

## EFFECT OF AGE AND BREEDING SEASON ON THE FREEZABILITY OF BUFFALO BULL SEMEN

M. Younis, H. A. Samad, Nazir Ahmad and C. S. Ali

Faculty of Veterinary Science, University of Agriculture, Faisalabad-38040, Pakistan

### ABSTRACT

The freezability of semen collected during the low (May-July) and the peak (September-November) breeding seasons from 18 Nili-Ravi buffalo bulls of young (3-4 years), adult (6-8 years) and old (12-15 years) age groups was studied. After-extension in lactose-fructose-egg-yolk-glycerol extender, semen samples were frozen and stored in liquid nitrogen. Post-thaw motility and liveability of spermatozoa were higher ( $p < 0.05$ ) in adult than in young and old bulls and during the peak than the low breeding season. Sperm abnormalities in frozen-thawed semen from bulls of young, adult and old groups differed significantly ( $p < 0.05$ ), and were higher ( $p < 0.05$ ) during the low than the peak breeding season. GOT and GPT activities in fresh, diluted and frozen-thawed seminal plasma from young and old bulls were higher ( $P < 0.05$ ) than that of adults. GOT and GPT activities in seminal plasma of fresh, diluted and frozen-thawed semen were higher ( $p < 0.05$ ) in the low than the peak breeding season. Due to freezing, GOT activity in seminal plasma of young, adult and old bulls increased by 29.80, 21.12 and 38.15%, respectively. The corresponding values for GPT activity were 16.22, 16.33 and 40.74%. It was concluded that the semen freezability was better in adult buffalo bulls than young or old bulls, and during the peak than the low breeding season.

### INTRODUCTION

The buffalo is a major dairy animal in Pakistan. According to a recent survey (Anonymous, 1996), there are approximately 20.2 million heads of buffaloes in the country. Most of these animals are kept in the central and northeast areas of Punjab. In this area, May to July is the hottest dry period of the year when green fodder is scarce and breeding activity of the female buffaloes is minimal. This period is usually termed as the low breeding season for the buffaloes. Most of the calvings in this species take place in July and August with the maximum breeding activity during the following September-November (Pedersen, 1989), which is the peak breeding season for females of this species.

Glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) enzymes are located in the mid-piece of spermatozoa and are concerned with their oxidative metabolism (Dhami and Kodagali, 1991). An increase in the activity of these enzymes in the seminal plasma is usually used as an indicator of sperm cell membrane damage caused during freezing (Bhosrekar *et al.*, 1991).

In a recent study on Nili-Ravi buffalo bulls kept under Pakistani climatic conditions, Younis *et al.* (1998)

recorded better quality for ejaculates collected during the peak than those collected during the low breeding season. Likewise, semen collected from young (3-4 years) and adult (6-8 years) bulls was of better quality than that collected from old (12-15 years) bulls. Bhosrekar *et al.* (1992) found that the percentage of motile spermatozoa in semen samples was higher in bulls of 3-10 years of age and declined thereafter. It is possible that these seasonal and age-related changes in the semen quality can affect its freezability.

However, little information is available regarding the freezability of semen collected from buffalo bulls of varying ages during the low and the peak breeding seasons in Pakistan. The present study was, therefore, carried out to determine the freezability of semen collected during low and peak breeding seasons from young, adult and old buffalo bulls using post-thaw motility, liveability, sperm morphology and extra-cellular release of GOT and GPT enzymes as semen assays.

### MATERIALS AND METHODS

#### Experimental Animals:

Eighteen clinically normal Nili-Ravi buffalo bulls were divided into 3 groups viz. young (3-4 years), adult

(6-8 years) and old (12-15 years), with six animals in each group.

#### Feeding and Management:

The experimental animals were kept under the naturally prevailing climatic conditions at the Semen Production Unit, Qadirabad, District Sahiwal. This study was carried out during low (May to July, 1995) and peak (September to November, 1995) breeding seasons of the female buffaloes.

