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Cryopreservation of Nili-Ravi buffalo bull semen: yearly postthaw analyses to depict climatic effects

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Abstract: The present study was aimed to observe the postthaw quality of Nili-Ravi buffalo bull semen cryopreserved during heat stress (hot summer months) and normal climatic conditions (fall, winter, and spring months). For this purpose, four (n = 4) adult regular donor Nili-Ravi buffalo bulls were selected to collect and cryopreserve their semen throughout the period of an entire year. The cryopreservation of semen was done following standard freezing protocol. The semen doses cryopreserved during heat stress and normal climatic condition were thawed to determine the sperm motility, motion kinetics, viability, plasma membrane integrity, acrosomal integrity, and morphological abnormalities (head, mid-piece, and tail). The results showed that sperm motility and sperm motion kinetics did not alter ($p > 0.05$) across heat stress and normal climatic period. The sperm head, mid-piece and tail abnormalities did not differ ($p > 0.05$); however, total sperm morphologic abnormalities were higher ($p < 0.05$) during heat stress period, whereas the lower ($p < 0.05$) sperm viability, sperm plasma membrane, and acrosomal integrity were observed during heat stress period. In conclusion, the total morphologic abnormalities, viability, and acrosomal and plasma membrane integrity of buffalo bull sperm cryopreserved during hot summer months are compromised due to heat stress.

Key words: Postthaw sperm quality, heat stress, cryopreservation, Nili-Ravi buffalo bulls

1. Introduction

Artificial insemination (AI) using cryopreserved semen is a well-known and worldwide applied technique used to enhance the genetic potential in bovine dairy herds. Until now, attempts are being made to maximize the utility of cryopreserved semen and to propagate the genetics of superior males through AI in different domestic animal species. The application and adaptation of AI using frozen-thawed semen for buffalo breeding is variable in different countries. However, poor freezability and fertility of frozen-thawed semen are the major constraints in adaptation of AI in buffalo [1, 2]. Several factors including age, season, heat stress, feeding regimens, cryopreservation techniques, and extender composition influence the quality of frozen buffalo semen [3-7].

Heat stress is one of the key factors that suppress spermatogenesis through the involvement of germ cells, Leydig and Sertoli cells by initiating the apoptotic and oxidative process in testes. Altered testicular biology in response to heat stress affects the sperm quality and subsequent fertilization and embryonic development [8]. Low level of libido and poor quality of fresh semen in buffalo bull due to heat stress [9] have been published under different climatic conditions [10-13]. It is indicated that fertility in buffalo is reduced whenever substandard fresh or frozen-thawed semen was used for insemination [1]. However, scanty information is available regarding the effect of climatic conditions including temperature detriments, humidity, and season on detailed postthaw quality of semen which is collected and cryopreserved during different months of the year. Therefore, the current study was designed to determine the post-thaw quality of Nili-Ravi buffalo bull semen, collected and cryopreserved during heat stress (hot summer months) or normal climatic conditions (fall, winter, and spring months).

2. Materials and methods

The experimental procedures were adopted after granting permission from institutional bioethical committee (PMAS-UAAR/2017-18/17/89).

2.1. Study area, climatic conditions, and husbandry practices

The study was conducted at a private semen production unit (SPU; Vital Sires Pvt. Ltd), Faisalabad, Punjab-Pakistan.

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The city Faisalabad 'experimental site' is situated in the subtropical zone (31°15′0″N, 73°03′0″E) with an altitude of 185 m above sea level. During experimental period, the data regarding climatic conditions were obtained from local office of the Pakistan Meteorological Department, Faisalabad. The temperature-humidity index (THI) was calculated for the assessment climatic intensity by using dry bulb temperature (db; °C) and relative humidity (RH; %) in the following formula [14]:

$$THI = db\ °C - [(0.31 - 0.31 \times RH) \times (db\ °C - 14.4)]$$

The calculated values of THI were referred as normal or comfort zone (<22.2), moderate intensity of heat stress (22.3 to 23.3), severe intensity of heat stress (23.4 to 25.6), and extremely severe intensity of heat stress (<25.7) (Table 1). The results of meteorological data indicated that April, May, June, July, August, and September fall into the category of severe-to-extremely-severe heat stress periods, whereas January, February, March, October, November, and December fall into the category of normal-to-moderate climatic conditions (Table 1). In the current study, impact of severe and extremely severe heat stress vs. normal and moderate climatic conditions on postthaw semen quality were compared. For this purpose, four regular semen donor Nili-Ravi buffalo bulls (n = 4) which were maintained at the same station from time of their puberty were selected. As this area is also considered home tract of Nili-Ravi buffalo, normal heat intensity and moderate heat stress were graded as comfort zone for these bulls.

The bulls were kept in individual pens in standard area along with loafing area. They were provided seasonal green fodder, roughages, and concentrates according to their body weight. All the bulls had free access to clean and fresh drinking water. The vaccination and deworming were followed according to annual schedule.

2.2. Semen collection and processing

The semen was collected twice a week from each bull throughout the period of 1 year by using an artificial vagina. The bulls were sexually stimulated by allowing once or twice false mountings prior to each collection. Following semen collection, the ejaculates were transferred to the lab and evaluated for macroscopic (volume, pH, and color) and microscopic (motility, concentration, and morphology) attributes. Afterwards, only qualifying ejaculates were further diluted with TCG-based semen cryodiluent (Tris 199.8 mM, citric acid 69.74 mM, glucose 55.5 mM, glycerol 7%, egg yolk 20%, streptomycin 1000 µg/mL and benzyl penicillin 1000 IU/mL) and final sperm concentration was kept 50 × 10^6/mL through dilution. The diluted semen was packaged into 0.5-mL French straws and processed for cooling. The cooling of extended semen samples, from 37 to 4 °C, was done in a duration of 2 h and kept for equilibration process in the next 2 h. The semen samples were frozen by placing them above the liquid nitrogen fumes and then preserved in liquid nitrogen (–196 °C) until postthaw analysis.

2.3. Semen evaluation for postthaw sperm quality

The cryopreserved semen straws were transported in liquid nitrogen containers from SPU (Vital Sires Pvt. Ltd.) to the

Table 1. Average values of ambient temperature (db; °C), relative humidity (RH; %), and temperature-humidity index (THI) in relation to heat stress intensity observed in the vicinity of semen production unit (SPU).

Month	Ambient temperature (db; °C)	Relative humidity (RH; %)	Temperature-humidity index (THI)	Intensity of heat stress
January	14.0	68.0	14.0	Normal
February	18.0	59.0	17.5	Normal
March	24.0	55.0	22.7	Moderate
April	30.0	42.0	25.4	Severe
May	33.0	36.0	29.3	Extremely severe
June	38.0	55.0	34.7	Extremely severe
July	38.0	75.0	36.2	Extremely severe
August	37.0	69.0	34.8	Extremely severe
September	33.0	69.0	25.6	Severe
October	29.0	59.0	23.2	Moderate
November	26.0	62.0	22.2	Normal
December	23.0	69.0	22.1	Normal

The values indicate the heat stress intensity ranging from normal to extremely severe (<22.2: absence of heat stress; 22.2 to <23.3: moderate heat stress; 23.3 to <25.6: severe heat stress; and 25.6 and more: extreme severe heat stress) [13].

Semen Analysis and Cryopreservation Laboratory, Faculty of Veterinary Sciences, Bahauddin Zakariya University, Multan. In the present study, a total of 192 frozen semen straws (four straws per bull randomly chosen per each month) from January through December 2019, were evaluated for post-thaw quality. The frozen semen straws were thawed by placing them in water bath at 37 °C for 30 s. The straws were cut to collect semen contents in prewarmed Eppendorf tubes for postthaw evaluation. The thawed samples were evaluated for sperm motility, motion kinetics, viability, plasma membrane integrity, acrosome integrity, and morphological abnormalities.

