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Cross Breeding Promotes Deterioration of Semen Quality in Cattle Bulls

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ABSTRACT

Nine healthy mature bulls having average age 3 ± 0.14 years, were selected in three different groups *i.e.,* Sahiwal, Holstein-Friesian and crossbred (Sahiwal x Holstein-Friesian) to compare the semen quality. Semen samples were individually diluted with egg yolk extender and tested for individual motility, progressive motility, plasma membrane integrity, acrosomal and DNA integrity, seminal pH and dead sperm percentage in fresh and thawed semen. Data were subjected to one way analysis of variance. High sperm individual motility (85.12 ± 0.51%), progressive motility (20.25 ± 0.47%), plasma membrane integrity (82.25 ± 0.43%), acrosomal integrity (79.91 ± 0.51%), and DNA integrity (98.54 ± 0.14%) were observed in fresh semen in Sahiwal bulls, which were significantly ($P < 0.01$) higher compared with the other cattle bulls. High dead sperm percentage (51.87 ± 0.50%), deterioration of plasma membrane integrity (38.29 ± 0.45%), pH (6.83 ±0.01) and DNA integrity (92.45 ± 0.28%) were observed in frozen semen in cross-bred bulls, which showed significant ($P < 0.01$) differences with the other cattle bulls. The results imply that Sahiwal bulls showed the best semen qualitative characteristics in fresh and frozen semen conditions followed by Holstein Friesian bulls. Moreover, the semen quality declined in the crossbred bull.

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IMK and AK conducted th experiments, ZU and HR arranged and analyzed the data, RUK and MSQ designed the experiments and supervised the research, TU analyzed the data and wrote the paper.

Key words
Cattle bulls, Pure breed, Crossbred, Semen quality.

INTRODUCTION

In the tropical and subtropical countries cross breeding was practiced during mid-seventies in order to improve herd productivity by cleaving the desired characteristics of higher dairy potential of *Bos Taurus* and enhanced resistance to heat stress and local diseases of *Bos Indicus* (Usman *et al*., 2014). The idea was to generate crossbred offsprings with early maturity, superior dairy characteristics and adaptability to harsh local environmental conditions (Usman *et al*., 2013; Sattar and Mirza, 2009). It has been suggested that over 50% crossbred juvenile bulls invested for semen collection had complications with quality of semen, cryo-tolerance and libido (Mukhopadhyay *et al*., 2010). Indigenous or pure breed bulls had comparatively higher freezing ability and fertility of spermatozoa than crossbred bulls (Gulia *et al*., 2010). Due to the poor semen (initial) quality nearly 50-55% of the ejaculates collected from the crossbred bulls, were unsuitable for freezing (Sudheer and Xavier, 2000). Various efforts have been made in order to isolate the etiology of poor quality semen production in crossbred bulls at seminological, andrological, genetic and probable ecological stages with varied success (Gulia *et al*., 2010). Due to the presence of some defective genes in bovine semen carrying special effect resulted in primary deformities. These deformities were comprised of acrosome shortcomings (ruffled, incomplete and knobbed), head deficiencies (decapitated, round head, abnormal head condensation, rolled head and nuclear crest), mid-piece defects (corkscrew, pseudo-droplet and dag defects) and tail abnormalities (Chenoweth, 2005). It was reported that cross bred progeny is more prone to reproductive problems (Bitew and Prasad, 2011). One of the important factors of sub fertility in cattle is the use of substandard semen in artificial insemination (Gnoth *et al*., 2005). Generally the fertility is more vital in case of bull compared with cow, because one bull might be used for breeding nearly up to 40 cows through natural service or possibly hundreds thousands by means of artificial insemination. Though 20-40% bulls may have demoted fertility very few are entirely sterile (Coulter and Kastelic, 1999). A bull is considered to be selected for artificial insemination if its semen sample contains number of motile and live spermatozoa to reach the site of fertilization. Various tests have been conceived to assess the quality of semen in terms of viability, motility,

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concentration, acrosomal integrity and morphology (Amann and Hammerstedt, 1993; Pace *et al*., 1981). The present study was conducted to compare the semen quality of pure and crossbred cattle bulls under fresh and frozen conditions and to identify the best breed for the purpose of artificial insemination.