The experimental bulls were housed individually in pens with open space, having sufficient cross ventilation and protection against heat during summer. Each bull was fed 35 to 65 kg of good quality seasonal green fodder daily. In addition, two to three kg of concentrate was offered per bull per day.

#### Semen Collection, Processing and Freezing:

Semen (two ejaculates) from each buffalo bull was collected once a week using an artificial vagina. Immediately after collection, the two ejaculates were pooled and evaluated at 37°C for sperm cell concentration and percentage of individually motile spermatozoa (Nazir, 1988). Samples with at least 60% motile spermatozoa were selected for processing.

Selected samples were diluted at 30°C by slow and one step dilution method (Nazir, 1988), using lactose-fructose-egg-yolk-glycerol extender, with a final concentration of 30 million spermatozoa per 0.5 ml and were filled in 0.5 ml french straws. After an equilibration period of six hours at 5°C, freezing of straws was performed in a wide mouth freezing chamber containing liquid nitrogen. A wire net, temperature of which was lowered down by dipping it into liquid nitrogen for a while, was used for holding the straws in vapours of liquid nitrogen 5cm above the surface of liquid nitrogen. The freezing process was completed within 8 minutes (Ahmad *et al.*, 1980). Then the wire net containing the straws was gradually lowered down into liquid nitrogen and stored for at least 24 hours.

#### Evaluation of Frozen Semen:

Frozen straws were thawed at 37°C for 15-20 sec and examined for post-thaw motility, liveability and sperm morphology. In order to study the liveability of spermatozoa, the thawed samples were stored at 37°C and examined for sperm motility every hour till the death of all spermatozoa.

The sperm morphology was studied by the eosin-nigrosin staining technique. For this purpose, one drop of frozen-thawed semen was mixed with 4-5 drops of eosin-nigrosin stain and incubated at 37°C for one

minute. A thin smear was then made on a clean microscopic slide, dried in air at room temperature and examined under oil immersion by using phase contrast microscopy (X 1000). Two slides were made for each sample and 100 spermatozoa were examined for their morphology in random fields per slide. Then the percentage of morphologically abnormal spermatozoa was assessed (Jainudeen *et al.*, 1982).

#### Estimation of Transaminase Activities:

The extracellular GOT and GPT activities were measured in fresh, diluted and frozen-thawed semen. For this purpose, the respective semen samples were centrifuged at 3000 rpm for 10 minutes and the supernatant was used for enzyme assay by photometric method, recommended by the International Federation of Clinical Chemistry (Anonymous, 1980), using Randox GOT and GPT Kits.

Each kit consisted of a 100ml vial containing substrate and 10 vials containing reagent powder. To reconstitute, 10ml of the substrate was added into a reagent vial containing reagent powder, mixed gently and stored at 4°C for use within 48 hours. A 500ul of this mixture was poured into a 3ml vial to which 50ul of seminal plasma was added. The final mixture was incubated in a water bath at 37°C for one minute and fed to the photometer (Microlab, Merk). The photometric reading gave the enzyme activity in units per litre of seminal plasma.

#### Statistical analysis:

In order to see the magnitude of variation in attributes of frozen-thawed semen and conception rates among bulls of three age groups and the two seasons, the data were subjected to analysis of variance, using factorial experiment under completely randomized design (Snedecor and Cochran, 1989). The factors included were season and age for the motility and liveability and season, age and fractions of the semen (fresh, diluted and frozen-thawed) for GOT and GPT activities. Duncan's Multiple Range test (Duncan, 1955) was applied for multiple mean comparisons, where the analysis of variance revealed a significant effect.

## RESULTS AND DISCUSSION

#### Post-Thaw Motility:

In this study, the overall post-thaw motility averaged  $41.81 \pm 0.49\%$ . Similar post-thaw motility of 40.34-44.23% was recorded by Chaudhry (1971) and Heuer (1981). However, Chaudhry (1989) and

Chinnaiya and Ganguli (1990) reported lower values (34-38%) whereas Bhosrekar et al. (1992) reported relatively higher post-thaw motility (49.45%) than the present study. These variations can be attributed to differences in the composition of extenders and the methods used for deep freezing and thawing of semen by these workers.

