Effect of Buffer Composition, Sephadex Grade and Column Size on Filtration Based Quality Improvement of Semen from Murrah Buffalo Bull

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ABSTRACT : Sixty semen ejaculates collected at weekly interval from four Murrah Buffalo bulls over a period of seven months (Nov.1999 to May 2000) were used in the present study. Three buffer medium (sodium citrate, TES and Tris) were used for soaking of sephadex. Three grades of sephadex (G-15,G-100, and G-200) were used for preparation of columns. Columns of three different height (one, two and three cm) were used for separation of semen. Twenty semen ejaculates were used in each project. In the first experiment each semen ejaculates was divided into four parts. One part was kept as control and other three parts were passed thought one cm column of sephadex G-15 prepared in three different buffers. There was significant ($p<0.05$) increase in percent progressive sperm motility and percent live spermatozoa and decrease in percent abnormal spermatozoa and percent spermatozoa with damaged acrosome as well as sperm numbers after filtration through all the three columns. Sperm quality obtained in the filtrate of column prepared in Tris buffer was better in comparison to other two buffers. So the Tris buffer was used in the second trial. Twenty semen ejaculates were used in this experiment. Each semen ejaculate was divided into four parts. One part was kept as control (non-filtered) and other three parts were passed through columns of different grade of sephadex (G-15, G-100 and G-200). Progressive sperm motility and live sperm percentage improved significantly while decline in percent abnormal spermatozoa and percent spermatozoa with damaged acrosome and sperm concentration was observed after filtration through all the columns as compared to control (non-filtered) semen. Since post filtration quality of semen was better in the sephadex G-100 column, therefore it was selected for the next experiment. In third experiment, Tris buffer and sephadex G-100 were used for preparing columns of different height (one, two and three cm) and twenty semen ejaculates were filtered. The quality characteristics of semen (percent progressive sperm motility, percent live spermatozoa and sperm concentration) after filtration through one cm column were significantly ($p<0.05$) higher than after filtration through columns of two and three cm height. However non -significant ($p>0.05$) difference due to height of columns was observed for percent abnormal and percent damaged acrosome but 1 cm column comparatively gave better result than of 2 and 3 cm column height. ***(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 2 : 165-171)***

Key Words : Murrah Buffalo, Sephadex, Filtration, Acrosomal Integrity

INTRODUCTION

There are 163.14 million buffaloes in the world out of which 92.09 million are present in India FAO (1999). Murrah buffalo is one of the best breeds of buffalo present in our country. Artificial breeding of buffaloes on large scale using semen from bulls with superior germplasm can solve the problem of low productivity. One of the major constraints in the success of artificial insemination for the extensive exploitation of productive potential of buffalo has been its inherent inferior semen quality.

The success of artificial insemination technique is associated with effective prolongation of fertile life of spermatozoa obtained from genetically superior bulls under *in vitro* storage condition. During last three decades, 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. In spite of this improvement in freezing technology, the post-thaw semen quality of buffalo bulls is not as good as that of cattle (Dhami et al., 1995). A high percentage of abnormal and dead spermatozoa in buffalo bull semen can have an adverse effect on their fertilizing potential (Heuer and Tahir, 1982). In addition to this, dead and abnormal spermatozoa have been known to exert toxic and lytic effect on motile sperm cells in the ejaculate (Shannon and Curson, 1972), which leads to lower fertility. Therefore, the removal of non motile, dead and abnormal spermatozoa from the semen prior to its freezing might be another approach to increase post thaw motility and survivability of buffalo spermatozoa. The retention of sperms in sephadex reflects complex binding force between sephadex and plasma membrane of sperms. There is interaction between charge of spermatozoal membrane and sephadex. Both these charges are altered through the interaction with the buffer used for the preparation of sephadex column, to suspend the sperm or for illusion of sperms (Landa et al., 1980). Mogas et al. (1998) indicated that the interaction between dog spermatozoa and sephadex G-15 particle is based on loose contact and only sperm cells with alteration in the

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acrosomal membrane were affectively trapped in sephadex column.

