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UMER FAROOQ

SYED AAMER MAHMOOD

IJAZ AHMAD

NAZIR AHMAD

MUSADIQ IDRIS

See next page for additional authors

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Evaluation of postthaw sperm parameters and fertility of Cholistani service bulls

Umer FAROOQ[1,*], Syed Aamer MAHMOOD[2], Ijaz AHMAD[3], Nazir AHMAD[4], Musadiq IDRIS[1], Muhammad Tariq ABBAS[1]
[1]University College of Veterinary and Animal Sciences, The Islamia University of Bahawalpur, Pakistan
[2]Semen Production Unit, Livestock and Dairy Development Department, Karaniwala, Bahawalpur, Pakistan
[3]The Imperial University, Lahore, Pakistan
[4]University of Agriculture, Faisalabad, Pakistan

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Abstract: The present study was conducted with the objective of evaluating Cholistani service bulls on the basis of their postthaw sperm parameters and fertilizing potential through artificial insemination under the prevailing local climate. Semen from each experimental bull (n = 6) was collected at weekly intervals over a 12-month period, frozen, thawed, and evaluated for postthaw sperm parameters. Fertility rate of postthaw samples was determined by inseminating 1200 Cholistani cows in 2 trials: Trial 1 had 600 cows with 100 inseminations per bull to assess the effect of individual bulls and Trial 2 had 600 inseminations with 150 insemination doses frozen per season to assess effect of seasons. The results revealed a significant effect ($P < 0.05$) of bulls and seasons on all the postthaw semen parameters studied. The overall fertility rate recorded in the present study was 69.7% (837/1200), being 68.5% (411/600) and 71.0% (426/600) in the first and second trials, respectively. The highest fertility rate of 75.3% (113/600) was noticed in the hot dry summer and the lowest of 67.3% (101/600) in the hot wet summer (67.3%). However, differences in fertility rate within bulls and within seasons were statistically nonsignificant ($P > 0.05$).

Key words: Cholistan Desert, fertility rate, service bulls

1. Introduction

The approach of attaining development in the livestock sector through indiscriminate crossing of indigenous livestock with exotic bulls or their semen in developing nations has merely resulted in dissolution of these thermo- and disease-resistant breeds with exotic ones (1). However, the dawn of this century has shown a sharp twist in realizing the role of locally adapted indigenous livestock of a region for sustainable agriculture in rural and suburban livelihoods and many countries have consequently initiated conservation programs for their indigenous livestock (2,3).

Ample work reported for Sahiwal cattle of Pakistan has rightly given this breed international recognition as a major milch breed of Pakistan. However, the potentials of other indigenous cattle breeds such as Cholistani still remain unknown. Cholistani is a large-sized humped flabby breed (*Bos indicus*) with elite specimen producing 15 to 20 L of milk daily. The average body weight varies from 500 to 600 and 300 to 400 kg in males and females, respectively (4). Owing to its speckled red, black, or brown body it is called 'flea-bitten' by the nomads of the Cholistan Desert. It is being reared by the nomadic pastoralists of the Cholistan Desert, Pakistan, and the details of livestock production systems in Cholistan were published earlier by our group (4). It is a major contributor to the socioeconomic status of Cholistani nomads. Cholistani cattle were incorporated in the Livestock Census 2006 of Pakistan for the first time to stay as a breed with an estimated 0.6 million heads in the Cholistan Desert of Pakistan.

Our group has also highlighted some aspects of performance traits for this indigenous cattle breed being reared by the nomads of the Cholistan Desert with preliminary data in 2010 (4). Later we directed our research towards assessing the reproductive profile of Cholistani bulls being reared for semen collection/breeding and published results on their hematochemical profile, libido and serum testosterone levels, physiochemical properties of fresh semen, and various readily measurable reproductive attributes such as scrotal circumference and paired testicular volume (5–9). The results elaborated the fact that this breed has an innate ability of being well adapted to the harsh, hot desert climate. This adaptability helps them maintain their reproductive abilities at an acceptable level even during stressful seasons of hot dry and hot humid summers (7). The present study is a

* Correspondence: umer.farooq@iub.edu.pk

continuation of our work conducted with the objective of evaluating Cholistani service bulls on the basis of their postthaw sperm parameters and fertilizing potential through artificial insemination (AI).