2.3.1. Sperm motility and sperm kinetics

Computer-assisted sperm analyzer (CASA) system (SCA®, Microptic S.L. Viladomat, Barcelona, Spain) was used for sperm motility and sperm motion kinetics assessment. The CASA specifications were appropriate for evaluation of buffalo sperm motility (10–80 µm particle size, 85% progressive motility threshold along 75 micron per second average path velocity). The included sperm motion kinetics were curvilinear velocity (VCL, µm/s), average path velocity (VAP, µm/s), straight-line velocity (VSL, µm/s), straightness of average path (STR, %), linearity of curvilinear path (LIN, %), wobbler (WOB, %), beat cross frequency (BCF, Hz), and amplitude of lateral head displacement (ALH, µm). The diluted semen samples (5 µL) were placed in warmed glass slides over heated specimen stage (37 °C). About 500 sperm (approximately 100 sperms per field per 2 s) characteristics were recorded from different points over the slides for each sample at magnification of 10×.

2.3.2. Sperm plasma membrane integrity

The sperm plasma membrane integrity was determined through hypoosmotic swelling test (HOST). The thawed semen sample was taken and put into prewarmed HOST solution. After an incubation period of 15 min at 37 °C, wet mounts were prepared and observed under phase contrast microscope. A total of 200 sperms were counted and graded as intact (sperm with coiled tail) and nonintact (no coiling of tail region).

2.3.3. Sperm viability

Assessment of sperm viability was carried out through eosin-nigrosin staining procedure. After placing and mixing the drops of eosin-nigrosin and semen over warmed glass slides, thin and uniform smears were prepared. The smears were air dried and observed using bright field microscope at 200×. This technique differentiates the dead and live sperm upon head coloration (colored heads were considered to indicate dead sperms and colorless heads were considered to indicate live sperms).

2.3.4. Sperm acrosome integrity

To observe the sperm acrosome integrity, a portion (50 µL) of diluted semen was mixed in 500 µL of 1% formal citrate containing 2.9% (w/v) trisodium citrate dehydrate for sperm fixation. Afterwards, the smears were prepared from this semen fixative suspension and observed under phase-contrast microscope at 1000× (oil immersion lens). About one hundred sperms were counted for normal acrosome or abnormal acrosome.

2.3.5. Sperm morphological abnormalities

The head, mid-piece, and tail sperm morphological abnormalities were classified over dried smear (diluted semen and 1% nigrosin-eosin stain) using bright filed microscope at 1000× (oil emersion lens).

2.4. Statistical analyses

The data were analyzed by using SPSS Statistics software (v. 17.0.1, SPSS Inc., Chicago, IL, USA). The climatic condition (comfort zone vs. heat stress) was the main factor; hence, Student's t-test was applied to compare the microscopic sperm variables between comfort zone and heat stress conditions. A value of $p < 0.05$ was considered significant.

3. Results

The results presented in Table 2 indicate heat stress period influence on cryosurvival of buffalo bull semen with respect to motility and sperm kinetics. The postthaw sperm progressive motility, total motility, and motion kinetics (VCL, VAP, VSL, LIN, STR, WOB, BCF, and ALH) did not alter ($p > 0.05$) across heat stress and normal climatic conditions. The viability, plasma membrane, and acrosomal integrities of sperm samples preserved during heat stress period were lower ($p < 0.05$; Figure). The head, mid-piece, and tail morphologic abnormalities of sperm either cryopreserved in normal or heat stress period remained

Table 2. Sperm motility and motion kinetics in buffalo bull semen cryopreserved under comfort zone (normal and moderate heat intensity) and heat stress (severe and extremely severe) conditions.

Variables	Comfort zone	Heat stress
Progressive motility	38.0 ± 1.6	36.1 ± 1.9
Total motility	70.3 ± 1.9	68.7 ± 2.1
VCL	57.3 ± 1.6	56.6 ± 1.9
VSL	28.4 ± 0.8	27.7 ± 1.1
VAP	35.6 ± 1.3	36.1 ± 1.2
LIN	42.5 ± 0.6	42.1 ± 0.8
STR	67.0 ± 0.5	65.9 ± 0.9
WOB	59.5 ± 0.7	59.3 ± 0.5
ALH	2.3 ± 0.06	2.3 ± 0.05
BCF	6.8 ± 0.2	7.1 ± 0.1

