Investigations on Fertility Related Biomarkers in Buffalo Semen to Reduce Male Factor Losses (MFLs)

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2016

Investigations on Fertility Related Biomarkers in Buffalo Semen to Reduce Male Factor Losses (MFLs)

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

By

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ISLAMABAD, PAKISTAN

2016

IN THE NAME OF ALLAH

THE MOST BENEFICIAL

THE MOST MERCIFUL

AND

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CERTIFICATE

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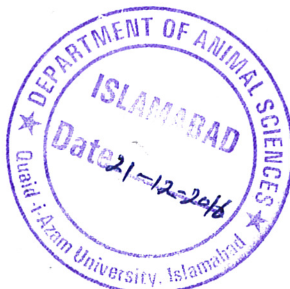

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DECLARATION

I hereby declare that the material contained in this thesis is my original work. I have not previously presented any part of this work elsewhere for any other degree.

Hussain Ahmed

DEDICATION

DEDICATED TO MY MOST HUMBLE

SUPERVISOR,

CO-SUPERVISOR

AND

LOVING PARENTS

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ACKNOWLEDGEMENTS

From the depth of my heart I express my deep sincere gratitude to the Almighty omnipresent **Allah Ta'ala** for the Blessings He had bestowed upon me, for answering my prayers, for giving me the strength to plod on despite my constitution wanting to give up. Countless salutation upon the **Holy Prophet Muhammad** (peace be upon him), who gave us path to recognize our self and the Creator.

I am immensely pleased to place on record my profound gratitude and heartfelt thanks to my supervisor, **Professor Dr. Sarwat Jahan,** Chairperson, Department of Animal Sciences, for her guidance during my research, for the continuous support of my PhD study and research, for her patience, motivation and enthusiasm.

Very special thanks to my Co supervisor, **Dr. Sayed Murtaza Hassan Andrabi,** Principal Scientific Officer, Animal Sciences Institute, Animal Reproduction and Genetics Program (ARGP), National Agriculture Research Centre, Islamabad. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my study. I ever remain grateful to him.

I am grateful to **Dr. Muhammad Shahab, Dr. Saeed-ul-Hassan Khan, Dr. Sajid Malik Dr. Abid Mehmood** (PSO, ARGP), **Dr. Muhammad Anwar** (PSO, ARGP) and **Dr. Shafiq Haider** (SO, ARGP) for their help and valuable suggestions during the period of my study.

I am very thankful to **Pakistan Science Foundation (PSF)** for the financial support under the Natural Sciences Linkages Program (NSLP-213).

I would like to extend my thanks to the supporting staff at ARGP, ASI, NARC, Islamabad including **Muhammad Mumtaz, Ghulam Muhammad, Tanveer Abbas, Shehryar Khan** and **Muhammad Anis**.

I would like to extend my thanks to my friends, **Hizbullah, Dr. Latafat Ameen Khan, Dr. Shakeel Ahmed, Dr. Hafiz Khaleel, S.A.H Shah, Dr. Shafiq Haider** (SO, ARGP) **Dr. Chaman Lal, Babar Khan** and **Wasim Akram** for their help and nice company.

I am very thankful to the non-teaching staff viz. **Muhammad Saeed (Lab Attendant), Naeem Masih** and **Samiullah (Office Clerks)** for their cooperation. At the end I am very thankful to all those who directly or indirectly contributed in the successful completion of my thesis.