An efficient and promising technique which was reported to remove abnormal and dead sperms is the filtration of semen through the columns of sephadex . The factors affecting the efficiency of sperm 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; Vyas et al., 1991; Anzar and Graham, 1993; Anzar and Graham, 1995; Anzar et al., 1997; Mustafa et al., 1998), but little/meagre 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 i) to study the efficiency of different buffers in separating the motile and non motile spermatozoa from buffalo bull semen through sephadex column, ii) to study the efficiency of different grades of sephadex on the viability and acrosomal morphology of buffalo bull spermatozoa, iii) to study the effect of different height of sephadex column on the survivability and acrosomal integrity of buffalo bull spermatozoa.

MATERIALS AND METHODS

Location of study site

The study was conducted at the Chaudhary Charan Singh, Haryana Agricultural University animal farm, Hisar. This area is located in a semi-arid region of sub-tropical India at longitude 75°-46E', latitude 29-10N'. The annual rainfall is approximately 300 mm.

The experimental animals and management

The Murrah Buffalo is the most important breed of domestic Indian buffalo and is distributed through out the India. The home tracts of Murrah is spreads in Haryana, Delhi and Punjab and are found in Rohtak, Hisar, Gurgaon and parts of Utter Pradesh west to river Jamuna. The Murrah buffalo cows are one of the most efficient milk and butter fat producers in India. Average milk yield is 1,500 to 2,500 kg per lactation with average fat 7.0 percent (Kaura, 1961). Four healthy Murrah buffalo bulls, aged 8 to 11 years and body weight 650 to 700 kg were selected for the present investigation. The bulls were maintained under conventional farm conditions. They were housed individually in a open shed bull pen. Each bull was fed a daily ration of 2 kg concentrate mixture (Barley 47%, de oiled rice polish 20%, ground nut cake 20%, gram 10%, salt 2% and mineral mixture 1%). Wheat bhusa and water were supplied *ad. lib.* to these bulls through out the investigation. Green fodder was offered depending upon its seasonal availability.

Collection of semen and its evaluation

Semen from 4 Murrah buffalo bulls was collected weekly for 7 month using artificial vagina and examined for its quality characteristics viz., volume, consistency, mass activity and sperm concentration. Live, dead and abnormal spermatozoa were estimated using eosin- nigrosin stain (Campbell et al., 1960). Giemsa stain (Watson and Martin, 1972) was used for determining the damaged acrosomes. In all, sixty ejaculates were collected from four Murrah bulls during the experimental period.

Preparation of buffers

Sodium citrate buffer : 2.9 gm of sodium citrate (anhydrous) was dissolved in little volume of distilled water. The volume was made to 100 ml by adding distilled water and pH was adjusted to 6.8.

TES buffer : A total of 7.4 percent TES (N-Tris (hydroxy methyl) methyl-2-amino ethane sulphonic acid) was titrated with 3.9 percent Tris (Tri hydroxy methyl amino methane) and volume was made to 100 ml with distilled water. The pH of buffer was adjusted to 6.8 with 10% Tris solution.

Tris citric acid buffer : 3.786 gm of Tris salt (Tri hydroxy methyl amino methane) and 2.115 gm of citric acid (anhydrous) were dissolved in 80 ml distilled water and pH of the buffer was adjusted to 6.8 with 10% citric acid solution. The volume was made to 100 ml with distilled water.

Preparation of sephadex column

Slurry of sephadex G-15 (20 percent w/v) Sigma chemical Co. USA, was prepared by allowing the sephadex to swell in sodium citrate, TES and Tris buffers for overnight at room temperature (22 to 25°C). The filtration column 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 column and also to prevent the loss of sephadex slurry. Use of coarse strand of glass wool was avoided to obtain an evenly packed plug. Under gentle but constant stirring sephadex slurry was layered over the glass wool. The corresponding buffer i.e. Sodium citrate, TES and Tris were added twice or thrice in each syringe and allowed to drain by gravity and to pack the sephadex gel column. These columns were prepared just before filtration and kept in a vertical position at room temperature.

Filtration of semen sample

Twenty semen ejaculates collected from four Murrah buffalo bulls (five ejaculates per bull) were utilized for each trial. Each semen ejaculates (appox. 4.0 ml) was divided into four parts (one ml each). In case of semen volume being

less than 4 ml, then second ejaculate was collected. One part of semen was kept as control (non-filtered). The second, third and fourth aliquot of semen was applied to the top of each of the sephadex G-15 columns of one cm height prepared using sodium citrate, TES and Tris buffers separately. The filtrate was collected in a graduated test tube placed at the bottom of each column. One ml each of sodium citrate, TES and Tris buffer was added into each column to complete the process of elusion/drainage of semen. In the control semen sample one ml normal saline was added simultaneously. The sperm concentration, percent progressive sperm motility, percent live and percent dead spermatozoa and spermatozoa with damaged acrosome were recorded before (control) and after filtration.