Adult buffalo bulls had higher ( $P < 0.05$ ) post-thaw motility than young and old bulls, whereas difference between the latter two groups was non-significant (Table 1). The semen collected during the peak breeding season yielded higher ( $P < 0.05$ ) post-thaw motility than that collected during the low breeding season (Table 1). Nazir (1988) and Chinnaiya and Ganguli (1990) made similar observations on frozen-thawed semen of buffalo bulls. According to Heuer (1981), post-thaw motility of spermatozoa differed non significantly during the low and the peak breeding seasons, if the bulls were kept under improved managerial practices.

Table 1: Post-thaw motility, liveability and abnormal spermatozoa in frozen-thawed semen during the low and the peak breeding seasons (means  $\pm$  SEM)

| Age groups                      | Low breeding season | Peak breeding season | Overall mean      |
|---------------------------------|---------------------|----------------------|-------------------|
| <b>Post-thaw motility (%)</b>   |                     |                      |                   |
| Young                           | 39.72 $\pm$ 0.86    | 41.94 $\pm$ 1.08     | 40.83 $\pm$ 0.70b |
| Adult                           | 42.50 $\pm$ 0.83    | 48.06 $\pm$ 0.82     | 45.27 $\pm$ 0.74a |
| Old                             | 38.61 $\pm$ 0.89    | 40.00 $\pm$ 1.21     | 39.30 $\pm$ 0.75b |
| Mean                            | 40.28 $\pm$ 0.54B   | 43.33 $\pm$ 0.76A    | 41.81 $\pm$ 0.49  |
| <b>Liveability (hours)</b>      |                     |                      |                   |
| Young                           | 4.07 $\pm$ 0.20     | 5.69 $\pm$ 0.19      | 4.86 $\pm$ 0.20b  |
| Adult                           | 5.10 $\pm$ 0.13     | 6.27 $\pm$ 0.60      | 5.60 $\pm$ 0.12a  |
| Old                             | 3.86 $\pm$ 0.23     | 5.12 $\pm$ 0.22      | 4.73 $\pm$ 0.21b  |
| Mean                            | 4.34 $\pm$ 0.13B    | 5.73 $\pm$ 0.13A     | 5.04 $\pm$ 0.11   |
| <b>Abnormal spermatozoa (%)</b> |                     |                      |                   |
| Young                           | 37.56 $\pm$ 1.43    | 29.98 $\pm$ 3.69     | 33.77 $\pm$ 1.05a |
| Adult                           | 31.14 $\pm$ 0.88    | 23.69 $\pm$ 0.76     | 27.42 $\pm$ 0.85b |
| Old                             | 38.18 $\pm$ 1.38    | 34.56 $\pm$ 3.89     | 36.36 $\pm$ 0.87a |
| Mean                            | 35.63 $\pm$ 0.85A   | 29.41 $\pm$ 0.76B    | 32.43 $\pm$ 0.53  |

Values with different lower case letters in a column and upper case letters in a row for each parameter differ significantly ( $p < 0.05$ ).

#### Liveability of Spermatozoa:

In the present study, the overall liveability of frozen-thawed spermatozoa stored at 37°C averaged 5.04  $\pm$  0.11 hours. Rao et al. (1986), Nazir (1988) and Bhosrekar et al. (1992) reported similar findings of sperm liveability ranging from 4.27 to 6.13 hours in

frozen-thawed buffalo semen. According to Khokhar et al. (1987), the liveability of buffalo spermatozoa was 3.46 hours which is less than that reported in the current study. The difference might be due to variations in the methods used for handling of semen.

Spermatozoa collected from adult buffalo bulls had higher ( $P < 0.05$ ) liveability than young and old bulls, the difference between the latter two groups was non-significant. Higher ( $P < 0.05$ ) liveability of spermatozoa was recorded during the peak than the low breeding season (Table 1).