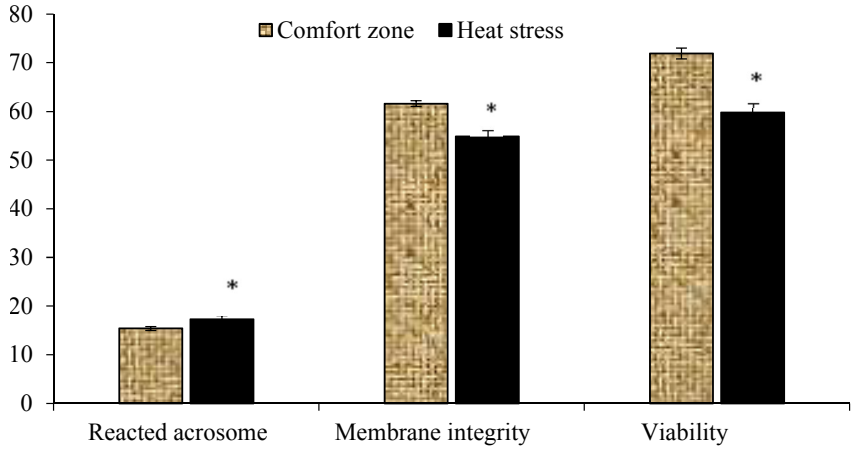

Figure. Variations in sperm viability, plasma membrane integrity, and reacted acrosome in cryopreserved during summer and comfort zone conditions. Asterisk (*) between the columns indicate significant difference ($p < 0.05$).

similar ($p > 0.05$). In contrast, there was a significant rise ($p < 0.05$) in total morphologic abnormalities of sperm preserved under heat stress conditions (Table 3).

Table 3. Sperm abnormalities present in buffalo bull semen cryopreserved under normal and heat stress conditions

Sperm abnormalities	Comfort zone	Heat stress
Head	6.4 ± 0.5	6.6 ± 0.4
Mid-piece	15.9 ± 1.5	18.2 ± 1.4
Tail	5.2 ± 0.5	5.1 ± 0.4
Total	27.7 ± 0.5 [b]	29.9 ± 0.6 [a]

Different superscripts (a, b) indicate significance difference ($p < 0.05$) between the parameters.

4. Discussion

The current study was aimed to observe postthaw sperm quality in buffalo bulls whose sperm were preserved under different climatic conditions. Detrimental effects of seasons on raw semen quality have been elaborated earlier in buffalo breeds located in different regions; however, there is paucity of information regarding Nili-Ravi buffalo bull semen in the context of season and cryopreservation [7]. The current data reveals that motility or motion kinetics of postthaw semen is not influenced by preservation either in normal or heat-stressed conditions. The individual morphologic abnormalities (head, mid-piece, or tail) also showed a similar trend; however, the total morphologic abnormalities and structural deformities (plasma membrane and acrosome) were increased when semen was collected and preserved under heat-stressed conditions.

In the current experiment, the impact of high THI was restricted to plasma membrane or acrosomal integrity in postthaw semen. It is seen that THI values (<25.6; severe heat stress) influenced the postthaw plasma membrane and acrosome integrity but severe or moderate type heat stress did not affect any other postthaw sperm attribute. This change might be related to conditions of buffalo bulls used in this experiment because these bulls were maintained in the same semen production unit from prepubertal life and spent enough time there to acclimatize to the local ambient conditions. In addition, the intricate anatomical and physiological systems of the scrotum and testes (pendulous scrotum, thermosensitivity of scrotal muscles or presence of pampiniform plexus in spermatic cord) could be another factor that regulated the heat stress mechanism in testes of buffalo bulls [15]. It has also been documented that chronic type of heat stress is combated by expression of heat shock proteins in testicular architecture [16]. It is noteworthy that the local thermal stress detrimentally influenced the fresh and postthaw semen quality in exposed male animals rather than the climatic conditions [17]. The observed positive or negative effects on semen quality might be closely related to cryopreservation, membrane structural composition, or low lipid contents in feeding ration rather than the environment. The mentioned mechanisms could be quantified by comparing the bulls exposed to high THI conditions and by applying the scrotal insulation during heat stress conditions in buffalo bulls.