Based on the results obtained from the first trial, Tris buffer was selected for the next experiment. Slurries of the sephadex G-15 (20% w/v), G-100 (3.3% w/v) and G-200 (1.8% w/v) were prepared by allowing them to swell in Tris buffer as done in first trial. The columns of one cm height were made in five ml disposable syringes by using slurry of different grades of sephadex. The filtration was carried out in the same way as in the previous experiment. Based on the results of this trial, sephadex G-100 was found to be better in improving semen quality followed by sephadex G-200 and sephadex G-15. So sephadex G-100 was used in the final trial. Tris buffer and sephadex G-100 was used for preparing column of different heights (one, two and three cm) in disposable syringes of five ml capacity. Filtration was done as mentioned above. All the above seminal attributes were recorded before (control) and after filtration of semen through sephadex columns of various heights.

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, consistency, sperm concentration, mass activity, percent progressive motile sperms, percent live sperms, percent abnormal sperms and percent damaged acrosomes. The data showed significant difference ($p<0.01$) in all the above parameters except percent damaged acrosomes in the semen among buffalo bulls.

The average mean values of filtered buffalo semen through sephadex G-15 column of one cm height using different buffers are given in Table 2. Percent progressive motile sperm and percent live sperm increased significantly ($p<0.05$) after filtration of semen through columns prepared in different buffers as compared to non-filtered semen. A significant ($p<0.05$) decrease in the sperm concentration, percent abnormal sperms and percent sperms with damaged acrosome was obtained after filtration of semen through sephadex columns using different buffers (sodium citrate, TES and Tris) as compared to non-filtered semen. Among the different buffers, Tris buffer improved above mentioned seminal parameters significantly ($p<0.05$) and hence it was used in the second trial.

In second trial, three grades of sephadex (G-15, G-100 and G-200) and Tris buffer was used as medium for

Table 1. Micro and macroscopic seminal characteristics of Murrah buffalo bulls (Mean±SE)

Seminal attributes	Bull No.				
	11 (20)	815 (20)	1,082 (20)	1,085 (20)	Over all (80)
Semen vol. (ml)	3.13^{b}±0.88	4.16^{a}±0.93	2.95^{b}±0.93	3.11^{b}±1.09	3.34±0.25
Consistency (0-3 d)	2.09^{b}±0.23	2.46^{a}±0.21	1.58^{c}±0.12	2.39^{a}±0.17	2.13±0.20
Sperm con. ($\times10^{6}$/ml)	785.5^{c}±40.23	1131.5^{a}±30.29	726.7^{c}±34.32	995.0^{b}±40.39	909.7±69.39
Mass activity (0-5)	2.55^{b}±0. 13	3.73^{a}±0.17	2.40^{b}±0.21	3.25^{a}±0.23	2.98±0.22
Prog. motile sperm (%)	50.75^{c}±2.22	74.00^{a}±0.82	46.00^{c}±3.29	68.5^{b}±2.65	59.81±3.53
Live sperm (%)	64.83^{b}±3.77	78.56^{a}±0.47	56.19^{c}±3.42	75.37^{a}±1.49	68.73±5.11
Abnormal sperm (%)	16.79^{bc}±1.50	17.62^{b}±0.46	21.74^{a}±0.43	14.85^{c}±1.65	17.75±1.45
Damaged acrosome (%)	13.45^{a}±1.56	13.82^{a}±1.95	12.47^{a}±1.87	11.52^{a}±1.51	12.81±0.52

Figure in parentheses indicate number of semen ejaculates.
^{a,b,c} Means with different superscript in same row differ significantly ($p<0.01$).