2. Materials and methods

2.1. Study area and seasons

The present study was conducted at the Semen Production Unit (SPU), Karaniwala, Bahawalpur, located in the Cholistan Desert of Pakistan. Sprawling over an area of 26,000 km^2, this desert is located at 27°42′N to 29°45′N and 69°52′E to 75°24′E at an altitude of about 112 m above sea level (4). The climate of this area is arid subtropical and continental with low sporadic rainfall, high temperature, low relative humidity, high rate of evaporation, and strong summer winds. It is considered to be one of the driest and hottest areas of Pakistan with an extensively lengthy summer season extending from May to October. The early part of summer (hot dry summer) extends from May to July with maximum temperature exceeding 45 °C. In the later part (hot wet summer), which extends from August to October, monsoon winds bring in negligible rains. Thus, the area receives minimum rains and remains water-scarce and drought-stricken. November, December, and January are the coldest months with temperatures ranging between 17 and 20 °C (6,7).

The study was conducted over a 12-month period from November 2012 to October 2013. Keeping in view the prevailing climatic conditions of the study area, four seasons of 3 months in duration each were defined as i) cool dry winter (November, December, January), ii) temperate spring (February, March, April), iii) hot dry summer (May, June, July), and iv) hot wet summer (August, September, October). The climatic data on ambient temperature, relative humidity, and rainfall in the study area during the study period was collected on a daily basis from the Regional Meteorological Center of Lahore, Pakistan (7).

2.2. Experimental animals

The study included adult Cholistani service bulls (n = 6) (KWC-19, KWC-21, KWC-24, KWC-25, KWC-27, and KWC-28), ranging between 6 and 10 years of age and having a mean body weight of 527.55 ± 4.5 kg (Table 1). They were housed individually in 10 × 12 m separate pens with 4 × 4 m shelter at the center. These bulls had clinically normal reproductive tracts and were giving semen of acceptable quality for AI. Their feeding regimen included good-quality seasonal fodder at the rate of 10% of their body weight, with 2 to 3 kg of concentrate (Anmol Wanda; Table 2) per bull per day. Water was provided ad libitum. Vaccination against hemorrhagic septicemia and foot and mouth disease was carried out as per schedule. Preventive measures against worm infestation were undertaken twice a year or whenever necessary (7).

2.3. Semen collection and initial evaluation

Semen from each bull was collected at weekly intervals at homosexual mount using an artificial vagina having a final temperature of around 42 °C. Two ejaculates were collected from each bull at each collection. However, some bulls occasionally failed to give a second ejaculate. Thus, a total of 543 ejaculates were obtained for further processing. Immediately after collection, each ejaculate was kept at 37 °C and evaluated for ejaculatory volume, mass motility (10× with phase contrast microscope; Olympus BH-2, Tokyo, Japan), individual sperm motility (40× with phase contrast microscope; Olympus BH-2), sperm concentration (photometrically at 560 nm wavelength with Bovine Photometer n° 1119, IMV, France), and percentages of morphologically normal spermatozoa and those with intact acrosome (100× with phase contrast microscope; Olympus BH-2). Details regarding semen collection and the results of initial evaluation of fresh sperm parameters were published elsewhere (7). Samples having >60% mass motility and individual sperm motility, 500 × 10^6 spermatozoa per mL concentration, and >70%

Table 1. Profile of Cholistani service bulls (n = 6) of the Semen Production Unit (SPU), Karaniwala, Bahawalpur (Cholistan), Pakistan, incorporated in the study.*

Bull #	Date of birth	Age at start of research in Nov. 2012	Date of entering SPU
KWC-19	30-11-2002	9 years, 11 months	24-08-2002
KWC-21	07-03-2003	9 years, 07 months	24-08-2002
KWC-24	14-12-2006	5 years, 11 months	08-06-2006
KWC-25	15-06-2006	6 years, 04 months	08-06-2006
KWC-27	15-05-2006	6 years, 05 months	08-06-2006
KWC-28	10-06-2006	6 years, 04 months	08-06-2006

*Data retrieved from the records of SPU Karaniwala, Bahawalpur, Punjab, Pakistan.