Hussain Ahmed

LIST OF ABBREVIATIONS

AI	Artificial insemination
ALH	Amplitude of lateral head displacement
AV	Artificial vagina
BCF	Beat cross frequency
CASA	Computer aided sperm motion analyzer
DNA	Deoxyribonucleic acid
HP	High mitochondrial transmembrane potential
JC–1	"5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide
LIN	Linearity
LP	Low mitochondrial transmembrane potential
LRS	Livestock Research Station
mOsmol	Milliosmole
MV	Medium velocity
NARC	National Agricultural Research Center
NV/DACR	Nonviable with damaged acrosome
NV/HP	Nonviable with high mitochondrial transmembrane potential
NV/IACR	Nonviable with intact acrosome
NV/LP	Nonviable with low mitochondrial transmembrane potential
PBS	Phosphate buffered saline
PI	Propidium iodide
PM	Progressive motility
RV	Rapid velocity
STR	Straightness
SV–PMI	Supra-vital plasma membrane integrity
TCA	Tris-citric acid
V/DACR	Viable with damaged acrosome
V/HP	Viable with high mitochondrial transmembrane potential
V/IACR	Viable with intact acrosome
V/LP	Viable with low mitochondrial transmembrane potential
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight line velocity

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GENERAL ABSTRACT

Background: Fertility of cryopreserved water buffalo (*Bubalus bubalis*) spermatozoa through artificial insemination (AI) is reported to be affected by seasonality. The quality of frozen–thawed semen is one of the most influential factors affecting the probability of pregnancy. This study was designed to investigate various semen quality parameters to predict the *in vivo* fertility of buffalo bull during low and peak breeding seasons. In this study, we have also investigated the effect of seasons on structural-functional parameters and *in vivo* fertility of buffalo bull spermatozoa. In the last study, we have investigated the effect of hydrogen peroxide (H_2O_2) on semen quality parameters having the capability of prediction of fertility with ultimate aim to validate them for buffalo bull spermatozoa.

Materials and Methods: Semen was collected from five mature water buffalo bulls with artificial vagina maintained at 42 °C during low and peak breeding seasons. After collection, semen samples were transferred to the laboratory immediately for initial evaluation. Sperm progressive motility was assessed using phase contrast microscope (x 400) connected with closed circuit monitor and sperm concentration was measured by using the specific spectrophotometer at a wavelength of 546 nm. Qualifying ejaculates having > 1 mL volume, > 60 % sperm progressive motility and > 0.5 x 10^9 spermatozoa/mL concentration from each bull were diluted in Tris–citric acid egg yolk glycerol extender (TCA). For cryopreservation, semen of each bull was extended in TCA extender at 37 °C, cooled to 4 °C in 2 hr, and equilibrated for 4 hr in cold cabinet (4 °C). Extended semen was then packed in polyvinyl French straws (0.54 mL) and frozen in a programmable cell freezer. Finally, semen straws were plunged into liquid nitrogen (–196 °C) for storage until analyses. For *in vivo* fertility, data of at least 100 inseminations per bull were collected under controlled field conditions per season. In order to study the effect of H_2O_2 on semen quality parameters like computer-aided sperm motion analysis (CASA) aspects, subjective motility (SM, %), supra–vital plasma membrane integrity (SV–PMI, %), viability/ mitochondrial transmembrane potential and viability/ acrosome integrity of buffalo bull spermatozoa, qualifying ejaculates from five buffalo bull were diluted at the rate of 25 x 10^6/mL) in PBS–0.1% BSA extender containing 10 µM H_2O_2, equilibrated for 5 min followed by measurement of semen quality parameters at different time intervals (0 min, 30 min, 60 min, 90 min and 150 min). Furthermore, for studying the effects of H_2O_2 on sperm DNA fragmentation indices, semen samples were diluted in

PBS–0.1% BSA extender containing 0.0 mM, 25 mM, 50 mM, 75 mM and 100 mM H_2O_2 respectively, equilibrated for 1 hr to induce damage and then followed by neutral comet assay protocol.

