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PRACTICAL TRIALS FOR FREEZING SEMEN OF BUFFALO AND FRIESIAN BULLS: Effect of various regimens of freezing, different milk extenders and types of straws packages on post-thawing semen characters

(With 5 Tables and 4 Figures)

By

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محاولات عملية لتجميد السائل المنوى الجاموسى والبقرى (تأثير نظم التجميد ونوع المخففات وكذلك نوع قصيبات التعبئة على صفات السائل المنوى بعد الاسالة)

كامل مصطفى ، مها زياده ، جمال مصطفى

تناول البحث دراسة تأثير أربعة أنواع من مخففات اللبن (البقرى والجاموسى ولبن الأغنام والماعز) مع دراسة خمس معدلات للتجميد أثناء فترتين (عشرة وخمسة عشر دقيقة) مختلفتين من التعرض لبخار النتروجين وتم أيضا دراسة تأثير نوعين مختلفتين من قصيبات التعبئة سعة ¼ مللى (قصيبات النظام الفرنسى وقصيبات النظام الألمانى). أستهدف البحث دراسة تأثير هذه المعاملات المختلفة على حركة وحيوية السائل المنوى الجاموسى والبقرى بعد التجميد والاسالة. أيضا تأثير هذه المعاملات على سلامة الحيامن والمستدل عليه من انزيم ال ALT و AST . بعد تجميع السائل المنوى الجاموسى والبقرى وتقييمه وتخفيفه واضافة الجليسرول وتركه عند درجة ٥ منوية لاستعادة توازنه ثم تعبئته فى القصيبات المختلفة وسعتها ٠,٢٥ مللى ثم تجميد القصيبات وماتحتويه من سائل منوى مخفف بالطريقة التقليدية وبطرق أخرى وهى وضع القصيبات على مسافات ٢ و٤ و٦ و٨ سم من سطح النتروجين السائل ويتم هذا فى صندوق من المطاط الأسفنجى. تترك القصيبات معرضة لبخار النتروجين لمدة ١٠ دقائق و ١٥ دقيقة. توصلت الدراسة الى أن أفضل النتائج (على وجه العموم) تحققت عند استخدام صندوق المطاط الاسفنجى مقارنة بالطريقة التقليدية المستخدم فيها تنك كبير من النتروجين. بالنسبة للسائل المنوى البقرى وجدنا أن أحسن نتائج للتجميد تحققت مع العينات التى تعرضت لمدة عشر دقائق على مسافة ٢ و ٤ سم من سطح النتروجين وكذلك التى تركت لمدة ١٥ دقيقة على مسافة ٢ و ٨ سم. أما بالنسبة للسائل المنوى الجاموسى وجد أن أحسن النتائج تم الحصول عليها عند التجميد على

مسافة ٢ و ٤ سم لمدة ١٠ دقائق وكذلك ٢ و ٦ سم لمدة ١٥ دقيقة. أعلى نسبة حركة وحيوية مع أقل نسبة تكسير فى قلنسوة الحيامن المستدل عليها بانزيم AST و ALT للسائل المنوى البقرى قد تحققت عند تعرض القصيبات لمدة ١٥ دقيقة لبخار النتروجين وكانت ٥٤,٩١ و ١١٢,٥٨ و ١١٦,٨٦ و ٦١,٧٣ على التوالى. أما الحيامن الجاموسى فكانت أعلى نسبة حركة ٣٤,٣٣ وأعلى حيوية ٦٧,٨٤ وأقل نسبة تكسير فى قلنسوة الحيامن المستدل عليها بانزيم AST هى ١١٦,٤٤ وقد تحققت أيضا عند تعرض القصيبات لمدة ١٥ دقيقة لبخار النتروجين . كما أظهرت الدراسة أن استخدام اللبن الجاموسى كمخفف للسائل المنوى الجاموسى والبقرى أفضل عن غيره من المخففات الأخرى المستخدمة فى الدراسة. ووجد من تقييم السائل المنوى البقرى بعد الاسالة أنه لايوجد اختلاف معنوى باختلاف نوع القصيبات المستخدمة (قصيبات النظام الفرنسى وقصيبات النظام الألمانى). كما أثبتت الدراسة وجود علاقة عكسية بين انزيمات AST و ALT , وبين نسبة حركة وحيوية الحيامن البقرى والجاموسى.

SUMMARY

In the present investigation, the influence of 4 types of milk extenders (cow, buffalo, sheep and goat milk), 2 exposure time to liquid nitrogen (LN) vapour (10 and 15 minutes), 5 freezing rates and 2 kinds of semen packages (0.25 ml ministraws and minitubes) on the motility and viability index of post-thawed cattle and buffalo semen. The released aspartate aminotransferase (AST) and alanine aminotransferase (ALT) into the extracellular of seminal plasma had been also estimated as an indicator of sperm cells membranes damage. After semen collection, evaluation, dilution, glycerolization and equilibration, the semen was loaded into 0.25 ml ministraws and minitubes. Packaged semen was frozen by conventional (control) and at 4 different distances (2, 4, 6 and 8 cm) from LN inside foam box, for 10 and 15 minutes as exposure time to LN vapour. Among both animal species (Friesian and buffalo bulls), most freezing regimens which applied in the field by using foam box produced better semen quality (motility, viability, AST and ALT) as compared to control. For Friesian semen, among all different types of freezing regimens, freezing at 2 and 4 cm above LN for 10 minutes and 2, 8 cm for 15 min were superior ($P<0.05$) to other regimens, while, in buffalo semen, freezing at 2 and 4 cm for 10 min and 2, 6 cm for 15 min gave the highest means of post-thawing motility, viability and lowest mean of AST level. The maximum means of post-thaw motility, viability with minimum acrosomal damage as indicated by low scores of AST and ALT were recorded as 54.91 ± 1.24, 112.58 ± 4.02, 116.86 ± 1.65 and 61.73 ± 1.66, respectively in Friesian semen and 34.33 ±

3.19, 67.84 ± 8.09 and 116.44 ± 5.84 in buffaloe semen when 15 min, as exposure time to semen was employed. Post-thawed semen quality in buffalo milk was significantly better in Friesian ($P<0.05$) and buffalo semen ($P<0.01$) than other milk diluents. However, buffalo milk gave good results with Friesian semen than Buffalo semen. The post-thawed semen quality was slightly similar for the two semen packages (straws and minitubes). Changes in AST and ALT were highly negative correlated with motility and viability of the post-thawed Friesian semen and buffalo semen, so, they were considered suitable for the objective measurement of spermatozoal damage and could be used as marker enzymes in the development of semen processing techniques and of semen diluents.

