 1999), which limits the survival of spermatozoa during incubation (Fiser et al., 1991). The significant decline of the percent progressive motile spermatozoa during post-thaw incubation in the present study may be due to inability of spermatozoa to generate ATP through mitochondrial respiration as a consequence of mitochondrial aging (Cummins et al., 1994 and Viswanath and Shannon, 1997). In addition to this toxic effect of dead sperms associated with the liberation of amino oxidase activity (Shannon and Curson, 1972) can also be responsible for decline in motility and viability of sperm cells. The prolong incubation of sperms also causes the deterioration and changes in the acrosome of spermatozoa (Saacke and White, 1972). In this context it has been proposed by Samper and Crabo (1993) that some sperm membrane proteins such as clusterin in the stallion sperm are unmasked after capacitation and play an important role in the interaction between sperm and sephadex beads and similar mechanism may be operating in the semen of other species including buffalo bull. Graham and Graham (1990) reported that filtration did not improve the fertility of bull already exhibiting high non-return rate but did improve the 60 to 90 day non-return rates of most lower fertility bulls. Heuer and Tahir (1982) stated that artificial insemination with filtered semen increases conception rate in buffalo than non-filtered semen. In addition to this, the improvement in the fertility was also reported by Fayemi et al. (1979) in case of ram and Kanakaraj et al. (1996) in case of bull semen.

It is concluded that filtered frozen-thawed semen has significantly higher post-thaw motility and acrosomal integrity and better thermal resistance than non-filtered frozen thawed semen.

ACKNOWLEDGEMENTS

Authors are thankful to Professor cum Head, Department of Animal Production Physiology, College of Animal Sciences, CCS HAU, Hisar. The support rendered by Drs V.K.Sharma and B.G.Desai is also acknowledged.

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