Table 2. Influence of buffer medium on filtration of Buffalo bull semen

Seminal attributes	Control	Sodium citrate	TES	Tris citric acid
Sperm con. ($\times10^{6}$/ml)	969.00^{a}±35.94	742.00^{c}±37.15	759.50^{bc}±37.36	773.80^{b}±36.67
Prog. motile sperm (%)	62.00^{c}±2.63	73.50^{b}±1.50	77.50^{ab}±1.52	78.50^{a}±1.41
Live sperm (%)	72.21^{c}±2.02	81.19^{b}±1.36	84.31^{ab}±1.17	87.55^{a}±1.36
Abnormal sperm (%)	17.44^{a}±1.12	10.94^{b}±0.87	8.16^{bc}±0.63	7.48^{c}±0.66
Damaged acrosome (%)	8.62^{a}±0.52	5.73^{b}±0.29	4.86^{b}±0.53	4.70^{b}±0.29

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

sephadex soaking and for preparation of columns of one cm height. The mean values (±SE) for the sperm count, percent progressive motility, percent live, percent abnormal sperm and percent sperm with damaged acrosomes of non-filtered and filtered semen are presented in Table 3. The percent progressive sperm motility and percent live sperms improved significantly ($p<0.05$) in the filtrate obtained from sephadex G-15, G-100 and G-200 columns as compared to non-filtered semen. No significant difference among the different grades of sephadex was obtained for percent progressive sperm motility as well as percent live sperms. There was a significant ($p<0.05$) decrease in the sperm concentration, percent abnormal sperms and percent damaged acrosomes in the filtrates of G-15, G-100 and G-200 columns as compared to non-filtered semen. Among the different sephadex grades the filtrate of sephadex G-100 had significantly ($p<0.05$) higher sperm numbers than sephadex G-200 and G-15 grades. The comparative seminal characteristics were better in the filtrate of sephadex G-100 column followed by G-200 and G-15 columns. In the third final trial filtration of semen was carried out through columns of different height (one, two and three cm) using sephadex G-100 and Tris buffer. The mean and SE of seminal attributes obtained after filtration of semen are presented in Table 4. There was a significant ($p<0.05$) increase in the percent progressive sperm motility and percent live sperm and significant ($p<0.05$) decrease in sperm concentration, percent sperm abnormality and percent sperm with damaged acrosomes in the filtrate of all the column as compared to non-filtered semen. The decrease in the sperm concentration after filtration of semen through the column of one cm height was from 855.5 to 703.50 million/ml, abnormal spermatozoa from 19.08 to 8.85% and sperm with damaged acrosomes was from 14.85 to 4.99% and increased in the progressive sperm motility from 58.20 to 82.75% and live sperm from 70.23 to 89.75% respectively. Among the different column size, the filtrate of one cm column height had significantly ($p<0.05$) higher percent progressive motility, percent live sperms and sperm concentration and lower ($p<0.05$) percent abnormal sperms as compared to two and three cm column height. However, no significant difference was obtained for percent damaged acrosomes among the filtrate of different height (one, two and three cm) columns. Although there was decrease of 17.77 to 29.34 percent in sperm concentration but it was compensated with increase in progressive sperm motility and decrease in sperm abnormality and damaged acrosomes.

DISCUSSION

The seminal characteristics obtained in the present study were within the range as also reported by (Sengupta et al., 1963; Raizada, 1979; Dhami et al., 1992; Goyal, 1993; Rao et al., 1993; Panghal, 1996; Gupta et al., 1997; Prajapati et al., 1998; Younis et al., 1998). The variation in percent progressive sperm motility 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, health of animals and the pre-ejaculatory stimulus.

The increase in percent progressive sperm motility and percent live spermatozoa and decrease in sperm concentration, percent abnormal sperms and percent damaged acrosome was noticed after filtration of buffalo

Table 3. Influence of different sephadex grades on filtration of Buffalo bull semen

Seminal attributes	Control	G-15	G-100	G-200
Sperm con. ($\times10^6$/ml)	$957.5^{a}\pm54.56$	$724.50^{c}\pm51.30$	$779.00^{b}\pm53.59$	$772.50^{b}\pm54.66$
Prog. motile sperm (%)	$57.50^{b}\pm3.92$	$71.30^{a}\pm3.41$	$77.00^{a}\pm2.91$	$73.30^{a}\pm3.12$
Live sperm (%)	$64.96^{b}\pm3.63$	$76.29^{a}\pm3.11$	$82.63^{a}\pm2.27$	$78.06^{a}\pm2.98$
Abnormal sperm (%)	$18.18^{a}\pm2.98$	$11.75^{b}\pm1.55$	$10.11^{b}\pm1.23$	$10.33^{b}\pm0.99$
Damaged acrosome (%)	$13.01^{a}\pm1.11$	$8.81^{b}\pm0.85$	$6.22^{b}\pm0.73$	$6.88^{b}\pm0.81$

[a,b,c] Means with different superscript in same row differ significantly ($p<0.05$).