Results: In experiment 1, we have investigated various semen quality parameters to predict the *in vivo* fertility of buffalo bull during low breeding season. Pearson's correlation coefficients showed that sperm progressive motility (PM, %), rapid velocity (RV, %), average path velocity (VAP, µm/s), straight line velocity (VSL, µm/s) and rapid subpopulation 1 (%) of buffalo bull were significantly correlated with *in vivo* fertility during low breeding season ($r = 0.64$, $P < 0.01$; $r = 0.57$, $P < 0.01$; $r = 0.52$, $P < 0.01$; $r = 0.56$, $P < 0.01$ and $r = 0.73$, $P < 0.001$). Moreover, sperm SV–PMI and viable spermatozoa with intact acrosome (V/IACR, %) were significantly correlated with *in vivo* fertility ($r = 0.74$; $P < 0.001$ and $r = 0.88$; $P < 0.001$), whereas nonviable spermatozoa with damaged acrosome or low mitochondrial transmembrane potential (NV/DACR or NV/LP, %) were negatively associated with *in vivo* fertility during low–breeding season ($r = 0.79$; $P < 0.001$ and $r = 0.75$; $P < 0.001$). Comet length (CL, µm) parameter of neutral comet assay was negatively associated with *in vivo* fertility during low-breeding season ($r = –0.60$, $P < 0.05$). Step forward regression analyses showed that the prognostic value to predict the *in vivo* fertility by the equation of VAP, VSL, curvilinear velocity, rapid subpopulation 1, SV–PMI, V/IACR, and viable with high mitochondrial transmembrane potential (V/HP, %) accounted for 81.30 % ($P < 0.001$) during low breeding season.

In experiment 2, we investigated various semen quality parameters to predict the *in vivo* fertility of buffalo bull during peak breeding season. Pearson's correlation coefficients showed that sperm PM, RV, VAP, VSL, straightness (STR, %) and rapid subpopulation 1 (%) of buffalo bull were significantly correlated with *in vivo* fertility during peak breeding season ($r = 0.81$, $P < 0.01$; $r = 0.85$, $P < 0.01$; $r = 0.64$, $P < 0.05$; $r = 0.73$, $P < 0.05$; $r = 0.57$, $P < 0.05$ and $r= 0.65$, $P < 0.05$). Furthermore, sperm SM, SV–PMI, V/IACR and V/HP were positively correlated with *in vivo* fertility during peak breeding season ($r = 0.79$; $P < 0.01$, $r = 0.88$, $P < 0.01$, $r = 0.84$, $P < 0.01$ and $r = 0.81$, $P < 0.01$), whereas NV/DACR or NV/LP were negatively correlated with *in vivo* fertility during peak breeding season ($r = –0.81$, $P < 0.01$ or $r = –0.81$, $P < 0.01$). Tail length (TL, µm) were negatively correlated with *in vivo* fertility during peak breeding season ($r = –0.70$, $P < 0.05$). Moreover, the best predictive equation (R^2 adjusted=83.50 %, $P < 0.000$) of fertility

for frozen–thawed buffalo semen included PM, RV, VAP, VSL and SV–PMI during peak breeding season.

In experiment 3, we investigated the effect of season on structural-functional parameters and *in vivo* fertility of buffalo bull spermatozoa during peak and low breeding seasons. Analysis of variance showed that ejaculate volume (mL), concentration of spermatozoa (mL), total motility (TM, %), PM, VAP, VSL, STR, linearity (LIN, %) rapid subpopulation 1, SV–PMI, V/IACR, V/HP and DNA integrity in fresh semen were significantly higher ($P < 0.05$) during peak than low breeding season. At post–thawing, sperm TM, rapid subpopulation 1, SV–PMI, V/IACR, V/HP and DNA integrity were significantly higher ($P < 0.05$) during peak than low breeding season. *In vivo* fertility of frozen–thawed buffalo spermatozoa processed during peak breeding season was significantly higher ($P < 0.05$) than semen samples cryopreserved during low breeding season in a fertility trial carried out during low breeding season (58.98 % vs. 52.49 %). In experiment 4, we have studied the effect of H_2O_2 on semen quality parameters having the capability of prediction of fertility with ultimate aim to validate them for buffalo bull spermatozoa. It was found that H_2O_2 at a dose of 10 μM diminished PM, RV, motion kinematics (VAP, VSL and curvilinear velocity), SV–PMI, V/HP and V/IACR of buffalo bull in a time dependent manner as compared to control. Moreover, exposure of H_2O_2 significantly increases ($P < 0.05$) comet length, tail length, tail DNA, tail moment and olive moment in a dose dependent manner as compared to control. Similarly, exposure of H_2O_2 significantly increases ($P < 0.05$) DNA fragmentation in a dose dependent manner than control.

