SEASONAL VARIATIONS IN QUALITY OF CRYOPRESERVED NILI RAVI BUFFALO SEMEN

Rabea Ejaz[1]*, Saima Qadeer[2], Iram Maqsood[3], Asima Azam[3] and S. Akhter[4]

[1]*Department of Zoology, Rawalpindi Women University, Rawalpindi - 46300, Pakistan*
[2]*Department of Zoology, Division of Science and Technology, University of Education, Lahore-54000 Pakistan*
[3]*Department of Zoology, Shaheed Benazir Bhutto Women University Peshawar, 25000, Pakistan*
[4]*Department of Zoology, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi-46300, Pakistan*
*Corresponding Author: Email: rabeaejaz29@gmail.com

ABSTRACT

Present study was aimed to assess the seasonal variations in quality of cryopreserved buffalo semen. Three adult buffalo bulls of same age were selected to collect semen using artificial vagina (42°C). *Tris* citric acid extender was used to dilute semen ejaculates (>0.5 billion per mL conc., >1 mL vol. and >60% motility). Semen samples were collected in consecutive three weeks routinely in different seasons, winter (December and January), dry summer (May and June). Extended semen chilled to 4°C (in 2h) was placed for equilibration at 4°C for a period of 4 hours. Plastic straws 0.5 mL (4°C) were used to fill the equilibrated semen. Liquid nitrogen gas vapours were used to further freeze the semen for 10 minutes and then dipped in liquid nitrogen for storing. After 24 hours thawing was done at 37°C for 30 seconds and semen quality was assessed. Sperm progressive motility, sperm live/dead ratio, plasma membrane integrity, DNA integrity, and viability remained the same in both winter and summer season. However, number of morphologically abnormal sperms were higher in summer ($P<0.05$) compared to winter season. In conclusion, quality of cryopreserved buffalo semen remained poor in summer season in terms of morphologically normal sperms.

Key words: seasonal variations, cryopreservation, extender, buffalo, semen quality

INTRODUCTION

Pakistan has best breeds of buffalo and cattle (Khan *et al*., 2007). Pakistan has a long summer period that lasts from May to September with average temperature range from 30 to 45°C and very humid and hot period from mid July to mid September. This environment can adversely affect reproductive efficiency of bulls (Barth and Waldner, 2002). Buffalo sperm are more vulnerable to cryodamages than cattle sperm (Raizada *et al*., 1990). These hazards can be reduced by optimizing the freezing and cooling rates and by using suitable semen extender (Kumar *et al*., 1992).

Seasonal variations seem to effect sexual function either by changes in ambient temperature or by photoperiod, as spermatogenesis is very sensitive to a brief increments in scrotal temperature (Barth and Waldner, 2002). Among swamp buffaloes, morphological abnormalities, specifically defects in tail, are affected by the season, with the highest occurrences observed during rainy season and the lowest during the summer (Koonjaenak, 2007). In Thai swamp buffalo, sperm quality, including sperm motility both before and after thawing, is observed to fluctuate whole year under the environmental conditions of Thailand. Specifically, sperm concentration is found to be the highest during rainy season and the lowest during summer (Koonjaenak *et al., 2005).* Semen quality can be influenced by temperature or stress (Johnston and Branton, 1953). In dairy bulls semen quality is highly affected by seasonal variations as in most of the bulls more abnormal tails, proximal droplets, abnormal sperm head and total sperm morphological abnormalities are more in summer than winter (Sekoni and Gustafsson, 1987).

Photoperiod plays a crucial role in influencing the reproductive and sexual performance of buffalo bulls (Vale, 1997). Numerous studies have highlighted that the season has an impact on the libido and the quality of buffalo semen. (Sansone *et al.,* 2000). Buffaloes are highly susceptible to heat stress, which results in a decline in semen quality (Sansone *et al.,* 2000). There is significant difference in seasonal variations of sperm abnormalities between summer and winter. Indeed, as summer is the hottest season of the year, it leads to highest occurrences of abnormal tails, proximal droplets, total sperm morphological abnormalities and abnormal sperm heads are higher during summer than winter, autumn or spring (Sekoni and Gustafsson, 1987). Keeping in view the impact of season on semen quality the present study was designed to assess effect of season on post-thaw quality of cryopreserved buffalo semen.