Table 4. Influence of different column heights on filtration of Buffalo bull semen

Seminal attributes	Control	1 cm	2 cm	3 cm
Sperm con. ($\times10^6$/ml)	$855.50^{a}\pm33.59$	$703.50^{b}\pm34.61$	$657.00^{bc}\pm33.91$	$604.50^{c}\pm31.88$
Prog. motile sperm (%)	$58.30^{c}\pm4.09$	$82.75^{a}\pm2.97$	$75.25^{b}\pm3.25$	$72.00^{b}\pm3.27$
Live sperm (%)	$70.23^{c}\pm2.79$	$89.75^{a}\pm2.19$	$81.25^{b}\pm2.38$	$78.34^{b}\pm2.51$
Abnormal sperm (%)	$19.08^{a}\pm3.29$	$8.85^{b}\pm2.67$	$11.43^{b}\pm2.67$	$11.23^{b}\pm3.00$
Damaged acrosome (%)	$14.85^{a}\pm1.10$	$4.99^{b}\pm0.39$	$5.76^{b}\pm0.34$	$6.05^{b}\pm1.24$

[a,b,c] Means with different superscript in same row differ significantly ($p<0.05$).

semen through sephadex column using different buffers in the present investigation. The retention of sperm in the sephadex column may be due to interaction between buffers and sephadex beads and between buffers and abnormal spermatozoa and cells with damaged acrosomal membrane. In this context Heuer and Tahir (1982), Kumar (1989) and Chauhan et al. (1993) also observed the same results.

From results it is clear that G-100 grade of sephadex had better power of separation of immotile, dead and damaged acrosomes bearing spermatozoa as compared to sephadex G-15 and G-200 grades. It could be due to firm nature, beads diameter and swelling capacity of different grades of sephadex. The significant ($p<0.05$) increase in percent progressive sperm motility, percent live sperm and decrease in sperm concentration, percent sperm abnormality and percent damaged acrosomes over the control in the semen filtered through different grades of sephadex column support the observations of Chauhan et al. (1993) Goyal et al. (1996) Panghal and Tuli (1999) and Kumar et al. (1999). 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 cells incubated in *in vitro* capacitation media (Samper and Crabo, 1993). On other hand, bovine spermatozoa must have normal acrosome to pass through the sephadex column.

Results of the present investigation revealed that height of sephadex column plays a significant role in retaining the dead, abnormal, non motile and sperm with damaged acrosomes in the column. Increased height of column causes a deleterious effect on the progressive sperm motility because sperm has to travel a long distance and they are suppose to impede through zig-zag path formed by beads of sephadex and it leads to increased abnormalities and decline in the motility of spermatozoa. In addition to this in long sephadex column the recovery rate of semen is also poor and this leads to a significant decrease in the sperm concentration in the filtrate. The above discrepancies may be avoided by lowering the height of sephadex column as reflected in the present investigation. In accordance to our findings Heuer and Tahir (1982) Chinnaiya et al. (1988) Kumar (1989) and Vyas et al. (1991) reported the similar results.

Filtration of fresh buffalo semen through 1 cm column of sephadex G-100 grade prepared in Tris buffer preferentially trapped more dead, non-motile and abnormal sperm as well as sperm having damaged acrosomes. 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. The sephadex filtration of bull and stallion semen also had similar improvement in progressive sperm motility, live sperms and decline in sperm concentration, damaged acrosomes and abnormal sperms (Spreckels, 1994; Vyas et al., 1991; Vyas et al., 1992).

The quality determination of sperm cells by means of sephadex filter test is a promising alternative to other conventional semen assay. This test is highly correlated to microscopic counts of spermatozoa having normal acrosomal morphology and live counts of freshly collected semen (Heuer and Tahir, 1982). Roberts (1972) was of the opinion that spermatozoan's own progressive sperm motility was responsible for separation of weak/ non motile sperm through filtration. Many researchers have reported promising relationship between fertility and percent progressive sperm motility (Linford et al., 1976). The intactness of acrosome has a significant relationship with fertility (Saacke, 1970). 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. It is possible to speculate that the presence of a similar mechanism could form basis of sperm and sephadex interaction in Murrah buffalo bull semen.