Key words: *Buffalo - Semen - Friesian - Bull semen - Freezing*

INTRODUCTION

The basic method for freezing spermatozoa have been considerable advances in the cryopreservation technology particularly in the freezing of bovine semen which used in AI. Buffalo spermatozoa are more sensitive to cold shock as well as osmotic shock than bull spermatozoa (Tuli et al, 1982 and Bhosrekar et al, 1990). So, systemic studies on buffalo semen are needed to understand the problems of freezing. However, the application of the technology in developing countries is facing a serious challenge. For conventional freezing techniques specific training and expensive equipment are necessary. These equipment vary in cost depending on how much automation is desired, the accuracy of cooling required and the temperature to which controlled cooling is needed. Hence, there is a need for modification and improvement a simple, suitable, economically viable and practical techniques for freezing and handling bovine (Yassen et al, 1985; Mathur et al, 1990; Dhami, 1992 and Dhami et al, 1992) and buffalo (Tuli et al, 1982 and Raizada et al, 1988) semen which would yield an acceptable level of post-thaw motility and fertility under tropical field conditions. Deep freezing over liquid nitrogen in static vapour in wide mouth container have been the accepted method for cryopreservation of bovine semen (Bhosrekar et al, 1994). Most studies on semen viability to date have used subjective motility measures to assess the effects of processing and of different diluents. Milk could be used as an efficient, inexpensive and readily available substitute for the costlier diluents in deep-freezing of bovine semen (Dhami

and Sahni , 1993). Motility may not necessarily be a good indicator of potential fertilizing ability of spermatozoa (Smith et al, 1993). When bovine (Graham and Pace, 1967; Pace and Graham, 1970 and Pangawkar et al, 1988), buffalo (Tuli et al, 1982) and porcine (Crabo et al, 1971) spermatozoa are frozen, cellular damage occurs which causes leakage of various enzymes such as alkaline phosphatase (AKP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactic dehydrogenase (LDH), hyaluronidase and acrosine as well as other materials (Salisbury et al, 1978). These enzymes are essential for metabolic processes which provide energy for survival, motility and fertility of spermatozoa and so, it play an important role in the processes of fertilization (McRorie and Williams, 1974). The estimation of these enzymes have been recommended as markers for semen quality, since, they indicate sperm damage during the process of freezing and thawing (Tuli et al, 1982; Jani, et al, 1983; Dhami and Kodagali, 1988; Dhami and Kodagali, 1990; Belorkar, et al, 1991 and Singh, et al 1992). Several investigators (Robbins, et al, 1976; Gilbert and Almguist, 1978; Jani, 1982; Pandit and Garg, 1983; Ahmed, 1984; Chandler, et al, 1984; Yassen, et al, 1985; Rao, et al, 1986; Sahni and Mohan, 1988; Ziada, 1989; Dhami, 1992; Belorkar et al, 1993) have evaluated the influence of various diluents, cooling rates, equilibration periods and freezing-thawing procedures on the freezability, acrosomal morphology, enzyme leakage and fertility but without constant results. Therefore, the present study was undertaken to investigate the comparative efficiency of different regimens of freezing in foam box and by conventional method as well as different types of milk extenders and semen packages on the post-thaw progressive motility, viability, AST and ALT release in frozen semen of Friesian and buffalo bulls, in order to determine a suitable diluent and an economically viable and practical protocol for freezing that would yield optimal freezability.

MATERIAL AND METHODS

Animals:

The present work was carried out over October 1996 to March 1997. The study utilized 5 Holstein-Friesian bulls, 2½ - 3½ years old, housed at Tonsy farm and 6 buffalo bulls, between ages 3-7 years, bred at ARRI. These animals maintained under identical nutrition and management conditions.

Semen diluents:

Four types of milk based diluents (cow, buffalo, sheep and goat) were used as extenders for semen dilution. The first two types of milk extenders Friesian were used for cow semen, while the last three types were used for buffalo semen. The milk was boiled and cooled by keeping in a refrigerator over night, fat layer was removed and this milk rewarmed in a close glass vessel at 92-95 °C in a water bath for 10-12 min., then cooled in refrigerator and the remaining fat was removed again and the milk was filtered through cotton gauze. Milk and fresh egg yolk were combined to obtained 80% milk and 20% egg yolk mixturing by volume. Antibiotics were added to diluents according to Ziada (1989). This diluent was splited into two fractions A&B. Glycerol was added to fraction 'B' to reach concentration 14% glycerol.

Semen collection and processing:-

Semen was collected, using an artificial vagina. Ejaculates with good wave motion and initial sperm motility not less than 70% were used for processing. Aliquots of pooled semen from bulls (for each species) were partially equally diluted at 30-35 °C using fraction A. The partially diluted semen was cooled to 5 °C within 45-60 min. Further dilution, using fraction 'A' was performed at 5 °C to a concentration of 12 x 10^7 sperm/ml. Glycerolization was carried out at 5 °C by addition of an equal volume of fraction B to the fraction A-diluted semen. The glycerolized semen (final concentration of 6 x 10^7 sperm/ml) was left at 5 °C for 2 hrs for equilibration then loaded into 0.25 ml PVC ministraws (IMV, L'Aigle, France) and 0.25 ml minitubes (Minitub GmBH, Tiefenbach, Germany) at 5 °C in a cold handling cabinet, by automatic packaging machine. Friesian bulls-diluted semen was packaged into both 0.25 ml ministraw and minitubes, while diluted buffalo semen was packaged only into 0.25 ml straws. The ministraws and the minitubes were then placed and arranged horizontally on freezing racks at 5 °C. After equilibration, 1/5 of the total packaged semen was frozen by conventional method (control) according to Ziada (1989). The remaining straws and minitubes were suspended in liquid nitrogen vapour inside an foam box container (54 x 35 x 18 cm, containing 10 liters of liquid nitrogen) at heights of 2, 4, 6 and 8 cm above the level of LN, for 10 and 15 min. The frozen straws and minitubes were stored in the liquid nitrogen for at least 72 hr. before thawing.

Semen evaluation:-

The frozen semen was thawed in a water bath at 37 °C for 30 seconds. The pre-freeze as well as the post-thaw motility was recorded using phase-contrast microscope fitted with a biotherm stage (37 °C). The thawed straws and minitubes were soon transferred to a water bath maintained at 37 °C, sperm motility was reassessed after 1, 2 and 3 hours of thermal stress to determine the viability index according to Milovanov (1962). In all pre-and post-freezing seminal transaminase (ALT and AST) activity were assayed within 1-2 hr after collection/dilution or thawing, according to (Reitman and Frankal, 1957).

Statistical analysis:-

All data obtained on motility, viability index and enzyme leakage were analyzed statistically, using COSTATE, computer program, version 3.03; copyright, 1986 Cottort software.

RESULTS

Results are presented in (Tables 1, 2, 3, 4 & 5 and Fig. 1, 2, 3 & 4).

DISCUSSION

The mean values of post-thaw Friesian semen characters (motility, viability, AST and ALT enzymes leakage) frozen by different regimens using buffalo and cow milk based diluent and loaded into 0.25 ml ministraws and minitubes are given in tables 1 and 2. Table 3 presented semen characters of buffaloes. Effect of semen packages, animal species and diluents are shown in Figures 1, 2, 3 and 4. The analysis of variance and correlation's are presented in table 4 and 5, respectively.

EFFECT OF FREEZING REGIMENS:-

In Friesian semen, the maximum ($P<0.01$) post thaw motility (63.33±1.66) associated with highest viability (153.33±4.16) were seen in buffalo-milk-diluted semen, loaded into 0.25 ml ministraws, when semen frozen at 2 cm above LN for 15 min., while the minimum motility (16.67±6.67) accompanied with lower viability index (44.17±15.45) were

observed in cow-milk-diluted semen packaged in 0.25 ml ministraws when frozen 8 cm above LN for 10 min (Table 1).

Meanwhile, the highest ($P<0.01$) motility (63.33±1.66) of post-thaw buffalo semen accompanied with highest viability index (161.67±7.12) obtained when buffalo-milk-diluted semen frozen at 2 cm above LN for 10 min and the lowest motility (5.00±0.00) and viability (4.17±1.66) were obtained when ministraws frozen at 8 cm above LN and maintained for 10 min (Table 3). Irrespective to the types of diluents and semen packages, the overall means revealed that, the best motility obtained for cow semen frozen by regimens 7 and 10, it was 57.5±2.17 and 57.91±1.56, respectively (Table 1). On the other side, for buffalo semen, regimens 2 and 9 achieved higher ($P<0.01$) percentage of motility, 50.55±5.73 and 45.55±6.74, respectively. This freezing regimens have to be balanced in such away that the damage to the sperm cell is minimized. However, the post-thaw quality of Friesian bull semen (motility, viability, AST and ALT enzymes) was significantly ($P<0.01$) better when semen maintained in LN for 15 min than 10 min (Tables 1 and 2).