MATERIALS AND METHODS

Fresh semen assessment

Selection of bulls

Nine physically and andrologically healthy mature bulls of three different groups *i.e.* Sahiwal, Holstein-Friesian and crossbred (Sahiwal x Holstein-Friesian) at Semen Processing Unit in the Cattle Breeding and Dairy Farm Harichand were selected in order to compare the semen quality of pure and crossbred cattle bulls. The average age of bulls was 3 ± 0.14 years. All the experimental bulls were kept under the same shade and same feeding regime. Semen was collected from each bull early in the morning twice a week (Monday and Thursday). The experiments were continued for four weeks.

Extender preparation

For the extension of sperm cell life, extender was prepared using Tris-citric egg yolk extender solution having a normal pH and standard osmotic pressure of 320 mOsmol Kg-1 for cattle. This extender consists of 1.56% of citric acid (Fisher Scientific, Loughborough, Leicestershire, UK), 3.0% buffer extender (Tris-hydroxymethyl), (Research Organics, Cleveland, OH, USA) and distilled water used as a solvent. Glycerol level 7% (Merck, Darmstadt, Germany); fructose (Scarlau, Barcelona, Spain) 0.2%; egg yolk 20% were used in extended solution. Antibacterial agent's procaine penicillin 300 IU/mL, streptomycin sulphate (1 mg/mL) and benzyl penicillin 100 IU/mL were also used in extended solution (Ansari *et al*., 2011).

Semen collection

Semen was collected in a graduated tube from each experimental bull with a gap of 10 to 15 min using a teaser bull. The semen ejaculates having more than 60% motile spermatozoa, more than 1 mL semen volume and more than 0.5 billion/mL sperm concentration were considered for further semen quality tests (Ansari *et al*., 2011). For initial semen quality evaluation tests, the semen collected was immediately shifted to water bath at 34°C.

Semen evaluation

The following parameters were evaluated in fresh diluted semen of each bull.

Determination of seminal pH: The pH was measured soon after dilution of fresh collected semen through pH meter. First of all, the semen sample was shacken properly and the normal color of the semen was checked. Later on, semen drops were taken and spread equally on pH paper. After 15 seconds the color of pH paper became uniform and impregnated, the color was then compared with the pH chart and the semen pH was decided considering 6.7 as the normal pH of semen (Haugen and Grotmol, 1998).

Determination of sperm individual motility and progressive motility: A single drop of diluted fresh semen on pre warmed glass slide was taken and cover slip was placed over it. The individual motility percentage of sperm was visualized with a compound microscope at 400X at 34°C (Qureshi, 2011). Under microscopy, 100 spermatozoa were counted at five different fields. All those sperms, which showed any type of movement, were considered as motile spermatozoa while those sperm, which move in straight direction, were concluded as progressive motile.

Determination of sperm plasma membrane integrity: Sperm plasma membrane integrity percentage was evaluated via hypo osmotic swelling test (Qureshi, 2011). A 0.1 mL diluted fresh semen was taken and mixed with 1 mL HOST solution which consists of 0.5 mL of D-Fructose (1.47%), sodium citrate (2.7%) and Eosin 0.5% (w/v). This mixed solution was incubated at 37°C for 40 min. After an incubation time, 5µL of sample with the help of micropipette was taken and kept on pre warmed glass slide. Cover slip was placed and visualized at 40X under compound microscope. A total of 100 spermatozoa were counted at five different fields. The HOST solution provided hypotonic media for spermatozoa, which caused coiling and swollen tail sperm due to different osmotic potential. Those sperm cell which showed swollen, coiled tail and unstained head represent intact plasma membrane integrity, while those with unswollen, uncoiled tail and pink head showed inactive sperm plasma membrane integrity.

Determination of dead spermatozoa: For dead sperm ratio, Eosin and Nigrosin stain was prepared, mixing 1% Eosin and 5% Nigrosin in 3% sodium citrate dehydrate solution (Zemjanis, 1970). Nigrosin provides a background for sperm cell visualization. Whereas, Eosin goes into the dead sperm cell membrane and highlightspink color for identification of dead sperm. A small drop of fresh diluted semen was taken on clean pre warmed slide and then added a comparatively large drop of stain, mixed it well enough by means of applicator stick for 30 seconds. A thin smear then was made, and air dried for 1 to 3 min. After drying, observed the slide under the compound

microscope at 100X. The spermatozoa with unstained heads were considered live while those sperms which had stained heads or partial stained were considered dead spermatozoa. A total of 100 spermatozoa were counted at 5 different microscopic fields, and dead sperm percentage was calculated.