Table 2. Ingredients and nutritive value of concentrate (Anmol Wanda).

Ingredients	Inclusion level (%)
Cotton seed cake	18
Maize gluten 30	15
Canola meal	10
Maize grain broken	14
Wheat bran	25
Molasses	16
Mineral mixture[a]	2
Total	100
Nutritive value	
Crude protein	16
Total digestible nutrients	68

[a]100 kg of mineral mixture included: DCP 70.81 kg, NaCl 18.91 kg, $MgSO_4$ 8.64 kg, $FeSO_4$ 0.89 kg, $ZnSO_4$ 0.22 kg, $CuSO_4$ 0.03 kg, KI 8.77 g, $CoCl_2$ 0.89 g, and $NaSiO_3$ 1.50 g.

morphologically normal spermatozoa and those with intact acrosomes were included in the study.

2.4. Extension and freezing of semen

After initial assessment, the two ejaculates collected from each bull on each collection day were pooled and diluted at 25 to 30 °C by a slow one-step dilution method, using TRIS-fructose-egg yolk-glycerol extender (Tris 24.2 g, citric acid 13.4 g, fructose 10.0 g, glycerol 70.0 mL, streptomycin + penicillin 1.0 g, distilled water up to 1000 mL) to achieve the final concentration of 30×10^6 spermatozoa per insemination dose of 0.5 mL. The diluted semen was cooled to 4 °C, filled into 0.5-mL French straws, stored at 4 °C for an equilibrium period of 6 h, and frozen by a rapid method of freezing using a wide-mouthed freezing chamber containing liquid nitrogen (8). A total of 59,667 doses were frozen, being 11,484, 11,010, 18,771, and 18,402 for temperate spring, cool dry winter, hot dry summer, and hot wet summer, respectively. The frozen straws were stored in containers filled with liquid nitrogen for at least 24 h before their evaluation for postthaw quality or use in the fertility trial.

2.5. Evaluation of postthaw sperm parameters

Frozen samples were thawed at 37 °C for 30 s in a water bath and evaluated for postthaw sperm parameters in terms of sperm motility, viability, morphology, acrosomal integrity, plasma membrane integrity (PMI), and DNA integrity. Spermatozoa motility was assessed under phase contrast microscope (10), whereas viability was assessed through the vital staining technique (11). For the assessment of spermatozoa morphology and acrosomal integrity, each thawed semen sample was mixed with 1% formaldehyde citrate. At least 200 spermatozoa were examined under a phase contrast microscope and percentages of morphologically normal sperm and those with intact acrosome were recorded.

A hypoosmotic swelling test was applied to assess PMI of spermatozoa in each sample. Thawed semen was mixed with hypotonic solution and incubated at 37 °C for 45 min. After this, 0.5 µL of sample from the above incubated mixture was examined under a phase contrast microscope, as described previously (10).

For the estimation of DNA integrity of spermatozoa, acridine orange staining technique was used as described previously (12). A fluorescent microscope (Labomed Lx 400, USA) was used to examine the stained slides. Sperm heads showing green fluorescence were considered as having intact DNA, while those with red fluorescence were taken as having damaged DNA.

2.6. Fertility trial

Frozen thawed semen samples were used for an in vivo trial to compare their fertility on the basis of fertility rate. The primary source of water in the Cholistan Desert is rain water, which is stored in natural depressions or man-made ponds (called 'Tobas' in the local dialect) for use by humans and their animals (4,13). Various such ponds were identified within the desert having Cholistani cows settled near them. A total of 1200 inseminations were performed in Cholistani cows in 2 separate trials. In the first trial, 600 cows were inseminated with 100 inseminations per bull to assess the effect of individual bulls. Similarly, for the assessment of effect of seasons, 600 inseminations were carried out with 150 insemination doses frozen per season in the second trial. Cows in the 2nd to 4th lactations, having calved at least 60 days before insemination with clinically normal reproductive tracts and showing signs of true estrus (mucus discharge, restlessness, and standing heat), were selected for insemination. All livestock in the Cholistan Desert are reared under nomadic pastoral production system (4). Semen straws (0.5 mL) were thawed at 37 °C for 30 s and AI was performed with the help of an AI gun. Fertility rate attained in a single insemination was recorded on the basis of rectal palpation at days 50 to 60 after insemination.