The post-thawed buffalo semen showed no significant differences in the semen quality (motility, viability and AST) either maintained for 10 or 15 min above LN (Table 3). On the basis of post-thaw motility, Jansen (1989) reported that fastest freezing gave low survival of sperm, which is in similar trend to our study, when fast freezing was applied (10 min) for cattle and buffalo semen, the post-thaw motility was 47.16±1.81 and 30.33±3.31, respectively. On the other hand, when slow freezing (15 min) was employed post-thaw motility was 57.19±1.24 and 34.33±3.19, for cattle and buffalo semen, respectively. Also, our findings are agreement with those of Roy and Ansari (1973) and Bhandari et al (1982) who found that fast freezing of buffalo semen resulted in post-thaw motility ranged from 35.8-39% while slow freezing resulted in motility ranged from 38.3-40%. On the contrary, Bhandari et al (1982) reported fastest timing of freezing was the best. Anyhow, the cooling rates should not be too fast to cause cell death due to cold shock or too slow to cause death due to osmotic shock.

EFFECT OF DILUENTS:-

The pre-freeze motility, AST and ALT of Friesian semen were 75.00±2.89; 69.33±2.34; 51.67±5.82 and 73.33±1.66; 74.33±3.53 and 49.00±1.53, for buffalo milk and cow milk-diluted semen, respectively. The post-thaw motility and AST leakage of buffalo milk-diluted semen were

significantly superior ($P<0.05$) than cow milk-diluted semen (53.17±1.59; 118.55±2.21 Vs 48.92±1.63; 127.42±2.54). Whereas, no significant difference ($P<0.05$) in ALT leakage between buffalo milk and cow milk-diluted semen, it was 67.48±2.42 and 71.6±2.60, respectively, Fig. 1.

Also, the difference in the viability index between the two types of diluents was non significant, it was 105.08±4.58 and 103.13±4.51, for buffalo and cow milk-diluents, respectively. On the other side, the pre-freeze motility of buffalo semen and AST were 68.33±1.66 and 47.67±4.98; 71.67±1.66 and 53.00±2.89; 65.00±2.85 and 56.00±3.51, for buffalo, goat and sheep milk-diluted semen, respectively. Irrespective to the regimens of freezing, the deep freezing semen caused significant ($P<0.01$) decline in motility (41.33±3.99; 39.67±3.94 and 16.00±1.77) and significant ($P<0.01$) increase in AST leakage (94.77±5.98; 108.32±7.02 and 158.63±3.22), Fig. 2.

The difference betwen post-thaw motility of buffalo and goat milk-diluted buffalo semen on one hand was non significant and they were highly significant ($P<0.01$) than sheep milk-diluted semen on the second hand. Meanwhile, the post-thaw viability and AST were highly significant ($P<0.01$) between the three types of milk diluents (buffalo, goat and sheep milk). Moreover, the trend of pre- and post-thaw motility in all types of diluents revealed close parallelism with post-thaw viability index, these findings are supported with observations of Sukhija (1984) and Dhami, et al (1995), they found parrallesim between post-thaw motility and incubation survival of sperm as well as with conceptions.

The interactions between freezing regimens and diluents had not significantly affected the post-thaw motility and viability of Friesian spermatozoa (Table 4). This indicates that these variables work independently of one another in the freezability and post-thaw longevity of bovine spermatozoa . On the opposite side, for buffalo spermatozoa, the effect of interactions between freezing regimens and diluents were highly ($P<0.01$) significant on post-thaw motility, viability index and AST. Contrary to our findings Dhami et al (1995) found non significant interactions between diluents and cooling rates.

The use of different milk species that were employed appeared to be the first of its kind, since no comparable reports are available to compare between different types of milk species as extenders for semen dilution. Buffalo, goat and sheep milk are white in colour compared with cow milk, which is yellowish because of the presence of carotene (Saini and Gill, 1991).

Unlike cow milk is slightly acidic while goat milk is alkaline. This alkalinity is due to the higher protein content and a different arrangement of phosphatide (Saini and Gill, 1991). However, Dhami et al (1996) found equally efficacious in the cryoprotection of bovine semen between Tris and milk-based diluents.

Also, Dhami et al (1995) recorded that, the diluents used for freeze buffalo semen did not vary significantly with regard to any of the parameters studied (freezability and fertility) except the post-thaw aging motility which was higher in Tris than milk diluent, and this could perhaps be due to the rapid deterioration of buffering capacity of milk, to increased microbial growth and raising the acidity of the biological medium.The variation in the protective ability of these diluents (buffalo, cow, goat and sheep milk-diluents) against freezing damage to spermatozoa and the superior findings recorded with buffalo milk diluent may be attributed to its better buffering capacity and penetrance into the sperm cell medium, whereas, in the sheep milk-diluent, the inferior findings were probably due to its poor in the buffering capacity. The ability of buffering capacity which differ from milk species to other, resulting from differences in the milk composition which differ from species to other. Each 100 gm of goat milk contains approximately 194 mg Calcium and 270 mg phosphorous as compared with 160 mg and 145 mg for sheep (Posati and Orr, 1976 and Saini and Gill, 1991). Also goat milk contains higher proportions of phospholipids (phosphatidyl serine, phosphatidyl inositol, sphingomyelin and lysophospholipids) as well as low and medium chain fatty acids than sheep milk (Jenness 1980; IDF, 1986 and Jandal, 1996). The level of lactose in goat milk is higher than sheep milk (IDF, 1986). Buffalo milk may be contains high percentage of sugars, especially lactose, this sugar increase the cryoprotective ability of the diluent and gave more protection to sperm cells during freezing (Dhami, et al, 1995). Satish Kumar et al (1994) mentioned that, their post-thaw results appeared to be better in milk dilutor than Tris and sodium citrate might be due to presence of lactose already present in the milk. Also, buffalo milk-diluent may be contain antifreeze protein which had a beneficial effect on spermatozoal motility and could provide protection to cells from cryoinjury at the hypothermic stage, by blocking leakage of ions (Rubinsky et al ., 1991) and below freezing temperature by binding to ice crystal (DeVries 1988).

EFFECT OF SEMEN PACKAGES:

German minitubes, has been used to date for Freezing of bovine (Korvalan et al., 1986 and Gous, 1989) and buffalo semen (Afify, 1988; EL-Sheltawi, 1989 and Ziada, 1994), so, the experiments designed aimed to evaluate bull-semen freezing in minitubes.

Irrespective to the regimens of freezing and diluents, the post-thaw motility of Friesian semen packaged in ministraws and minitubes did not differ significantly, they were 49.25±1.89 and 52.83±1.29, respectively, but, the viability index was significantly ($P<0.01$) higher in ministraws than minitubes (116.04±4.97 Vs 92.17±3.45). Meanwhile, post-thaw AST and ALT leakage were significantly ($P<0.05$) increased in processed semen packaged into ministraws than minitubes (126.1±2.95; 73.03±3.00 Vs 119.87±1.73 and 66.05±1.83), Fig.3. ANOVA, showed that, there was interactions ($P<0.001$) between semen packages and freezing regimens concerning post-thaw motility, AST and ALT and non significant effect on the viability (Table 4). On the other hand, the interaction between semen packages and diluents had a significant ($P<0.05$) effect on viability index only (Table 4). The differences in the studied parameters could be due to the difference between the two packages in the ratio of surface area to volume. Ministraws as those used in the present study are 133 mm length, 2.0 mm in diameter, it would allow better exposure to LN vapour required for more rapid freezing compared to the minitubes which are 63 mm in length and 3 mm in diameter. In different comparative studies using ministraws and minitubes, some have found ministraws to be better than minitubes (Hunton et al., 1987), while others have found that semen frozen in ministraws was inferior to that in minitubes (EL-Sheltawi, 1989 and Maxwell et al., 1995). Concerning to the post-thaw motility as found in the current study is agreement with EL-Sheltawi (1989) and Maxwell et al (1995).