So in the present study percent progressive sperm motility increased and percent sperm abnormalities, percent sperm with damaged acrosomes decreased after filtration, which is advantageous for fertilization. The present finding also support theories of separation of normal active motile spermatozoa using sephadex column. In these experiments after filtration there was decrease in the sperm concentration as compared to non-filtered semen. This decrease was compensated by proportionate increase in the percentage of live, motile and morphologically active sperm which inturn could lead to improved conception rate through AI with filtered semen. So, the positive effect of buffer, sephadex grade and column height was strong enough to overlap the positive effect of these treatments separately.

In nutshell our results showed that filtration can be an effective tool for selecting quality of Murrah buffalo bull spermatozoa from fresh ejaculates. The next step will consist of determining the effectiveness of sephadex filtration techniques in improving the ejaculates in order to increase the post thaw quality of frozen buffalo bull semen.

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REFERENCES

Anzar, M. and E. F. Graham. 1993. Filtration of bovine semen. I. Development of a sephadex ion-exchange filter. Anim. Reprod. Sci. 31:187-195.

Anzar, M. and E. F. Graham. 1995. Role of sperm motility and acrosomal integrity in the filtration of bovine semen. Theriogenology 43:513-520.

Anzar, M., E. F. Graham and N. Iqbal. 1997. Post thaw plasma membrane integrity of bull spermatozoa separated with sephadex column. Theriogenology 47:845-856.

Campbell, R. C., J. L. Hancock and I. G. Shaw. 1960. Cytological characteristics and fertilizing capacity of bull spermatozoa. J. Agric. Sci. 55:91-99.

Chauhan, S. S., G. Mohan, S. Kumar and K. L. Sahni. 1993. Comparative evaluation of various grades of sephadex for improving the quality of buffalo semen. Indian. J. Anim. Sci. 63:246-250.

Chinnaiya, G. P., P. A. Sarma and O. B. I. Reddy. 1988. Standardization of semen filtration technique through different media to improve its quality. J. Anim. Reprod. 10:56-60.

Dhami, A. J., V. R. Jani, K. L. Sahni and G. Mohan. 1995. Freezability and fertility of heterospermic semen of Friesian and Murrah bulls using Tris and milk extenders. Indian Vet. J. 72:1273-1276.

Dhami, A. J., K. L. Sahni and G. Mohan. 1992. Effect of various cooling rates (from 30°C to 5°C) and thawing temperatures on the deep freezing of *Bos Taurus* and *Bos Bubalis* semen. Theriogenology 38:565-574.

FAO. 1999. Quarterly Bulletin of Statistics. 12(3/4):145.

Goyal, R. L. 1993. Effect of separation procedures on freezability of buffalo spermatozoa. Ph.D. Thesis, Chaudhary Charan Singh Haryana Agricultural University, Hisar (India).

Goyal, R. L., R. K. Tuli, G. C. Georgie and D. Chand. 1996. Comparison of quality and freezability of water buffalo semen after washing or sephadex filtration. Theriogenology 46:679-686.

Graham, E. F. and J. K. Graham. 1990. The effect of whole ejaculate filtration on the morphology and fertility of bovine semen. J. Dairy Sci. 73:91-97.

Graham, E. F., M. K. L. Schmehl and B. K. Evensen. 1978. An over view of column separation of spermatozoa. Proc. of the 7th NAAB Technical Conf. on Artificial Insemination and Reproduction, 13-15 April, Madision, WI, National Association of Animal Breeders, Columbia, MO, pp. 69-73.

Gupta, H. P., K. L. Sahni and G. Mohan. 1997. Physico-chemical characterization of epididymal semen of buffalo bulls. Indian. J. Anim. Res. 31:111-114.

Heuer, C. and M. N. Tahir. 1982. Experiment on deep freeze preservation of water buffalo semen using sephadex filtration as semen assay. Proc. Buffalo Seminar on Reproduction and Meat Production Tanuku: pp. 55-63.

Kaura, R. L. 1961. Indian breeds of livestock. Prem publishers, Golaganj, Lucknow. pp. 40-41.

Kumar, N. 1989. Studies on filtration through different media and freezing of buffalo bull semen in certain extenders. M. V. Sc. Thesis, Punjab Agricultural University, Ludhiana, India.