MATERIALS & METHODS

Preparation of Extender

*Dr. Rabea Ejaz, Assistant Professor, Department of Zoology, Rawalpindi Women University, Rawalpindi, Pakistan. Contact No. +923365534679; Email: rabeaejaz29@gmail.com

Tris-citric extender was prepared by using citric acid (1.56g) and tris–(hydroxymethyl) aminomethane (3.0g) in 74 mL distilled water was used as buffer for extenders, fructose 0.2% wt/vol; glycerol 7.0 mL and 100 mL distilled water. Antibiotics, streptomycin sulphate (1000 ug ml^{-1}) and benzyl penicillin (1000 IU ml^{-1}) were added in experimental extender (Akhter *et al.,* 2012; Ejaz *et al.,* 2021).

Animal Selection and Semen Collection

A total of three mature Nili- Ravi buffalo bulls kept at Semen Production Unit, Qadirabad, were selected for this study. Semen samples were collected in consecutive three weeks routinely in different seasons, winter (December and January) summer (May and June).

Initial Evaluation

Two repeated ejaculates were collected by using artificial vagina from three adult buffalo bulls of well known fertility and same age. Ejaculates from each bull were directly transported to laboratory for evaluation. Sperm motility was assessed microscopically and sperm concentration was evaluated by haemocytometer. Qualified ejaculates having more than 60% motile spermatozoa, minimum standard of 1mL volume, and 0.5 billion sperm/mL of ejaculate concentration was used for further dilution.

Semen Processing

Semen samples were diluted in extenders to achieve a conc. of 50×10^6 sperm mL^{-1}at a temperature of 37°C. Semen was then cooled to 4°C within a span of 2 h and kept at this temperature for 4h. Subsequently the semen was filled into 0.5 mL straws (IMV, France) using a suction pump in a cold cabinet at 4°C. These straws were placed in the vapors of liquid nitrogen, approximately 5 cm above the level of LN_2, for a duration of 10 minutes. After this, they were placed in the liquid nitrogen for long term storage (-196°C). Following 24h storage period, the semen straws were placed in a water bath set at 37°C for a period of 30 seconds.

SEMEN QUALITY ASSAYS

Sperm Motility (%)

A small amount of semen was put onto a pre- warmed microscope slide and assessed subjectively for sperm with progressive motility. The evaluation was conducted at a temperature of 37°C using a phase contrast microscope.

Sperm Plasma Membrane Integrity:

To assess plasma membrane integrity of sperm cells, the hypo-osmotic swelling (HOS) assay was used (Ejaz et al., 2017). HOS solution was prepared by mixing 0.735g sodium citerate and 1.351g fructose in 100 mL distilled water, resulting in an osmotic pressure of approximately 190 mOsm Kg^{-1}. HOS test was performed by mixing 500 µL of pre-warmed (37ºC) HOS solution to 50 µL of semen samples. The solution was incubated for 30-40 min. at 37ºC. After this, a drop of the semen sample was put under a phase contrast microscope. Sperm cells were observed for swelling, indicated by a coiled tail, which signifies an intact plasma membrane.

Sperm Viability and Liveability:

Trypan blue Giemsa stain was used to assess sperm viability (Akhter et al., 2008). Double stain procedure involves the application of two stains: Trypan blue as a supra vital stain to recognize dead and live sperm, and Giemsa to assess the integrity of acrosome membrane. Equal drops of semen and Trypan blue were placed on a glass slide at room temperature and were mixed. Slides were fixed in formaldehyde neutral red for 5 minutes after air drying. After rinsing in distilled water, a 7.5% Giemsa stain was applied for 4 h. Following this, the slides were mounted with Canada Balsam and spermatozoa were examined using a phase contrast microscope. During the staining process, trypan blue entered dead, non viable sperm with damaged membrane and were stained blue. On the other hand, intact and live sperm appear unstained (Tartaglione and Ritta, 2004).