EFFECT OF ANIMAL SPECIES:-

Comparison between Friesian and buffalo semen diluted in milk extender of the same species (i.e. cow semen diluted in cow milk and buffalo semen diluted in buffalo milk extenders) and of the same processing (freezing regimens and packages; 0.25 ml ministraws) revealed that, pre-freeze motility and AST were 75.0±2.89; 69.33±2.34 and 68.33±1.66; 47.67±4.98, for Friesian and buffalo semen, respectively. Post-thaw motility, viability index and AST which reported in this study were 46.5±2.67; 108.92±7.32 and

131.9±4.32 Vs 41.33±3.99; 94.1±10.63 and 94.77±5.98, for Friesian and buffalo semen, respectively. The differences in the same characters between 2 seminal species was significant (P<0.01). Similarly, Dhami et al (1992) found a significant (P<0.01) differences in pre-freeze and post-thaw motility within and between both cattle and buffalo semen and this effect of freezing was more drastic in Holstein Friesian bull spermatozoa. Also, Dhami and Sahni (1994) found that cooling rate had a significant effect (P<0.01) on the post-thaw sperm motility and after 1 hr of incubation of both cattle and buffalo semen. Moreover, the same authors agreement with our results and found a significant higher pre-freeze motility in cattle than in buffalo semen (72.78 Vs 67.76). On the contrary to our findings, Dhami et al (1992) reported that post-thaw motility was significantly (P<0.05) higher in buffalo than in cattle semen (49.4 Vs 44.9), but this may be due to the differences in the diluent and freezing regimens or different in temperature of thawing which were applied in their studies.

However, the reduction in the motility and increase in AST leakage of pre-and-post-freeze spermatozoa were better in buffalo than Friesian (27.00 and 47.10 Vs 28.5 and 62.57) and the difference between them was statistically significantly (P<0.05) for AST and non significant for motility. Similar reduction in the post-thaw motility of cattle and buffaloes (28.05 Vs 21.67) were reported by Dhami et al (1995). These findings indicated that much of the sperm cells enzymes leaked out into the extracellular medium with loss of viability as a result of cooling, equilibration and freezing-thawing of semen due to osmotic shock, structural damage and increase cell membrane permeability. These observations coincided well with the earlier reports on cattle (Graham et al, 1974; Pandit and Garg, 1983; Belorkar et al, 1993) and buffalo semen (Chinnaiya et al, 1979; Jagmohan and Sarma, 1988; Dhami and Kodagali, 1990 and Dhami and Sahni, 1995). Verma et al (1994) found, in spite of the fall in progressive motility followed post-thaw incubation for 4 hrs was greater for buffalo than for cattle spermatozoa but it did not differ significantly. The same authors demonstrated poor thermal resistance of buffalo spermatozoa as compared to cattle spermatozoa.

It is worthy to mentioned that, mean values of post-thaw motility, viability and AST for Friesian semen (52.0±2.52; 123.17±6.59 and 120.3±3.80) was better (P<0.05) than buffalo semen of the same treatment and processing (i.e. same diluent, which is buffalo-milk extender, same freezing regimens and same packages), Fig. 4. This means buffalo-milk

extender is suitable and preferable than cow-milk for Friesian semen processing.

Transaminases (AST and ALT) are located primarily in the mid-piece of the sperm cell (Mann and Mann, 1981) and the measurement of the release of these enzymes from the spermatozoa is considered to be a sensitive indicator of sperm damage occurring during freezing and thawing and had been used to evaluate the quality of frozen-thawed spermatozoa (Jani et al., 1983). The obtained data showed that, enzymes activity (AST and ALT) were highly significantly ($P<0.001$) negative correlated with post-thaw Friesian spermatozoal motility (ranged from -0.84 to 0.86) and viability (ranged from -0.46 to 0.50). For post-thawed buffalo spermatozoa the negative correlation's of enzyme activity (AST) was -0.95, for motility and -0.091, for viability. Positive correlation's were observed between motility and viability for Friesian and buffalo spermatozoa (Table 5). Further, the pre- and post-freezing activity of ALT and AST has significant positive interrelationships ($r=0.751$ to 0.995) with one and other. These correlation's were similar magnitude to those reported by Belorkar et al. (1988); Dhami and Kodagali (1990); Dhami and Sahni (1993) and Dhami et al. (1995). Comparable to other results reported by many investigators (Crabo et al., 1971; Chinnaiya et al., 1979 and Belorkar et al, 1993), the high levels of transaminases (ALT and AST) recorded in our study, may be attributed to the fact that, whole milk, skim milk or whey has a natural biological secretion contained an appreciably high amount of enzymatic activity and to the number of microflora which present normally in the milk may also have contributed to high enzyme activity in fresh and frozen-thawed diluent (Dhami and Sahni, 1993).

The fertility rate which is the ultimate goal of frozen semen could be predicted on the basis of post-thaw motility, freezability, post-thaw longevity and for AST and ALT leakage's. These observations compared favorably with those of Saacke and White (1972); Uwland (1984) and Dhami et al (1995) who reported significant positive correlation's between post-thawed incubation and aging (5 °C, 24 hrs) survival of sperm and its fertility rates. Also, Dhami et al. (1996) found highly correlation between post-thaw motility and conception rate (post-thaw motility 44% Vs 37%, conception rate 71% Vs 56.6%). Similarly, Dhami and Kodagali (1990) and Pandit and Garg (1983) found negative association between sperm cell AST leakage and its fertility in bovine.

Based on our findings, we suggested that, semen frozen at 2 cm above LN for 15 min (Friesian semen) and 10 min (buffalo semen) gave the best results. Buffalo milk based diluent appeared to be the best diluent for cryopreservation of both buffalo and cattle semen. Concerning to, the post-thaw motility, no significant differences between Friesian semen packaged in either ministraws or minitubes. Estimation of AST and ALT leakage can be used as indicator for assessment of cattle and buffalo semen. However, further large-scale of studies on fertility trails using different regimens of freezing, diluents as well as semen packages are needed before a practical protocol can be recommended.

REFERENCES

Afify, A. M. (1988): Factors affecting conception following AI in buffaloes. Ph. D. Thesis, Cairo University.

Ahmed, K. (1984): Effect of thaw rates on survival buffalo spermatozoa frozen in straws. J. Dairy Sci., 67: 1535-1538.

Belorkar, P. M., Dhami, A. J. and Kodagali, S. B. (1988): Leakage of transaminases during freezing of crossbred bulls semen. Indian Vet., J. 65: 809-812.

Belorkar, P. M., Dhami, A. J. and Kodagali, S. B. (1993): Effect of extenders and seasons on the freezability, GOT leakage and fertility of crossbreed bulls semen. Indian J. Dairy Sci., 46: 198-202.

Belorkar, P. M., Dhami, A. J. and Kodagell, S. B. (1991): Physiological norms and correlations of seminal characters. Biochemical constituents, freezability and fertility in crossbreed bulls. Indian Vet. Med. J., 15: 191-197.

Bhandari, N., Chauhan, R. A. and Mathew, A. (1982): Note on the effect of rate ofreezing on the freezability of buffalo spermatozoa. Indian J. of Anim. sci., 52: 1237-1238.

Bhosrekar, M. K., Purohit, J. R. and Mangurkar, B. R. (1990): Studies on the effect of additives to semen diluent. Indian J. of Am. Rep. 11: 85-88.