#### Sperm Morphology:

In the present study, the overall percentage of abnormal spermatozoa in frozen-thawed semen averaged 32.43  $\pm$  0.53. Previous studies have shown that abnormal spermatozoa in frozen-thawed semen varied from 25.5 to 44.4% (Rao et al., 1986; Khokhar et al., 1987).

The incidence of abnormal spermatozoa in frozen-thawed semen was higher ( $P < 0.05$ ) in old than in young or adult bulls, whereas the difference between bulls of the latter two groups was non-significant (Table 1). In the low breeding season, there was higher percentage of abnormal spermatozoa ( $P < 0.05$ ) than the peak breeding season (Table 1). Higher percentage of sperm defects including micro, swollen and giant heads are associated with poor fertility (Heuer et al., 1987). The same holds true for various acrosomal abnormalities.

In the present study, the overall percentage of abnormal spermatozoa in frozen-thawed semen averaged 32.43  $\pm$  0.53. Pre-freeze evaluation of these semen samples revealed an average of 12.97  $\pm$  0.48% abnormal spermatozoa (Younis et al., 1998). Thus, there was an increase of 150% in the sperm abnormalities due to freezing. This highlights the importance of improving the existing technique used for freezing of buffalo bull semen in the country.

In the present study, the post-thaw quality of semen samples collected from adult buffalo bulls was better in terms of post-thaw motility, liveability and sperm morphology than those collected from young or old bulls. This might have been due to the fact that the freshly collected ejaculates from the same bulls were also of better quality in adult than in old animals in terms of initial sperm motility and live and morphologically normal spermatozoa (Younis et al., 1998). Bhosrekar et al. (1992) found that the percentage of motile spermatozoa in semen samples was higher in bulls of 3-10 years of age and declined thereafter. Age related changes in the genital tract e.g. testicular degeneration and fibrosis of seminiferous tubules may be blamed for

poor quality of semen in old animals. Furthermore, it appears that ejaculates showing better pre-freeze quality also prove better at post-thaw evaluation.

Table 2: GOT and GPT activities (units/litre) in fresh, diluted and frozen-thawed seminal plasma for the low and the peak breeding seasons (mean  $\pm$  SEM).