2.7. Statistical analysis

Data on various postthaw sperm parameters were analyzed using SPSS 12 (SPSS Inc.) through one-way ANOVA. The difference between individual bulls and the four seasons was compared by Duncan's multiple range test. Differences in fertility rate within bulls and seasons were deducted using chi-square analysis. For all analyses, $P < 0.05$ was considered significant. Fertility rate has been given along with the 95% confidence intervals. Pearson's correlation coefficients were also calculated between various postthaw sperm parameters and fertility rate.

3. Results

The data on climatic conditions of the Cholistan Desert revealed that the highest (42.8 °C) and lowest (13.2 °C) mean temperatures were recorded for the hot dry summer and cool dry winter, respectively. Highest mean humidity (63%) was recorded for the hot wet summer with the lowest in the hot dry summer (37%). Similarly, highest mean rainfall (72 mm) was recorded for the hot wet summer, whereas no rain was noticed in the temperate spring. These variations are typical of the dry arid, tropic Cholistan Desert of Pakistan.

The overall mean values for postthaw sperm motility, viability, normal morphology, acrosomal integrity, PMI, and DNA integrity for Cholistani bulls in the present study were 58.6 ± 0.6%, 78.2 ± 0.6%, 78.3 ± 0.4%, 80.7 ± 0.3%, 75.6 ± 0.4%, and 98.4 ± 1.0%, respectively (Table 3). Bull-wise results showed that bulls KWC-24, KWC-25, and KWC-28 had the highest sperm DNA integrity ($P < 0.05$) at 97.2 ± 1.2%, 99.0 ± 0.9%, and 99.0 ± 0.8%, respectively (Table 3). Significant variation was observed in bulls for other sperm parameters as well, being lower for older bulls KWC-19 and KWC-21. Results of various postthaw sperm parameters as affected by various seasons (Table 4) indicated that sperm motility and viability were lowest ($P < 0.05$) during the temperate spring as compared to the other three seasons. Higher percentages ($P < 0.05$) of morphologically normal spermatozoa were noticed in the cool dry winter (80.8 ± 0.7%) and hot dry summer (80.4 ± 0.5%), whereas higher sperm DNA integrity ($P < 0.05$) was noticed in the hot dry summer (98.6 ± 1.1%) and hot wet summer (98.7 ± 1.1%), remaining lower in the cool dry winter and temperate spring.

The overall fertility rate recorded in the present study was 69.7% (837/1200) (95% CI, 67.1 to 72.3), being 68.5% (411/600) (95% CI, 64.7 to 72.2) and 71.0% (426/600)

Table 3. Effect of individual bulls on postthaw sperm parameters of Cholistani service bulls (n = 6).