Bhosrekar, M. R., Mokashi, S. P., Purohit, J. R., Gokhale, S. E. and Mangurkar, B. R. (1994): Comparative study on conventional control (programmable) freezer on the quality of buffalo semen. Indian J. of Anim. Ec. 64 (6): 583-587.

Chandler, J. E., Adkinson, R. W.and Nebel, R. L. (1984): Thawing optimums for the bovine spermatozoa processed by three methods and packaged in continental and french straws. J. Dairy Sci., 67: 398-404.

Chinaiya, G. P., Kaker, S. S. and Ganguli, N. C. (1979): Extracellular release of transaminases from buffalo spermatozoa on freezing of semen in extenders. Zbl Vet Med. 26: 402-407.

Crabo, B. G., Bowey, R. E., Brown, K. I., Graham, E. F. and Race, M. M. (1971): Extracellular GOT as a measure of membrane injury in spermatozoa during treatment. Current problems in fertility, planum press, New York, Ny, PP. 33-38.

De-vries, A. L. (1988): The role of antifreeze glycoproteins and peptides in the freeze avoidance of antarctic fishes. comp. biochem. physiol., 90: 611-621.

Dhami, A. J. (1992): Comparative evaluation of certain processing procedures in deep freezing of cattle and buffaloe semen under a tropical climate. Ph.D, thesis, Indian veterinary Research institute, Izatnagar.

Dhami, A. J. and Kodagali, S. B. (1988): Studies on seminal enzymatic profiles and its relationship with fertility of surti buffalo bulls. Indian J. of Anim. Reprod., 9: 24-29.

Dhami, A. J. and Kodagali, S. B. (1990): Freezability enzyme leakage and fertility of buffalo spermatozoa in relation to the quality of semen ejaculates and extenders. Theriogenology. 34: 835-863.

Dhami, A. J. and Sahni, K. L. (1993): Evaluation of different cooling rates, equilibration, periods and diluents for effects on deep-freezing, enzyme leakage and fertility of taurine bull spermatozoa. Theriogenology, 40: 1269-1280.

Dhami, A. J. and Sahni, K. L. (1994): A comparative evaluation of certain processing procedures (cooling, equilibration and thawing) in deep freezing of cattle and buffalo semen under tropical climate. Indian J. of Anim. Sci., 64(9): 933-938.

Dhami, A. J. and Sahni, K. L. (1995): Deep-freezing of cattle and buffalo semen with or without equilibration and its fertility trails-Acomparative study. Indian j. of Anim. Sci., 65(1): 59-64.

Dhami, A. J., Sahni, K. L. and Mohan, G. (1995): Effect of various extenders and additives on deep-freezing, enzyme leakage and fertility of bovine semen under tropical climate. Indian J. of Anim. Sci., 65(1): 20-27.

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

Dhami, A. J., Sahni, K. L., Mohan, G. and Jani, V. R. (1996): Effects of different variables on the freezability, post-thaw longevity and fertility of buffalo spermatozoa in the tropics. Theriogenology, 46: 109-120.

EL-Sheltawi, M. A. (1989): Evaluation of locally processed frozen semen. Ph. D., Thesis, Cairo University.

Gaus, j. (1989): Fortschritte I der spermaproduktion. Rinderproduktion, 100: 17.

Gilbert, G. R. and Almguist, J. C. (1978): Effect of processing procedures on post. thaw acrosomal retention and motility of bovine spermatozoa packaged in 0.3 ml straws at room temperature. J. Anim. Sci., 46: 225 (Abst.).

Graham, E. F. and Pace, M. M. (1967): Some biochemical changes in spermatozoa due to freezing. Cryobiology, 4: 75-84.

Graham, E. F., Crabo, B. G. and Schmehl, M. K. (1974): Utilization of enzyme assay in the developing techniques for freezing semen. Anim. Breed Abstr. 42: 4111 (Abstr.).

Hunton, J. R., Flecker, S. E. and Maxwell, W. M. (1987): Pregnancy rates following intra-uterine insemination with pellet or straw-frozen ram semen. J. Agric. Sci., 189-191.

IDF (1986): The manufacture and characteristics of ewe and goat milk. Bulletin of the International Dairy Federation, 202, 222.

Jagmohan and Sarma, P. A. (1988): Investigation on the effect of different freezing rates on physical and biochemical parameters of buffalo frozen semen. AI News, 3: 15-18.

Jandal, J. M. (1996): Comparative aspects of goat and sheep milk. Small Ruminant Research, 22; 177-185.

Jani, V. R. (1982): Studies on deep freezing of bull and buffalo semen for freezability, enzyme leakage and fertility. M. V. Sc. Thesis, Gujarat Agricultural University, Anand, Indian.

Jani, V. R., Prabhu, G. A., Darashri, H. J., Patel, M. M. and Kodagali, S. B. (1993): Release of transaminase from buffalo spermatozoa during deep freezing. Indian J. Anim. Reprod., 24: 30-32.

Janness, R. (1980): Composition and characteristics of goat milk. Review, J. Dairy Sci., 63: 1605-1630.

Jansen, H. B. (1989): The influence of cooling rates practised in Netherlands on survival of bovine spermatozoa packaged in 0.25 ml French straws. 11 th International Cong. on Anim. Reprod. and AI, University college, Dublin, Ireland.

Korvalan, P., Marinov, P. and Zlatarev, S. (1979): Some aspects of three methods of freezing bull semen. Biol. Immunol. Reprod.., 11: 88-91.

Mann, T. and Mann, C. L. (1981): Male reproductive function and semen. Springerverlage, Berlin, 43: 777 (Abstr.)

Mathur, A. C., Benjamin, B. R. and Sahni, K. L. (1990): Effect of various cooling rates on freezing of bullsemen. Indian J. Anim. Reprod., 11: 88-91.

Maxwell, W. M., Landers, A. J. and Evans, G. (1995): Survival and fertility of ram spermatozoa frozen in pellets, straws and ministraws. Theriogenology, 43: 1201-1210.

McRorie, R. A. and Williams, W. L. (1974): Biochemistry mammalian fertilization. Ann. Rev. Biocham., 43: 777 (Abstr.).

Milovanov, V. K. (1962): Biology of reproduction and AI of farm animals (Tranis title) monograph Scelkhoz Literature J. Moscow.

Pace, M. M. and Graham, E. F. (1970): The release of GOT from bovine spermatozoa as a test not hot for assessing test quality and fertility. Anim. Breed. Abstr., 39: 3249.

Pandit, R. K. and Garg, U. K. (1983): Freezability, GOT leakage and fertility of crossbred bull semen. Indian J. Anim. Reprod. 4: 36-38.

Pangawkar, D. R., Sharma, R. D. and Sharma, R. (1988): Phosphatase and transaminase activity in seminal plasma of bulls in relation to freezability of semen. Theriogenology, 29: 1393-1399.

Posati, L. P. and Orr, M. L. (1976): Composition of foods dairy and egg products: Raw, processed, prepared. Agri. Handbook No. 8-IUS, dept., of Agri. Res. Ser., Washington.

Raizada, A., Eattar, A., and Pandey, M. D. (1988): A comparative study of freezing buffalo semen in two dilution. Proceednys II World buffalo Cong. New Delhi, 3: 66-74.

Rao, A. V., Haranath, G. B., Eomasekharan, G. and Rao, J. R. (1986): Effect of thaw rates on motility, survival and acrosomal integrity of buffalo spermatozoa frozen in medium French straws. Anim. Reprod. Sci., 12: 123-129.

Reitman, S. and Frankal, S. A (1957): Colorimetric method for the determination of serum glutamic oxaloacetic transaminase and serum glutamic pyruvic transaminase. American J. of Clinical pathology, 28: 56-60.