| Age groups               | Low breeding season | Peak breeding season | Overall mean        |
|--------------------------|---------------------|----------------------|---------------------|
| <b>GOT (units/liter)</b> |                     |                      |                     |
| <b>Fresh</b>             |                     |                      |                     |
| Young                    | 267.00 $\pm$ 29.78  | 204.44 $\pm$ 12.21   | 235.72 $\pm$ 16.72b |
| Adult                    | 263.89 $\pm$ 13.43  | 187.00 $\pm$ 6.06    | 225.44 $\pm$ 9.74b  |
| Old                      | 318.61 $\pm$ 37.14  | 228.67 $\pm$ 21.13   | 273.64 $\pm$ 22.39a |
| Mean                     | 283.17 $\pm$ 16.54A | 206.70 $\pm$ 8.55B   | 244.94 $\pm$ 9.97   |
| <b>Diluted</b>           |                     |                      |                     |
| Young                    | 289.61 $\pm$ 29.61  | 241.72 $\pm$ 11.56   | 265.67 $\pm$ 16.18a |
| Adult                    | 284.83 $\pm$ 14.31  | 217.28 $\pm$ 6.37    | 251.06 $\pm$ 9.60b  |
| Old                      | 339.67 $\pm$ 37.91  | 262.94 $\pm$ 22.67   | 301.31 $\pm$ 22.71a |
| Mean                     | 304.70 $\pm$ 16.76A | 240.65 $\pm$ 8.95B   | 272.68 $\pm$ 13.53  |
| <b>Frozen</b>            |                     |                      |                     |
| Young                    | 327.33 $\pm$ 32.53  | 284.61 $\pm$ 13.89   | 305.97 $\pm$ 17.80a |
| Adult                    | 303.00 $\pm$ 15.97  | 243.22 $\pm$ 7.09    | 273.11 $\pm$ 9.98b  |
| Old                      | 358.33 $\pm$ 38.58  | 297.72 $\pm$ 26.33   | 378.03 $\pm$ 23.58a |
| Mean                     | 329.56 $\pm$ 17.59A | 275.19 $\pm$ 10.50B  | 302.37 $\pm$ 10.53  |
| <b>GPT (units/liter)</b> |                     |                      |                     |
| <b>Fresh</b>             |                     |                      |                     |
| Young                    | 66.44 $\pm$ 8.80    | 36.44 $\pm$ 5.80     | 66.44 $\pm$ 5.78b   |
| Adult                    | 35.44 $\pm$ 1.93    | 17.17 $\pm$ 1.16     | 42.31 $\pm$ 1.90c   |
| Old                      | 93.00 $\pm$ 15.76   | 49.39 $\pm$ 5.96     | 71.19 $\pm$ 9.08a   |
| Mean                     | 64.96 $\pm$ 6.76A   | 34.33 $\pm$ 3.19B    | 49.65 $\pm$ 4.02    |
| <b>Diluted</b>           |                     |                      |                     |
| Young                    | 74.00 $\pm$ 5.80    | 48.94 $\pm$ 6.76     | 66.31 $\pm$ 6.63a   |
| Adult                    | 51.50 $\pm$ 2.99    | 32.67 $\pm$ 1.92     | 38.19 $\pm$ 2.80b   |
| Old                      | 110.00 $\pm$ 16.32  | 64.50 $\pm$ 7.15     | 87.25 $\pm$ 9.67a   |
| Mean                     | 97.06 $\pm$ 18.38A  | 46.11 $\pm$ 3.39B    | 71.58 $\pm$ 9.67    |
| <b>Frozen</b>            |                     |                      |                     |
| Young                    | 92.78 $\pm$ 19.25   | 67.67 $\pm$ 7.16     | 77.22 $\pm$ 6.33b   |
| Adult                    | 65.78 $\pm$ 3.80    | 32.67 $\pm$ 1.92     | 49.22 $\pm$ 3.50c   |
| Old                      | 123.78 $\pm$ 17.06  | 76.61 $\pm$ 7.75     | 100.19 $\pm$ 10.06a |
| Mean                     | 94.11 $\pm$ 7.24A   | 56.98 $\pm$ 4.33B    | 75.55 $\pm$ 4.56    |

Values with different lower case letters in a column and upper case letters in a row for each parameter differ significantly ( $p < 0.05$ ).

#### Transaminase Activities:

The mean GOT activity in the seminal plasma increased by 29.80, 21.12 and 38.15% in young, adult and old bulls respectively, due to freezing (Table 2). Likewise, the mean GPT activity in the seminal plasma

increased by 16.22% in young bulls, 16.33% in adult bulls and 40.74% in old bulls during freezing (Table 2). These findings are in agreement to those reported by Khokhar *et al.* (1987), who stated that GOT and GPT activities in frozen-thawed semen were maximum after freezing. Iqbal (1987) stated that most of the transaminase release occurred between equilibration and 24 hr after freezing.

In this study, higher activities of GOT and GPT were recorded in all three fractions of seminal plasma from young and old than adult bulls ( $P < 0.05$ ). Similarly, ejaculates collected during the low breeding season had higher GOT and GPT activities ( $P < 0.05$ ) than those collected during the peak breeding season (Table 2). This was true for fresh, diluted and frozen-thawed fractions of the seminal plasma.

Based on the findings of this study it can be concluded that the semen from adult bulls had better freezability than that from young or old bulls. Moreover, the semen collected during the peak breeding season showed higher post-thaw motility and liveability and lower sperm abnormalities than that collected during the low breeding season. However, these findings are based on *in vitro* studies. Before making any recommendation, conception rates in buffaloes inseminated with frozen-thawed semen collected from bulls of different ages during the low and the peak breeding seasons should be studied.