Sperm Morphology

To assess sperm morphological abnormalities, a semen sample of 100 mL was fixed in 500 mL of 1% formal citrate. The formal citrate solution was prepared by dissolving 2.9 grams of Tris- sodium citrate dihydrate and 1 mL of 37% formaldehyde solution in 100 mL distilled water. For examination, two hundred sperm per extender per replicate were observed under phase contrast microscope (Olympus BX20; X1000, Tokyo, Japan) using oil immersion. Various types of sperm abnormalities were noted, including: head abnormalities (detached heads, micro and macro heads, pyriform heads, double heads), mid piece abnormalities (distal droplet, abaxial attachment and proximal droplet) tail abnormalities (tail bent on mid piece, tail coiled below the head, double tail, and tail without head) (Zafar et al., 1988).

Sperm DNA Integrity

To assess sperm DNA integrity, the assay method described by Ejaz et al. (2014) was followed. Air-dried semen smear was prepared on a glass slide. The smear was fixed in a mixture of 96% ethanol acetone in a 1:1 ratio at 4ºC for 30min. The fixed smear was hydrolyzed for 10-30 min. with 4N HCl at 25 ºC. After this, the smear was rinsed with distilled water three times for two minutes each time. The slides were then stained with toluidine blue in Mcllvaine buffer (sodium citrate phosphate) for 10 minutes. DNA integrity of spermatozoa was examined using light microscope under oil immersion at 1000X magnification. Sperm that stained light blue were considered to have intact DNA, while spermatozoa that stained dark blue were considered to have damaged DNA.

STATISTICAL ANALYSIS

Data on seasonal variations in quality of cryopreserved buffalo sperm was analyzed by the t-test. A significance level of less than 0.05 was used to define a confidence interval as statistically significant.

RESULTS

Data on effect of season on quality of cryopreserved Nili Ravi buffalo semen are shown in Table 1. Results of present study suggested that sperm plasma membrane integrity, progressive motility, live/dead ratio, viability and DNA integrity was not affected by season of the year. However, number of morphologically abnormal sperms were higher in summer ($P<0.05$) compared to winter season.

Table 1. Effect of seasons on post thaw sperm progressive motility, plasma membrane integrity, liveability, viability and chromatin integrity in cryopreserved buffalo semen.

Season	Sperm Motility (%)	Sperm Plasma Membrane Integrity (%)	Sperm Liveability (%)	Sperm Viability (%)	Sperm Chromatin Integrity (%)	No. of abnormal sperms (%)
Winter	45.3±1.20	59.7±1.6	73.1±1.4	37.6±2.8	97.06±0.24	6.2 ± 0.86^{b}
summer	47.6±2.4	59.5±1.1	62.8±2.4	36.1±2.4	97.03±1.4	9.2 ± 0.73^{a}

Columns with different superscripts differ significantly ($P<0.05$)

DISCUSSION

Use of frozen semen plays major role in scientific breeding of buffaloes. For production of good quality buffalo semen for use in artificial insemination, it is essential to study the effect of non genetic factors on semen production and to implement corrective measures accordingly. As hot and humid climate prevail in Pakistan. So, a detailed knowledge of variations in semen characteristics related to is very crucial. In present study a comparison was performed between frozen-thawed sperm parameters in the Nili-Ravi buffalo semen during summer and winter seasons.

According to present study there was no effect of season on the post thaw sperm motility. Results of the present study are in accordance with previous studies in which it is reported that sperm motility did not differ with season (Brito et al., 2002; Fonseca , 1995; Koonjaenak et al. 1987; Heuer et al., 1987; Sarder et al., 2000). Additionally, sperm motility is influenced more by mating frequency rather than seasonal changes (Chacón et al., 2002). However, results of a study revealed that motility differs significantly between seasons as well between bulls (Tiwari et al., 2011). Higher values in winter have also been reported by Sagdeo et al. (1991) in Surti buffalo bulls, Nitharwal et al. (2017) and Sinha et al. (2021) in Murrah bull. The disparities between the current study and other research findings could be attributed to variations in the study duration and differences in breed and age of the bulls involved.