Robbines, R. K., Eaacke, R. G. and Chandler, P. T. (1976): Influence of freeze rate, thaw rate and glycerol level on acrosomal retention and survival of bovine spermatozoa frozen in french straws. J. Anim. Sci., 42: 145-154.

Roy, D. J. and Ansari, M. R. (1973): Studies on freezability of buffalo spermatozoa using two freezing techniques. Indian J. Anim. Sci., 43; 1031-1033.

Rubinsky, B., arav, A. and Fletcher, G. L. (1991): Hypothermic protection-a fundamental property of antifreeze proteins. Biochem. Biophys. Res. Comm., 180: 566-571.

Saacke, R. G. and White, J. M. (1972): Semen quality tests and their relationship to fertility. Proc. 4 th Tech. Conf. AI and Reprod. 22-27.

Sahni, K. L and Mohan, G. (1988): Effect of various thawing temperatures on survival and vitality of frozen semen of buffalo and cow bulls. J. Anim. Sci., 58: 1077-1078.

Saini, A. L. and Gill, R. S. (1991): Goat milk: An attractive alternate. Indian Dairy man, 42: 562-564.

Salisbury, G. W., Vandemark, N. L., Lodge, J. R. (1978): Physiology of reproduction and AI of cattle. WH freeman and company. Ean Franncisco. 494-538.

Satish Kumar, Sahni, K. L. and Greesh Mohan (1994): Freezing of buffalo semen in different dilutors with different concentatrions of glycerol and different sugars in absence of yolk. Indian J. Dairy Sci., 47, 8.

Singh, J., Pangawkar, G. R. and Shrivastaa, A. K. (1992): Efficacy of certain extenders on leakage of phosphatase from buffalo spermatozoa during deep freezing. Indian J. Anim. Reprod., 13: 35-38.

Smith, J. F., Asher, G. W., Briggs, R. M., Morrow, C. J., Murray, G. R., Oliver, J. E., Veldhujen, F. A. and Upreti, G. C. (1993): Effect of diluent and storage time on pregnancy rate in ewes after intra-uterine insemination. Proc. N. Z. Soc. Anim. Prod., 53: 295-298.

Sukhija, S. S. (1984): Ph. D. Thesis. Haryana Agric. Univ. Hissar, India.

Tuli, R. K., Singh, M. and Matharoo, J. S. (1982): Effect of different extenders on glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) release from frozen buffalo semen. Theriogenology, 18: 55-59.

Uwland, I. (1984): Possibilities and limitations of semen evaluation for the prognosis of male fertility. Male in Farm Anim. Reprod. PP. 269-289.

Verma, H. K., Singh, M. M., Singh, G. D. and Pant, H. P. (1994): Effect of thaw rates on survival, post-thaw motility and thermal resistance of spermatozoa of cattle and buffalo. Indian J. of Anim. Sci., 64(7): 745-747.

Yassen, A. M., Fathelhab, A. Z. and Ibrahim, M. A. (1985): Freezability of buffalo bull semen in tris, UHT-mille and lactose yolle extenders: a comparative study. J. World Buffalo Cong. 1:189.

Ziada, M. S. (1994): Morpho-Biological studies on frozen buffalo semen. Ph. D. Thesis. Cairo University.

Table 1: Effect of various regimens of freezing, milk extenders and types of semen package on the post-thawed motility (Mot.) and viability (viab.) index in Holstein-Friesian semen (Mean ± SE):-

No. of freezing regimens	Time at LN vapor (min)	Heights above LN(cm)	Buffalo milk				Cow milk				Overall	
			Ministraws		Minitubes		Ministraws		Minitubes			
			Mot.	Viab. index	Mot.	Viab. index	Mot.	Viab. index	Mot.	Viab. index	Mot.	Viab. index
1		Control	46.67 ± 4.41	125.00 ±20.38	61.67 ± 3.33	81.67 ± 7.27	33.33 ± 7.27	64.17 ±22.49	48.33 ± 7.27	85.00 ±12.59	47.50 [a] ± 3.91	88.95 [a] ± 9.82
2	10	2	56.67 ± 3.33	152.50 ± 2.89	56.67 ± 4.41	85.00 ±12.51	46.67 ± 8.34	123.33 ±26.84	50.00 ± 5.00	100.8 ±12.28	52.50 [a] ± 2.71	115.4 [a] ±10.28
3		4	51.67 ± 6.01	133.33 ±14.25	58.33 ± 4.41	94.17 ± 9.83	45.00 ± 5.78	124.1 ±16.87	56.67 ± 1.66	117.50 ± 7.22	52.91 [a] ± 2.57	117.2 [a] ± 6.93
4		6	53.33 ± 3.33	135.83 ±13.34	56.67 ± 6.01	68.33 ±15.45	41.67 ± 1.66	101.67 ±12.28	46.67 ± 4.41	82.50 ±11.28	49.58 [a] ± 2.49	97.08 [a] ± 9.48
5		8	20.00 ± 5.78	50.00 ±13.00	53.33 ± 1.66	79.17 ±16.10	16.67 ± 6.67	44.17 ±15.45	43.33 ± 8.83	64.16 ±22.44	33.33 [b] ± 5.38	59.37 [b] ± 8.36
6		Control	63.33 ±1.66	139.17 ±14.54	56.67 ± 1.66	95.83 ±11.67	55.00 ± 2.89	111.67 ±21.68	46.67 ± 6.01	100.00 ±18.94	55.41 [a] ± 2.34	115.2 [a] ± 9.58
7	15	2	63.33 ± 1.66	153.33 ± 4.16	56.67 ± 6.01	84.17 ±17.35	56.67 ± 6.01	150.00 ±13.78	53.33 ± 1.66	112.50 ±13.78	57.50 [a] ± 2.17	121.4 [a] ± 9.98
8		4	53.33 ± 9.28	115.00 ±26.28	48.33 ± 8.34	84.17 ±16.68	53.33 ± 4.41	115.00 ±17.75	53.33 ± 6.01	119.17 ±20.65	52.08 [a] ± 3.16	108.3 [a] ± 9.78
9		6	55.00 ± 5.78	105.83 ±15.91	38.33 ±11.67	79.17 ±19.72	55.00 ± 5.78	115.83 ± 6.51	58.33 ± 4.41	91.67 ±10.24	51.66 [a] ± 3.95	98.12 [a] ± 7.31
10		8	56.67 ± 1.66	121.67 ±11.76	56.67 ± 6.01	118.33 ± 9.83	61.67 ± 1.66	139.17 ±12.28	56.67 ± 1.66	100.00 ±23.65	57.91 [a] ± 1.56	119.7 [a] ± 7.78
Overall	10		45.67 ± 3.96	119.33 ±10.93	57.33 ± 1.75	81.67 ± 5.29	36.67 ± 3.80	91.50 ±11.83	49.00 2.55±	90.00 ± 7.18	47.16 [b] ± 1.81	95.62 [b] ± 4.77
	15		58.33 ± 2.22	127.00 ± 7.64	51.33 ± 3.43	92.33 ± 6.97	56.33 ± 1.86	126.33 ± 7.09	53.67 ± 1.98	104.67 ± 7.28	54.91 [a] ± 1.24	112.5 [a] ± 4.02

Means,in the same column with different superscripts differ significantly ($P<0.01$).