## REFERENCES

- Ahmad, K., R.A. Chaudhry, W. Ahmad and Ala-uddin, 1980. Deep-freezing of buffalo spermatozoa by straw method. *Pakistan J. Agric. Sci.*, 17: 1-2.
- Anonymous, 1980. International Federation of Clinical Chemistry, Scientific Committee. *J. Clin. Chem. Clin. Biochem.* 18: 521-534.
- Anonymous, 1996. Pakistan in Figures. Federal Bureau of Statistics Division, Government of Pakistan, Islamabad.
- Bhosrekar, M.R., S. Mokashi, J.R. Purohit, S.B. Gokhale, and B.R. Mangurkar, 1991. Studies on the effect of deep freezing and seasons on the leakage of aspartate amino transferase into extracellular medium and sperm morphology of Murrah buffalo bulls. *Anim. Reprod. Sci.*, 25: 219-226.
- Bhosrekar, M.R., J.R. Purohit, S.B. Gokhale, and B.R. Mangurkar, 1992. Semen characteristics and behavioral patterns of buffalo bulls in relation to age and seasons. *Indian J. Anim. Sci.*, 62: 251-255.

- Chaudhry, R.A., 1971. Deep freezing of buffalo semen. 9th FAO/Swedish Int. Post-graduate Vet. Course Anim. Reprod. Royal, Vet. College, Stockholm, Sweden.
- Chaudhry, M.S., 1989. To study the effect of buffers on the freezability of buffalo bull semen. M.Sc. Thesis, Univ. Agric. Faisalabad, Pakistan.
- Chinnaiya, G.P. and N.C. Ganguli, (1990). Effect of age and season on the initial attributes and preservability of buffalo bull semen. Proceed. II World Buffalo Congr. India, PP: 80-86.
- Dhami, A.J. and I. Kodagali, (1991). Freezability, fertility and spermatozoa losses in Surti buffalo bulls. Indian J. Anim. Res. 24: 39-43.
- Duncan, D.B., (1955). Multiple range and multiple F. tests. Biometrics, 11: 1-42.
- Heuer, C., 1981. Results of experiment on deep-freeze preservation of buffalo semen. Report No.12, G.T.Z Project No.74-2041-7.
- Heuer, C., M.N. Tahir and H. Amjad, 1987. Effect of season on fertility of frozen buffalo semen. Anim. Reprod. Sci., 13: 15-21.
- Iqbal, M., 1987. Effect of extenders on the extra cellular release of transaminases from deep frozen buffalo spermatozoa. Proc. Int. Symp. Milk Buffalo Reprod. Islamabad. pp.278-294.
- Jainudeen, M.R., T.A. Bangso and S. Dass, 1982. Semen characteristics of the Swamp buffalo (*Bubalus bubalis*). Anim. Reprod. Sci., 4: 213-217.
- Khokhar, B.S., M. Singh and K.C. Chaudhary, 1987. Transaminases in cattle and buffalo semen in relation to fertility and seminal characteristics during moderate and colder seasons. Anim. Reprod. Sci., 13: 177-182.
- Nazir, M., 1988. Semen evaluation and sperm morphology. Monograph on reproductive pattern of Riverine buffaloes and recommendations to improve their reproductive performance at small farmer level. Pakistan Agricultural research Council, Islamabad, Pakistan.
- Pedersen, C.H., 1989. Infertility and disease surveillance using a milk recording scheme in the Sahiwal District of Pakistan. Trop. Anim. Hlth. Prod., 21: 263-272.
- Rao, N., G.B. Haranath, G.S. Sekhoram, and J.R.M. Rao, 1986. Effect of thaw rates on motility, survival and acrosomal integrity of buffalo spermatozoa frozen in French straws. Anim. Reprod. Sci., 12: 123-129.
- Snedecor, G.W. and W.G. Cochran, 1989. Statistical Methods. 7th Edn. Iowa State University Press, Ames, pp. 359-364.
- 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.