Bull no.	Number of doses frozen	Sperm motility (%)	Sperm viability (%)	Normal morphology (%)	Acrosomal integrity (%)	Plasma membrane integrity (%)	DNA integrity (%)
KWC-19	228.7 ± 7.2^{a}	57.5 ± 0.6^{a}	70.5 ± 0.7^{a}	69.1 ± 0.5^{b}	86.1 ± 1.0^{a}	78.5 ± 0.4^{b}	82.1 ± 0.9^{a}
KWC-21	220.2 ± 7.0^{a}	40.6 ± 0.34^{b}	55.5 ± 0.5^{b}	55.1 ± 0.4^{a}	60.2 ± 0.7^{c}	58.2 ± 0.09^{a}	82.0 ± 1.1^{a}
KWC-24	176.2 ± 5.4^{b}	58.1 ± 0.01^{a}	76.4 ± 0.2^{a}	75.2 ± 0.03^{b}	72.6 ± 0.7^{bc}	72.2 ± 0.02^{c}	99.0 ± 0.9^{b}
KWC-25	202.0 ± 5.2^{ab}	56.2 ± 0.5^{a}	70.7 ± 0.7^{a}	75.5 ± 0.8^{b}	80.3 ± 0.9^{b}	81.8 ± 1.0^{b}	97.2 ± 1.2^{b}
KWC-27	187.5 ± 3.4^{b}	51.8 ± 0.5^{ab}	71.1 ± 0.6^{a}	74.0 ± 0.7^{b}	80.0 ± 0.8^{b}	80.5 ± 0.8^{b}	93.2 ± 1.0^{c}
KWC-28	282.2 ± 7.1^{c}	50.0 ± 0.5^{ab}	67.2 ± 0.5^{a}	72.0 ± 0.6^{b}	78.7 ± 0.9^{b}	76.3 ± 0.5^{bc}	99.0 ± 0.8^{b}
Overall mean	215.8 ± 5.4	58.6 ± 0.6	78.2 ± 0.6	78.3 ± 0.4	80.7 ± 0.3	75.6 ± 0.4	98.4 ± 1.0

[ab]Means within a column without a common superscript differ ($P < 0.05$).

Table 4. Effect of various seasons on postthaw sperm parameters of Cholistani service bulls (n = 6).

Parameters	Temperate spring	Cool dry winter	Hot dry summer	Hot wet summer	Overall mean
Number of doses frozen	159.5 ± 9.1^{a}	152.9 ± 8.7^{a}	260.7 ± 10.0^{b}	255.5 ± 7.2^{b}	211.5 ± 8.4
Sperm motility (%)	54.0 ± 1.3^{a}	58.1 ± 1.2^{b}	61.3 ± 0.9^{b}	60.2 ± 0.9^{b}	58.6 ± 0.6
Sperm viability (%)	70.7 ± 1.0^{a}	78.8 ± 1.5^{b}	81.4 ± 0.7^{b}	80.7 ± 0.7^{b}	78.2 ± 0.6
Normal morphology (%)	72.7 ± 1.2^{a}	80.8 ± 0.7^{c}	80.4 ± 0.5^{c}	78.0 ± 0.7^{b}	78.3 ± 0.4
Acrosomal integrity (%)	81.3 ± 1.1^{b}	84.3 ± 0.5^{c}	77.5 ± 0.7^{a}	80.0 ± 0.4^{b}	80.7 ± 0.3
Plasma membrane integrity (%)	79.6 ± 1.1^{b}	75.3 ± 0.7^{a}	74.0 ± 0.6^{a}	74.2 ± 0.6^{a}	75.6 ± 0.4
DNA integrity (%)	98.1 ± 1.0^{a}	98.3 ± 0.9^{a}	98.6 ± 1.1^{b}	98.7 ± 1.1^{b}	98.4 ± 1.0

[ab]Means within a column without a common superscript differ ($P < 0.05$).

(95% CI, 67.3 to 74.6) for the first (effect of individual bulls) and second (effect of seasons) trials, respectively (Table 5). Bull-wise data revealed the highest rate for KWC-28 (77%) (95% CI, 68.7 to 85.2), being lowest for KWC-19 (58%) (95% CI, 48.3 to 67.6). Results on seasonal variation showed the highest rate in the hot dry summer (75.3%) (95% CI, 68.4 to 82.2) and the lowest in the hot wet summer (67.3%) (95% CI, 59.7 to 74.8). Differences in fertility rate within bulls and within seasons were, however, statistically nonsignificant ($P > 0.05$).

The results of correlation coefficients between various postthaw sperm parameters and fertility rates are presented in Table 6. All the attributes were positively correlated to the fertility rate at a significance level of $P < 0.01$. The PMI and DNA integrity, however, showed higher values of 0.539 and 0.313, respectively.

Table 5. Fertility rate of Cholistani service bulls (n = 6) as affected by individual bulls and seasons.