Table 2: Effect of various regimens of freezing, milk extenders and types of semen package on the post-thawed AST and ALT leakage in Holstein-Friesian semen (Mean ± SE):-

No. of freezing regimens	Time at LN vapor (min)	Heights above LN (cm)	Buffalo milk				Cow milk				Overall	
			Ministraws		Minitubes		Straws		Minitubes			
			AST U/ml	ALT U/ml	AST U/ml	ALT U/ml	AST U/ml	ALT U/ml	AST U/ml	ALT U/ml	AST U/ml	ALT U/ml
1		Control	121.67 ± 4.41	61.67 ± 4.41	114.67 ± 8.22	62.33 ± 6.18	159.00 ± 9.25	112.67 ±10.14	125.00 ±10.60	65.33 ± 8.58	130.0^{b} ± 6.29	75.50^{b} ± 7.26
2	10	2	108.33 ± 1.66	61.67 ± 1.66	118.00 ± 9.08	72.00 ± 5.13	129.67 ± 5.55	80.33 ± 9.50	114.33 ± 3.18	58.00 ± 7.10	117.5^{bc} ± 3.35	68.00^{bc} ± 3.83
3		4	125.00 ± 2.89	76.67 ± 6.01	121.67 ± 9.29	56.67 ± 6.94	124.33 ± 3.76	87.67 ± 9.50	121.00 ± 3.21	60.00 ± 8.90	123.0^{bc} ± 2.38	70.25^{bc} ± 5.09
4		6	120.00 ± 5.78	66.33 ± 8.42	109.67 ± 2.91	69.00 ± 6.01	138.67 ± 5.37	77.67 ± 6.39	129.67 ± 8.75	89.67 ± 7.69	124.5^{bc} ± 4.15	75.66^{b} ± 4.11
5		8	174.67 ± 5.18	128.67 ± 6.07	113.00 ± 3.51	76.33 ± 5.24	182.33 ± 5.37	107.00 ± 5.86	131.33 ± 9.29	77.33 ±10.14	150.3^{a} ± 9.15	97.33^{a} ± 7.26
6		Control	103.33 ± 6.01	50.67 ± 2.18	116.33 ± 6.75	58.33 ± 8.36	118.67 ± 6.34	64.67 ± 8.77	137.00 ± 5.86	70.67 ± 4.34	118.8^{bc} ± 4.49	61.1^{bcd} ± 3.57
7	15	2	111.67 ± 4.41	52.67 ± 1.45	113.00 ±10.61	61.00 ± 6.43	115.00 ± 8.15	58.33 ± 5.46	120.00 ± 5.78	65.33 ± 5.24	114.9^{c} ± 3.38	59.33^{cd} ± 2.54
8		4	110.67 ± 6.97	71.33 ±11.36	114.67 ± 5.49	65.67 ± 6.75	119.00 ± 6.99	60.00 ± 7.24	128.67 ± 9.14	68.00 ±10.13	118.2^{bc} ± 3.42	66.25^{bc} ± 4.06
9		6	107.67 ± 5.01	69.67 ± 4.38	132.67 ±11.71	79.67 ±10.36	124.00 ± 6.94	66.33 ± 9.91	110.00 ± 5.01	61.00 ± 9.55	118.5^{bc} ± 4.61	69.16^{bc} ± 4.30
10		8	120.00 ± 9.25	59.00 ± 2.31	114.33 ± 2.34	50.33 ± 5.21	108.33 ± 1.66	47.67 ± 3.28	112.33 ± 8.26	54.33 ± 6.12	113.7^{c} ± 2.49	52.83^{d} ± 2.30
Overall	10		129.93 ± 6.37	79.00 ± 7.14	115.40 ± 2.93	67.27 ± 2.92	146.80 ± 6.16	93.07 ± 4.96	124.27 ± 3.33	70.07 ± 4.53	129.9^{a} ± 2.83	77.35^{a} ± 2.81
	15		110.67 ± 2.44	60.67 ± 3.12	118.20 ± 3.64	63.00 ± 3.88	117.00 ± 2.80	59.40 ± 3.28	121.60 ± 3.78	63.87 ± 3.20	116.8^{b} ± 1.65	61.73^{b} ± 1.66

Means in the same column with different superscripts differ significantly ($P<0.01$).

Table 3: Effect of various regimens of freezing and milk extenders on the post-thawed motility, viability index and extracellular release of AST enzyme from Buffalo semen (Mean ± SE):-

No. of freezing regimens	Time at LN vapour (min)	Heights above LN (cm)	Buffalo milk			Goat milk			Sheep milk			Overall		
			Motility	Viability index	AST (U/ml)	Motility	Viability index	AST (U/ml)	Motility	Viability index	AST (U/ml)	Motility	Viability index	AST (U/ml)
1		Control	50.00 ± 2.89	103.33 ± 17.11	95.00 ± 5.78	50.00 ± 7.64	99.17 ± 21.73	81.67 ± 3.67	15.00 ± 0.00	26.67 ± 1.66	149.00 ± 5.13	38.33 ^{ab} ± 6.29	76.38 ^{bc} ± 14.79	108.55 ^{cd} ± 10.58
2	10	2	63.33 ± 1.66	161.67 ± 7.12	51.33 ± 1.76	60.00 ± 2.89	139.17 ± 7.95	69.33 ± 2.97	28.33 ± 3.33	48.33 ± 4.41	137.33 ± 4.41	50.55 ^{a} ± 5.73	116.38 ^{a} ± 17.63	86.00 ^{e} ± 13.19
3		4	53.33 ± 3.33	142.5 ± 6.29	82.33 ± 7.69	55.00 ± 2.89	110.00 ± 11.56	81.00 ± 4.62	13.33 ± 3.33	24.17 ± 4.40	170.33 ± 6.49	40.55 ^{ab} ± 6.99	92.22 ^{abc} ± 18.09	111.22 ^{cd} ± 15.12
4		6	20.00 ± 5.78	26.67 ± 6.82	136.33 ± 6.70	20.00 ± 0.00	25.83 ± 3.33	145.00 ± 4.72	10.00 ± 0.00	16.67 ± 0.83	168.66 ± 5.49	16.66 ^{c} ± 2.35	23.05 ^{d} ± 2.72	150.00 ^{b} ± 5.63
5		8	6.67 ± 1.66	15.83 ± 0.83	143.00 ± 1.53	5.00 ± 0.00	15.83 ± 0.83	175.33 ± 4.26	5.00 ± 0.00	4.17 ± 1.66	184.00 ± 3.21	5.55 ^{c} ± 0.55	11.94 ^{d} ± 2.03	167.44 ^{a} ± 6.44
6		Control	35.00 ± 8.67	62.50 ± 12.84	97.33 ± 3.84	35.00 ±7.64	58.33 ± 12.94	117.67 ± 9.54	26.67 ± 6.01	44.17 ± 9.61	139.00 ± 4.62	32.22 ^{b} ± 4.00	55.00 ^{c} ± 6.56	118.00 ^{c} ± 6.84
7	15	2	61.67 ± 1.66	144.33 ± 15.61	59.33 ± 5.49	53.33 ± 9.28	135.83 ± 33.89	87.33 ± 11.15	20.00 ± 2.89	34.17 ± 4.64	159.66 ± 2.03	45.00 ^{a} ± 6.97	104.77 ^{ab} ± 20.75	102.11 ^{cd} ± 15.38
8		4	55.00 ± 2.89	141.67 ± 11.76	78.00 ± 2.89	53.33 ± 4.41	86.67 ± 33.27	91.00 ± 9.08	11.67 ± 1.66	16.67 ± 2.20	161.00 ± 9.17	40.00 ^{ab} ± 7.26	81.66 ^{abc} ± 20.76	110.00 ^{cd} ± 13.44
9		6	58.33 ± 4.41	125.83 ± 12.02	70.00 ± 5.69	55.00 ± 8.67	99.17 ± 23.49	75.33 ± 9.31	23.33 ± 8.82	25.83 ± 6.01	144.66 ± 10.60	45.55 ^{a} ± 6.74	83.61 ^{abc} ± 16.86	96.66 ^{de} ± 12.79
10		8	10.00 ± 0.00	16.67 ± 0.83	135.00 ± 2.65	10.00 ± 0.00	16.67 ± 0.83	158.67 ± 5.79	6.67 ± 1.66	9.17 ± 1.66	172.66 ± 6.84	8.88 ^{c} ± 0.73	14.16 ^{d} ± 1.38	155.44 ^{ab} ± 6.12
overall		10	38.67 ± 5.91	90.00 ± 16.24	101.60 ± 9.38	38.00 ± 5.96	78.00 ± 13.72	110.47 ± 11.32	14.33 ± 2.23	24.00 ± 4.03	161.87 ± 4.83	30.33 ^{a} ± 3.30	64.00 ^{a} ± 8.28	124.64 ^{a} ± 6.51
		15	44.00 ± 5.46	98.20 ± 14.20	87.93 ± 7.30	41.33 ± 5.31	79.33 ± 14.13	106.00 ± 8.67	17.67 ± 2.75	26.00 ± 3.94	155.40 ± 4.26	34.33 ^{a} ± 3.19	67.84 ^{a} ± 8.09	116.44 ^{a} ± 5.84