Determination of sperm acrosomal integrity: Trypan-blue and Giemsa stain were conducted by the method of Kovacs and Foote (1992). In this protocol, trypan-blue was used to differentiate live and dead sperms and Giemsa stain was used for evaluation of sperm acrosomal integrity test. One drop of Trypan-blue (2.0%) was mixed with one drop of fresh diluted semen on a clean pre warmed slide, mixed by means of clean cover slip edge. The slide was then allowed to dry in air and formaldehyde-neutral red solution [86 ml IM HCl + 14 ml of Formaldehyde (Merck, Darmstadt, Germany) + 0.2g Neutral red (MP Biomedicals, Eschwege, Germany)] was used for 5 min for fixation. Slide was then washed with running distilled water and dipped in Giemsa stain (MP Biomedicals, Eschwege, Germany) (7.5%) for 4 h. After staining with Giemsa stain, slides were again rinsed with distilled water and kept in air to dry and cover slip was placed before covering with Balsam of Canada (Merck, Darmstadt, Germany). In each smear five different fields were evaluated to count a total of 100 sperms under phase contrast microscope at 1000X (LABOMED LX400).

Determination of sperm DNA integrity: A 200 µL of TNE buffer was mixed with 20 µL diluted fresh semen at 37 °C to get the final concentration of 2×10^6 spermatozoa/mL and clean glass slides were used for the preparation of three smears from each semen aliquot and air dried. The smears were dipped overnight in Carnoy's solution [methanol and glacial acetic acid (Merck, Darmstadt, Germany) in 3:1 ratio] for fixation. Slides were then allowed to dry in air and shifted to tampon solution (80 mM citric acid (Merck, Darmstadt, Germany) and 15mM Sodium phosphate (Sigma, NY, USA), pH 2.5) for 5 min. in water bath at 75 °C. After incubation in tampon solution, acridine orange (Sigma, NY, USA), pH 2.5) (0.2 mg/mL) was applied on slides. Slides were then covered with cover slip, while they were still soaked and evaluated under fluorescent microscope (LABOMED LX400). Acridine orange is fluorescent cationic dye having affinity for a nucleic acid and permeable to sperm cell interacts with single or double strand DNA by the process of intercalation or electrostatic attractions respectively. In each smear five different fields were evaluated to count a total of 100 sperms. Sperms DNA with green appearance indicated intact while sperms with disrupted DNA showed yellow green to red appearance (Evenson *et al*. 2002).

Post-thaw semen assessment

Semen evaluation

The semen was collected from each bull and diluted with Tris-egg yolk extender and kept in water bath for 10 min. After extension the concentration of motile spermatozoa in extended semen was $50x10^6$ per mL which was then forwarded to freezing process. The cooling was started from water bath temperature to 25°C and the changes were brought within 20 min. Further cooling started from room temperature to 4°C within 2 h and shifted to equilibration time for 4 h at 4°C (Leite *et al*., 2010). Straws filling were started with suction pump at cooled cabinet unit, kept on liquid nitrogen vapors for 10 min at 5 cm height and the straws were then plunged into liquid nitrogen and stored. After cryopreservation semen straws were thawed, incubated for 6 h and subjected to frozen semen quality tests (Ansari *et al*., 2011). After thawing the following tests were performed *i.e.,* the seminal pH detection, individual and progressive motility, dead sperm, plasma membrane integrity, acrosomal integrity and DNA integrity for each bull in frozen semen.

Statistical analysis

Results of the study were presented as means ± SEM. To compare the fresh and frozen semen quality, sperm motility, progressive motility, membrane integrity, acrosomal integrity, semen pH, and DNA integrity, data were analyzed using two factorial designs. Duncan multiple range test was used to find the significance. A P value ($P < 0.01$) was statistically considered significant.

RESULTS

The results of the present study showed that membrane integrity, progressive motility, individual motility, acrosomal integrity and DNA integrity percentage were significantly high in fresh and frozen semen in Sahiwal breed compared with crossbred bulls ($P < 0.01$, Table I). Whereas, the seminal pH and dead sperm percentage were significantly high in the fresh and frozen semen of cross bred bulls compared with Sahiwal breed bulls ($P < 0.01$, Table II).

DISCUSSION

The current research was conducted at Government Cattle Breeding and Dairy Farm Harichand Charsadda and National Agriculture Research Council (NARC) Islamabad to find the comparison between semen traits of fresh and frozen semen in pure and crossbred bulls.