Present study suggested that post thaw sperm plasma membrane integrity of cryopreserved buffalo semen remained same during winter and summer seasons. These results are similar to previous studies suggesting a non significant effect of season on plasma membrane and acrosomal integrity of buffalo sperm (Bhosrekar et al., 1992 and Koonjaenak et al., 2007). However, results of present study are contrary to previous studies reported by Mandal et al. (2000), Shukla and Misra (2007) and Nitharwal et al. (2017) suggesting significantly high number of sperm with functional plasma membrane and intact acrosome in winter season compared to summer in Murrah buffalo bulls. Further, in Thai swamp buffalo the sperm plasma membrane integrity and stability is observed to be highest in winter than rainy and summer seasons (Koonjaenak et al., 2007). The difference might be due to some other contributing factors as seasonal effects are collectively influenced by various factors such as food quality, relative humidity, ambient temperature, and day length (Mathevon et al., 1998). Further, the difference observed might be due to variations in freezing methods, breed, procedure of evaluation, extender used, and thawing rate.

In present study, sperm viability and liveability didn't affected by seasonal variations (P>0.05). The study aligns with previous research work conducted by Chacón et al. (2002) which also found a non significant effect of season on the viability and acrosome integrity of sperm. In this study, neither ambient temperature and humidity nor the specific month (season) significantly affected sperm viability. The results suggest that the Nili Ravi breed of buffalo, being adequately fed, demonstrates an inherent ability to adapt well to harsh climate conditions. This adaptability allows them to maintain their reproductive capabilities at optimum levels even during both the hot summer and cold winter seasons. These findings highlight the resilience and reproductive efficiency of the Nili Ravi breed in various climatic conditions.

No difference in post thaw sperm chromatin integrity was observed in present study. The reason behind is that sperm chromatin is tightly twisted due to presence of very compressed proteins especially protamine-1 (Martins et al., 2007) and it help prevent the sperm chromatin damage during cryopreservation. Moreover, chromatin of cryopreserved buffalo sperm is damaged in worst cases. During cryopreservation, formation of ice crystals is responsible for lipid phase transition that ultimately results in rearrangement of membrane components including membrane lipids (Muller et al., 2008).

In present study higher sperm morphological abnormalities were observed in summer. Results of present study are supported by the previous studies (Sansone et al., 2000) suggesting that semen quality is highly affected by seasonal variations in dairy bulls as summer is the hottest period and in most of the bulls more abnormal tails, proximal droplets, total sperm morphological abnormalities and abnormal sperm head are more in summer than during winter (Sekoni and Gustafsson, 1987). The permeability of calcium is affected due to changes in membrane structure during cryopreservation. It causes a destructive increase in intracellular calcium level that ultimately changes sperm morphological attributes (Parks and Lynch, 1992). Another probable reason for increased sperm abnormalities during summer season might be that the sexual function of buffalo bulls is affected through changes in ambient temperature (Sekoni and Gustafsson, 1987) or photoperiod (Barth and Waldner, 2002). The increase in scrotal temperature during summer badly affected spermatogenesis (Januskauskas, 1995) that results in increased number of abnormal sperms during summer compared to winter season. Certainly, a more extensive and comprehensive study is needed, spanning a longer period of time and involving a larger number of experimental animals. This wider study should encompass the evaluation of spermatozoa during various seasons to establish a stronger association between seasonal variation and semen quality in buffalo bulls. The results of this research would provide valuable insights and help improve management practices for Nili Ravi buffalo breeding, ensuring optimal reproductive outcomes under different environmental conditions.

CONCLUSION

In present study, seasonal variations didn't affect sperm motility, acrosome integrity, viability, DNA integrity, and plasma membrane integrity, on cryopreserved buffalo bull semen that indicates the superiority and better adjustment of Nili Ravi buffalo bulls in the extremes of both temperature and humidity. The findings from our study also revealed a significant effect of season on sperm morphology. Ejaculates collected during the winter season exhibited a higher proportion of normal sperms compared to other season. However, sperm morphological abnormalities increased in summer. This suggests that increased scrotal temperature in summer has negative effect on spermatogenesis. Further investigations may be required to determine the underlying factors responsible for the observed seasonal effects on sperm morphology.

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