Bull no.	Number of inseminations	Number pregnant (%)	P-value	95% CI
Effect of individual bulls				
KWC-19	100	58 (58)		48.3 to 67.6
KWC-21	100	67 (67)		57.7 to 76.2
KWC-24	100	70 (70)	0.38	61.0 to 78.9
KWC-25	100	71 (71)		62.1 to 79.8
KWC-27	100	68 (68)		58.8 to 77.1
KWC-28	100	77 (77)		68.7 to 85.2
Total	600	411 (68.5)		64.7 to 72.2
Effect of seasons				
Temperate spring	150	105 (70)		62.6 to 77.3
Cool dry winter	150	107 (71.3)	0.71	64.0 to 78.5
Hot dry summer	150	113 (75.3)		68.4 to 82.2
Hot wet summer	150	101 (67.3)		59.7 to 74.8
Total	600	426 (71)		67.3 to 74.6
Grand total	1200	837 (69.7)		67.1 to 72.3

Table 6. Pearson's correlation coefficient between various postthaw sperm parameters and fertility rate of Cholistani service bulls (n = 6).

Parameters	r-value
Sperm motility	0.301**
Sperm viability	0.253**
Normal morphology	0.204**
Acrosome integrity	0.303**
Plasma membrane integrity	0.539**
DNA integrity	0.313**

**Correlation is significant at $P < 0.01$ (2-tailed).

4. Discussion

The postthaw evaluation of semen from the Cholistani bulls in the present study included postthaw sperm motility, viability, normal morphology, acrosomal integrity, PMI, and DNA integrity. These parameters are considered to be more reliable and directly correlated to fertility than those of fresh semen (8). Extensive work has been reported from Pakistan on postthaw sperm parameters of Sahiwal service bulls (Zebu cattle) (14–17) and a comparison with our results on Cholistani service bulls indicates that the mean values for sperm motility, normal morphology, and acrosomal and DNA integrity were higher, whereas sperm viability and PMI were comparable to those for Sahiwal bulls. The difference in results could be attributed to variation in the handling of semen, number of bulls studied, degree of cryoinjury, environmental influence, and breed (8,14). Moreover, results can also vary from person to person, as sperm motility is usually assessed subjectively under microscope by naked eye.

It is generally accepted that during cryopreservation, the spermatozoa are subjected to chemical, physical, osmotic, and thermal stresses and the degree of damage normally depends upon breed, species, and method of cryopreservation (7,16). However, our results on postthaw sperm parameters of Cholistani service bulls when compared with those of fresh semen (for the same breed) published by our group earlier (7) reveal that the sperm of Cholistani bulls has an ability to withstand the stress during various stages of cryopreservation. A similar comparison with the results on Sahiwal bulls also indicates a higher postthaw PMI (75.6 ± 0.4%) in the present study as compared to 67.2 ± 4.1% (14) and 39.3 ± 3.2% (16) reported for Sahiwal bulls. This is indicative of the stable plasmalemma of Cholistani spermatozoa, which normally is considered to be the major site of damage during cryopreservation. A similar pattern of results was noticed for DNA integrity, as well.

The DNA packed inside the sperm chromosomes is being considered as the best indicator of fertilizing ability of spermatozoa (3). In the present study, overall DNA integrity of postthaw spermatozoa (98.4 ± 1.0%) is higher than that of earlier published reports. A study reported that percentage of sperm nuclear fragmentation in fresh and cryopreserved semen samples was 2.8 ± 0.4% and 10.7 ± 0.01%, indicating that 89.2% of the sperm were with intact DNA after cryoprotection (18). Similarly, another study (19) reported 8.3 ± 4.4% of fragmented DNA in spermatozoa of Sahiwal bulls, indicating that 91.6% of sperm had intact DNA, a value lower than that of the present study. More spermatozoa with intact postcryopreservation DNA recorded in the present study might be indicative of an inherent trait of the Cholistani breed to tolerate the damaging cryoprotective process.