Table I.- Membrane integrity, progressive motility, individual motility and acrosomal integrity (Mean ± SE) of fresh and frozen semen of Holstein-Friesian (HF), Sahiwal and crossbred bulls (Sahiwal x Holstein-Friesian).

Semen types	Breed	Membrane integrity (%)	Progressive motility (%)	Individual motility (%)	Acrosomal integrity (%)
Fresh semen	HF	81.66 ± 0.48^{ab}	17.87 ± 0.35^{b}	84.16 ± 0.54^{a}	71.50 ± 0.53^{b}
	Sahiwal	82.25 ± 0.43^{a}	20.25 ± 0.47^{a}	85.12 ± 0.51^{a}	79.91 ± 0.51^{a}
	Cross	80.62 ± .69^{b}	17.54 ± 0.42^{b}	82.08 ± 0.91^{b}	62.58 ± 0.67^{c}
	P value	0.001	0.001	0.001	0.001
Frozen semen	HF	69.20 ± 0.39^{b}	13.91 ± 0.33^{b}	71.08 ± 0.29^{b}	63.20 ± 0.31^{b}
	Sahiwal	78.58 ± 0.45^{a}	16.00 ± 0.30^{a}	76.54 ± 0.55^{a}	70.95 ± 0.47^{a}
	Cross	38.29 ± 0.45^{c}	8.70 ± 0.22^{c}	40.00 ± 0.35^{c}	35.83 ± 0.56^{c}
	P value	0.001	0.001	0.001	0.001

Mean values bearing different superscripts in a column differ significantly (P<0.01)

Table II.- Seminal pH, DNA integrity percentage and dead sperm percentage (Mean ± SE) of fresh and frozen semen for Holstein-Friesian (HF), Sahiwal and crossbred bulls (Sahiwal x Holstein-Friesian).

Semen types	Breed	pH	DNA integrity (%)	Dead sperm (%)
Fresh semen	HF	6.57 ± 0.01^{c}	95.08 ± 0.16^{c}	13.04 ± 0.44^{ab}
	Sahiwal	6.65 ± 0.01^{b}	98.54 ± 0.14^{a}	12.62 ± 0.44^{b}
	Cross-bred	6.67 ± 0.01^{a}	95.04 ± 0.22^{c}	14.12 ± 0.83^{a}
	P value	0.001	0.001	0.001
Frozen semen	HF	6.75 ± 0.01^{b}	93.37 ± 0.16^{b}	22.20 ± 0.32^{b}
	Sahiwal	6.77 ± 0.01^{b}	96.70 ± 0.16^{a}	19.04 ± 0.50^{c}
	Cross-bred	6.83 ± 0.01^{a}	92.45 ± 0.28^{c}	51.87 ± 0.50^{a}
	P value	0.001	0.001	0.001

Mean values bearing different superscripts in a column differ significantly (P<0.01)

Three different cattle breed bulls were selected in this research *i.e.* Sahiwal, Holstein-Friesian and cross-bred (Sahiwal x Holstein-Friesian), respectively. Each breed was tested for fresh and frozen semen condition to evaluate the semen quality. The data showed that sperm membrane integrity percentage of the semen was significantly ($P < 0.01$) high in pure breed (Sahiwal, Holstein-Friesian) compare with crossbred (Sahiwal x Holstein-Friesian) bull sperms. Sperm with intact plasma membrane plays a vital role to maintain and establish cellular homeostasis (Hammerstedt *et al*., 1990). Semen processing for future uses needs proper freezing and the spermatozoa are susceptible to freezing, which alters the sperm membrane activity. The degree of sperm membrane damage differs from any sort of changes in organization, membrane permeability, fluidity and lipid composition of sperm membrane bilayer (Thomas *et al*., 1998). It has been suggested that plasma membranes integrity in cross-bred bull sperms are affected more significantly than pure breeds during cryopreservation (Thippeswamy *et al*., 2014) which is in line with the results of the present study. Tyagi *et al*. (2003) demonstrated that reasons for culling the crossbred bulls from semen production stations were either their poor semen quality in fresh and frozen states or semen production was not suitable for further processing and cryopreservation and unacceptable for post thaw membrane integrity that satisfy the recommended standards for AI.