Means in the same column with different superscripts differ significantly ($P<0.01$).

Table 4: ANOVA showing the effects of freezing regimens, diluents, semen package and their interactions on motility, viability index and enzymes (AST-ALT) leakage in semen of Holstein-Friesian and Buffalo bulls:-

Source of variation	df	Motility		viability index		AST leakage		ALT leakage	
		MSS	F	MSS	F	MSS	F	MSS	F
				Holstein-Friesians semen					
Freezing regimens (F)	9	591.32	6.65***	4407.30	5.89***	1385.61	10.59***	1752.22	11.34***
Diluents (D)	1	541.88	6.09*	115.05	0.15ns	2358.53	18.03***	508.41	3.29ns
Semen package (P)	1	385.21	4.33*	17100.47	22.86***	1165.63	8.91**	1463.00	9.47**
Interactions									
F X D	9	155.76	1.75ns	753.13	1.00ns	311.74	2.38*	444.06	2.87**
F X P	9	412.99	4.64***	1356.37	1.81ns	1216.95	9.31***	836.29	5.41***
D X P	1	46.88	0.53ns	4532.55	6.06*	224.13	1.71ns	156.41	1.01ns
F X D X P	9	40.39	0.45ns	708.13	0.95ns	238.26	1.82ns	373.65	2.41*
Error	80	88.96		748.13		130.78		154.53	
				Buffaloes semen					
Freezing regimens (F)	9	2341.85	37.30***	12967.75	26.07***	6736.80	59.36***	-----	-----
Diluents (D)	2	6023.33	95.95***	39465.54	79.34***	34002.31	299.58***	-----	-----
Interactions								-----	-----
F X D	18	284.44	4.53***	2197.54	4.42***	855.92	7.54***	-----	-----
Error	60	62.78		497.39		113.50		-----	-----

* $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$ ns = Non significant

Table 5: Correlation coefficient between motility, viability index and enzyme leakage (AST and ALT) in frozen-thawed semen of Holstein-Friesian and Buffalo bulls:-

	Motility	Viability index	AST	ALT
		Correlation's: Holstein-Friesians semen		
Motility	-----	0.571***	- 0.860***	- 0.836***
Viability		-----	- 0.462***	- 0.496***
AST				0.776***
		Correlation's: Buffaloes semen		
Motility	-----	0.943***	- 0.948***	
Viability			- 0.913***	
AST			-----	

***. $P < 0.001$.

Fig. 1: Influence of diluents on pre-freeze and post-thaw Friesian semen quality:-

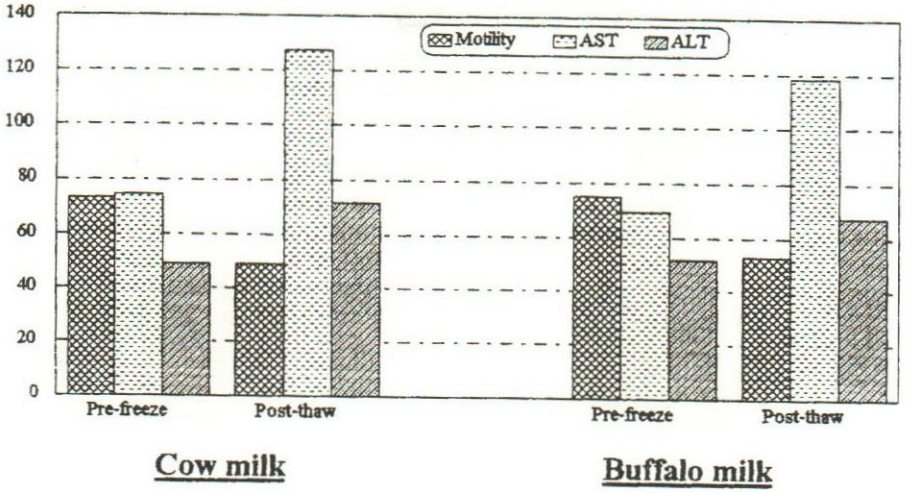

Fig. 2: Influence of diluents on pre-freeze and post-thaw buffaloes semen quality:-

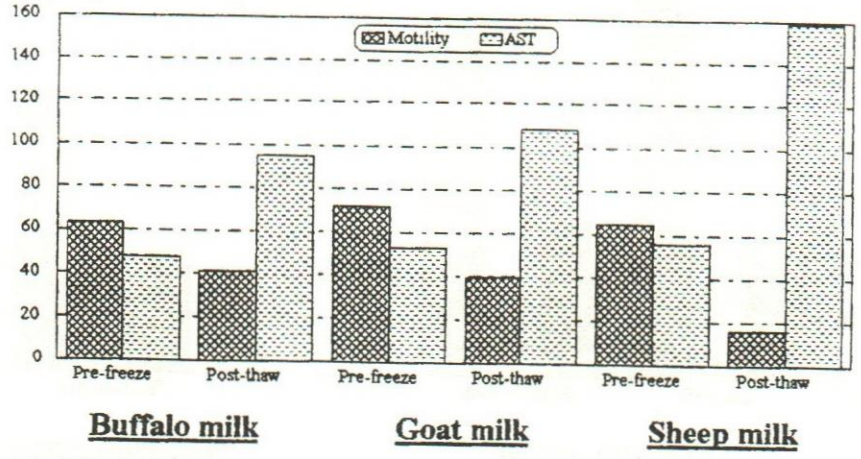

Fig. 3: Effect of package types on the post-thawed Friesians semen parameters:-

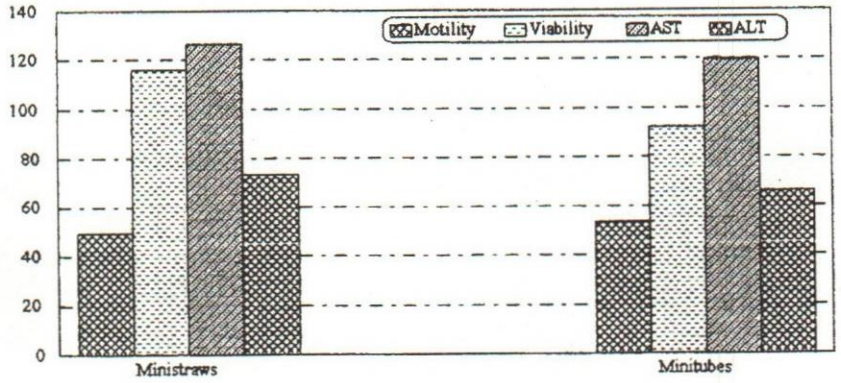

Fig. 4: Comparison between Friesians and buffaloes semen of the same processing:-