Kumar, N., G. R. Pangawkar, R. K. Sharma and J. S. Matharoo. 1999. Effect of sephadex filtration of buffalo bull semen on freezability and sperm penetration into homologous cervical mucus. Indian J. Anim. Sci. 69:91-93.

Landa, C. A., J. O. Almquist and R. P. Amann. 1980. Factors influencing sephadex separation of bovine and ovine spermatozoa. J. Dairy Sci. 63:277-282.

Linford, E., F. A. Glover, C. Bishop and D. L. Stewart. 1976. The relationship between semen evaluating methods and fertility in the bulls. J. Reprod. Fertil. 47:283-291.

Maki-Laurila, M. and E. F. Graham. 1968. Separation of dead and live spermatozoa in bovine semen. J. Dairy Sci. 51:965.

Mogas, T., T. Rigau, J. Piedrafita, S. Bonet and J. E. Rodriguez-Gil. 1998. Effect of column filtration upon the quality parameters of fresh dog semen. Theriogenology 50:1171-1189.

Mustafa, G., M. Anzar and M. Arslan. 1998. Separation of motile spermatozoa from frozen thawed buffalo semen: swim-up Vs filtration procedure. Theriogenology 50:205-211.

Norusis, M. J. 1988.SPSS/PC Student ware, SPSS Inc. Chicago, Illinois (USA).

Panghal, V. S. 1996. Freezability of buffalo semen after filtration through sephadex gel and glass wool columns. M. Sc. Thesis, Chaudhary Charan Singh Haryana Agricultural University, Hisar, India.

Panghal, V. S. and R. K. Tuli. 1999. Effect of filtration of Murrah buffalo semen on its freezability and seminal characters. Indian J. Dairy Sci. 52:233-236.

Prajapati, K. B., P. K. Nagpaul, M. K. Chauhan, D. V. Singh, T. K. Walli and V. S. Raina. 1998. Influence of exercise and by pass protein feeding on seminal attributes of Mehsana buffalo bulls. Indian J. Anim. Prod. Mgmt.14:10-15.

Raizada, S. 1979. Fertility of cattle and buffalo bulls vis-à-vis acrosomal abnormalities and rate of hyaluronidase release. M.Sc. Thesis, Punjab Agricultural University, Ludhiana, India.

Rao, A. V. N., G. B. Harnath and G. Somasekharam. 1993. Revivability and acrosomal integrity of buffalo spermatozoa deep frozen in straws using different equilibration and thaw rates. Indian J. Anim. Res.14:44-47.

Roberts, A. M. 1972. Gravitational separation of X- and Y-spermatozoa. Nature 238:223-225.

Saacke, R. G. 1970. Morphology of sperm and its relationship to fertility. Proc. NAAB III Tech, Conf. Artif. Insem. Reprod. pp. 17-30.

Samper, J. C. and B. G. Crabo. 1993. Assay of capacitated, freeze damaged and extended stallion spermatozoa by filtration. Theriogenology 39:1209-1220.

Sengupta, B. P., M. S. Mishra and A. Roy. 1963. Climatic environment and reproductive behaviour of buffaloes. I. Effect of different seasons on various seminal attributes. Indian J. Dairy Sci. 16:150-165.

Shannon, P. and B. Curson. 1972. Toxic effect and action of dead sperm on diluted semen. J. Dairy Sci. 55:615-620.

Spreckels, I. 1994. Investigations on the selection of spermatozoa by glass wool sephadex filtration of stallion semen with special reference to cryopreservation. Ph. D. Thesis University of Hannover, Germany.

Vyas, S., A. J. Dhami, G. Mohan and K. L. Sahni. 1991. Effect of sephadex and glass wool column filtration on quality and

storage (at 5°C) of crossbred bull semen. Indian J. Anim. Sci. 61:702-704.

Vyas, S., G. Mohan, A. J. Dhami and K. L. Sahni. 1992. Comparative evaluation of the different filtration techniques for improving the semen quality of cross bred bulls. Indian J. Dairy Sci. 45:237-240.

Watson, P. F. and I. C. A. Martin. 1972. A comparison of change in the acrosomes of deep-frozen ram and bull spermatozoa. J. Reprod. Fertil. 28:99-102.

Younis, M., H. A. Samad, N. Ahmad and I. Ahmad. 1998. Studies on semen quality of young, adult and old buffalo bulls during low and peak breeding seasons. Pakistan Vet. J. 18:134-140.