Bull-wise data on postthaw sperm parameters showed significant variation within bulls. Though the bulls of the present study were kept under almost similar management, they had age differences. The younger four bulls, aged 6 years, had better postthaw sperm parameters as compared to the older ones. These results are in line with previous work that reported that breeding bulls of 6 years old or less had satisfactory semen quality with no statistical difference between this age group (20). Bull-to-bull variation in the present study is also in line with previously published extensive work that elucidated that there is a gradual downfall in sperm parameters of service bulls with growing age (15–17). This has been attributed to age-related testicular atrophy and fibrosis (15,16). However, hereditary inclination cannot be overruled.

Results of seasonal influence on postthaw sperm parameters of Cholistani bulls revealed encouraging results during seasons of extreme weather (hot dry summer, hot wet summer, and cool dry winter). These results are not in line with any of the previously published reports that demonstrated that sperm parameters (both fresh and postthaw) of zebu bulls (*Bos indicus*) deteriorate during the hot dry summer and hot wet summer owing to the stressful extremes of climatic conditions during these seasons (15,17,21,22), being acceptable only during the temperate season. In the present study, the acceptable level of postthaw sperm parameters of the Cholistani bulls even in seasons of extreme climatic conditions is indicative of their adaptability to the harsh hot desert climate without any compromise of their reproductive ability (7,8).

Using frozen thawed semen of Cholistani service bulls, the overall fertility rate per single insemination recorded in the present study (69.7%) is quite satisfactory and higher than those of earlier reports on fertility rate from Pakistan, including rates as low as 27.1% in cattle inseminated under field conditions (23) and 60% in zebu bulls when thawing of frozen semen was carried out at lower than the recommended temperature (24). Contrary to our results, a higher fertility rate of 78.7% was recorded in Sahiwal cattle when a streptomycin and penicillin combination was used in semen extender (25). However, the same workers reported a 69.6% fertility rate when the combination of gentamycin, tylosin, and linco-spectin was added to semen extender. The number of animals used in that study was too low (100 inseminations per extender type) to draw any conclusion. Besides variation in postthaw semen quality, differences in geography, feeding, and management and the AI technique and protocol of fertility trials might be plausible causes of these variations among different studies (23). A previous study conducted to assess the sexual behavior of zebu bulls (Brahman) in the humid tropics of Costa Rica reported lower fertility rates of 28% and 37% for single-sire and multiple-sire mating groups, respectively (26). Relatively higher fertility rate in the present study is also supported by the evidence that among the frozen semen doses of Sahiwal, crossbred, exotic, and Cholistani breeds collected at the Frozen Semen Bank, Punjab, Pakistan, the Cholistani bull semen is of the highest demand.

Extensive work has been reported in the literature regarding seasonal influence on semen quality and freezability for *Bos taurus* and *Bos indicus* bulls (17,21,22). However, to the best of our knowledge, data regarding effect of season on fertility rate are quiet scanty. Higher fertility rate during the hot dry summer in the present study can be correlated to the better quality of semen recorded in that season.

Regarding the results of correlation coefficients between various postthaw sperm parameters and fertility rate, it was noticed that all the postthaw sperm parameters showed significant correlation with fertility rate, the values being relatively higher for PMI and DNA integrity than for other parameters. These results suggest that postthaw sperm parameters, especially PMI and DNA integrity of sperm, are reliable indicators of fertility, as has been suggested previously (27,28). In addition, this is supported by the highest fertility rate noticed in the present study for bulls KWC-24, KWC-25, and KWC-28 which revealed the highest DNA integrity of spermatozoa.

Based on the results described here, it appears that the degree of damage incurred during cryopreservation of semen in these bulls is quite low. Though season had a significant effect on various postthaw sperm parameters in the present study, they were acceptable, even in extreme climatic conditions. Age had detrimental effects on postthaw sperm parameters, especially on PMI and DNA integrity. The field fertility trial clearly revealed that Cholistani bulls have reasonably good fertility rates (per single insemination) throughout the year and across a wide a range of age. The government, researchers, and all the stakeholders need to devise a directional approach towards conservation and upgradation of the Cholistani breed of cattle based on similar patterns as those for Sahiwal cattle.

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