The sperm motility assessment is one of the prime variables to judge the quality of raw and cryopreserved semen in males. Its accurate judgment provides an initial clue for sperm fertilizing capacity; therefore, it is essential to maintain a considerable sperm motility following cryopreservation process. There are different factors like frequency of ejaculation, collection methods, age, season,

diluents types, and freezing procedures that influence the motility variables in fresh and postthaw semen in every species [2]. Therefore, the estimation techniques for motility and motion kinematics could provide the variability in observations. It is quite natural that the motility drops significantly after cryopreservation because of physical, mechanical, or chemical stress. The current data explains that motility reduction is similar regardless of whether semen was collected and cryopreserved under heat stressed conditions or comfort zone time. Generally, the seasonal effects are negligible with respect to motility, whereas the effects of cryopreservation on pre- and postthaw motility rate are quite prominent in buffalo semen [12]. In previous studies, it has been observed that season has either significant [3, 7] or no [4, 12] effect on buffalo bull sperm motility after cryopreservation. These variations might be related to breed of buffalo [9], extender composition [3], freezing protocol [18], and motility assessment techniques [12]. This fact should be clearly elucidated by analysis and expression of different proteins associated with sperm motility across the seasons prior to cryopreservation in the future.

The findings of postthaw sperm viability and plasma membrane integrity described the detrimental effects of heat stress and freeze-thaw process. In the present study, low percentages of viable sperm with intact membrane indicate sensitivity of sperm to cryopreservation and thermal stress conditions. The structural rearrangement of sperm plasma membrane in freezing-thawing process is a well-known phenomenon; however, fragility of sperm membrane under thermal stress could exaggerate the cryopreservation effects. An association of season and sperm plasma membrane integrity in raw semen has been highlighted in different buffalo breeds [8, 10, 11] but none have clearly shown sperm plasma membrane sensitivity to cryopreservation process. The evaluation of membrane contents and comparison of different techniques for sperm viability and membrane integrity could reveal the correlation of season or freezing protocol to sperm viability and membrane integrity in buffalo semen.

The normal sperm acrosomal integrity is a prime prerequisite for capacitation, acrosome reaction, and fertilization in in vivo conditions. The presence of enzymes in normal acrosome also facilitates the sperm penetration and fertilizing capacity. Damaged acrosome [10, 12] or no change [8] in acrosome integrity in response to hot summer season was observed in raw buffalo semen previously. In contrast to our findings, Nitharwal et al. [4] documented no change in acrosome integrity after cryopreservation across the normal vs. heat stress states. Previously, the relationship of plasma membrane fragileness and hot stress has been postulated. The fragile plasma membrane is more prone to damages under freezing and thawing procedure because of destabilization of membrane content in response temperature phase transition that affects the acrosome contents as well and increase the chances of cryocapacitation and premature acrosome reaction [19]. To combat this factor, different membrane stabilizers could be used across the season to minimize the damage induced by thermal and cryopreservation stress in buffalo semen.

In the present study, total sperm abnormalities have increased in semen preserved during months of heat stress, whereas head, mid-piece, or tail sperm abnormalities did not fluctuate in response to semen freezability or season of semen cryopreservation. Previously, effect of heat stress conditions on sperm abnormalities in fresh semen were observed [8, 10] and tail sperm abnormalities were greatly influenced by hot summer season compared to other seasons [10, 12, 20]. The rate of abnormal sperm in cryopreserved buffalo bull semen is also affected by season of preservation [7]; however, the variations in head, mid-piece, or tail sperm abnormities have not been compared in cryopreserved semen across the seasons. Such variations might be associated to acclimatization of bulls, freezing protocol, or methods used for sperm morphology evaluation.

In conclusion, hot summer months and cryopreservation do not influence the buffalo bull sperm motility, motion kinetics, and morphological variables. In contrast, altered sperm viability, plasma membrane, and acrosome integrity indicate detrimental effects of heat stress in Nili-Ravi buffalo bulls.

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