The research data showed that progressive motility percentage of the semen was significantly high in local Sahiwal bulls than crossbred, these results are in line with the study of Sudheer and Xavier (2000). The results of the present study are in agreement with various findings in the previous studies and found significant changes in progressive motility of sperm at different semen condition. The progressive motility can be defined as, those spermatozoa which move in a straight line direction in a microscopic field (Qureshi, 2011). Most damages to sperm occurs during semen freezing and thawing (ice crystallization and extra production of ROS) which deteriorate the plasma membrane and retarded the sperm movement (Aitken *et al*., 1998). In a study conducted on Frieswal crossbred (Friesian x Sahiwal) bulls, it was reported that 55% of the ejaculates produced by the crossbreed bulls were unsuitable for cryopreservation and the sperm progressive motility were unable to fertilize the ovum (Sudheer and Xavier, 2000).

In the present study, pure breed bull showed significantly higher individual motility than cross bred bull. We can define individual motility percentage as the spermatozoa with any movement in microscopic field called sperm individual motility (Qureshi, 2011). Sperm motility is the reliable tool that determines the fertility and viability of sperm (Janett *et al*., 2008). It is well known that ice crystal formation in mitochondria and axonemes

during cryopreservation impairs sperm individual motility (Courtens *et al*., 1989). A large number of Frieswal (Holstein-Friesian x Sahiwal) bulls were rejected from semen station mostly due to the poor semen quality and post thaw motility (Mandal *et al*., 2012).

The sperm acrosomal integrity percentage was higher for pure breed bulls than cross-bred bull semen samples. The cranial end of spermatozoa nucleus is overlapped by acrosome and double layered thin membranous sac during the last stage of sperm formation. Intact acrosome cap of frozen thawed semen is directly related to the fertility of bulls because intact acrosome damages the zona-pellucida by means of hydrolytic enzyme, during the process of fertilization (Saacke and White, 1972). Prior to fertilization, sperm undergoes capacitation. Cryopreservation induces disturbance in premature capacitation, resulting in low fertility of frozen semen (Watson, 2000). In crossbred bulls, the morphology of abnormal spermatozoa might possess damaged 50%-55% acrosome and at least of the ejaculates were unsuitable for freezing due to the poor initial semen quality (Sudheer and Xavier, 2000).

Normal pH of bull semen is 6.7. Storage of semen at room temperature may decrease the pH due to production of lactic acid from fructose (Qureshi, 2011). Semen pH may become alkaline when dead sperm percentage is high (Qureshi, 2011). The maximum average pH was obtained from crossbred bull semen after thawing the semen (Hossain *et al*., 2012). The presented data is supported by the above findings as for crossbred bull semen, having large number of dead sperm which results in higher pH as compared to the pure bred bull frozen semen samples.

The present data show that DNA integrity in fresh and frozen semen showed significantly higher value for pure breeds (Sahiwal, Holstein-Friesian) than crossbred (Sahiwal x Holstein-Friesian) bull semen. Sperm DNA is the molecular basis of heredity, constructed of a double helix held together by hydrogen bonding (Balhorn *et al*., 2000). DNA starts division and reunion during spermatogenesis of sperm in meiotic cell division (Rajendera *et al*., 2011). The different steps of cryopreservation *i.e.* dilution, cooling, equilibration time and thawing of frozen semen enhance production of reactive oxygen species (Baumber *et al*., 2005). DNA integrity is highly susceptible to increase production of ROS, which occurs at plasma membrane (Kankofer *et al*., 2005). The excessive ROS production is the major cause of damage to the sperm DNA integrity and this damage is observed comparatively more in crossbred bulls than pure (Garg *et al*., 2008). Some outdoor factors that also influence the sperm DNA structure include types of semen extenders, dilution methods, duration of incubation and thawing of cryopreserved semen (Hammadeh *et al*., 1999).

Dead sperm percentage value was significantly high for cross-bred bulls followed by pure breed bull semen samples. Sperm viability is one of the most important requirements of fertile semen (Hossain *et al*., 2012). Both freezing and thawing implicate tremendous alterations in cell water volume, which result in considerable mechanical stress on the membrane and consequently reduce sperm viability (Hammerstedt *et al*., 1990). The sperm plasma membrane is the primary site of damage induced by cryopreservation, which affects the viability of sperm (Hammerstedt *et al*., 1990). Average motility of sperm before and after freezing varied, which indicate that freezing of semen reduced sperm motility and increased dead ratio irrespective of breed.

CONCLUSION

Semen from cross-bred bulls showed more susceptibility to damage in fresh and cryopreservation than pure breed bull semen. Among the different cattle breed bulls Sahiwal showed the best semen quality traits.

Statement of conflict of interest

Authors have declared no conflict of interest.

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