Conclusion: It is concluded that assessment of CASA parameters and sperm structural and functional parameters viz. SV-PMI, viability/ acrosome integrity and viability/ mitochondrial transmembrane potential were able to predict the *in vivo* fertility of water buffalo bull during low and peak breeding seasons. Moreover, ejaculates collected/ processed during peak breeding season demonstrated better semen quality and *in vivo* fertility in a fertility trial conducted during low breeding season and are thus more suitable for AI than ejaculates collected during low breeding season. Finally, H_2O_2 negatively modulated semen quality parameters and may be used for monitoring the effectiveness of buffalo bull semen quality and must be included in optimization procedures. It is expected that application of these findings will improve the outcome of AI in water buffalo.

GENERAL INTRODUCTION

The domestic Asian or water buffalo (*Bubalus bubalis*) belongs to family Bovidae, sub family Bovini, genus Bubalus and species bubalis. It has been suggested that buffalo had been domesticated in the Indo–Pakistan sub–continent about 5000 years ago (Bhat & Qureshi, 1992). The domesticated buffalo has been broadly classified into Riverine and swamp types (Macgregor, 1941; Cockrill, 1974). The River buffalo has 50 chromosomes and is distributed in Pakistan, India, Middle East, while the Swamp buffalo has 48 chromosomes and is distributed in China, Bangladesh, the South-east Asian countries and North-eastern states of India (Cockrill, 1981). Buffalo is known for its love for water and hence is often called water buffalo. The swamp buffalo prefers stagnant water or mud and prepare its own wallow by digging any soft or marshy lands with its horns. The river buffalo prefers to immerse themselves in running water or ponds (Borghese & Mazzi, 2005; Barile, 2005; Minervini *et al*., 2013).

Breed characteristics and population

The Riverine buffaloes are usually black in colors having white markings on five different places i.e., forehead, face, muzzle, legs and tail switch (Sarwar & Ishaq, 1957; Cockrill, 1974). Buffalo has a significant role in the agricultural economy of many developing countries by providing milk, meat and draught power. The world population of buffalo is estimated to be 199 million (FAOSTAT, 2012) with more than 96 % in Asia, 2 % in Africa – mainly in Egypt, and 0.2 % in Europe – mainly in Italy, India has 56 %, Pakistan 14 % and China 13 % of the world buffalo population.

Role of livestock in the gross domestic product (GDP) of Pakistan

Livestock production constitutes a very important component and dynamic sector of the agricultural economy of many developing countries, a contribution not only includes food production but it is the source of multipurpose uses, such as fibre, skin, fuel and fertilizer, as well as revenue generation (FAO, 1992). Furthermore, Livestock is an important sector in Pakistan's economy and contributes about 11.90 % in the national gross domestic product (GDP) of Pakistan. Moreover, the livestock contributes 55.40 % in the agriculture sector's share of GDP in year 2012–2013 (Economic survey of Pakistan, 2013–2014). Livestock sector is considered to be a net source of invariable income for rural and middle

grade agri–business holders. It is considered as a source of employment generation in rural areas, helping reduce variation in income and ultimately poverty alleviation (FAO, 1993).

In this regard, buffalo is playing a leading role in the national economy by producing milk, meat and draught power. Out of total milk produced in the country, buffalo contributes about 68 %, followed by cattle (27 %) and sheep/goat/camel (5 %). Due to high fat contents of buffalo milk, it is the most preferred species in Pakistan. World milk production has doubled in the last few decades and it is noteworthy that in the last few years, buffalo have supplied about 12 % of the total world milk production. India and Pakistan have produced respectively 60 and 30 % of the world's buffalo milk. In India buffalo milk contributes 55 %, and in Pakistan 75 %, of their total milk production (FAO, 2004). Buffalo milk is high in total solids, fat, proteins and vitamins compared to cow's milk. Buffalo milk also contains less cholesterol and more tocopherol, which is a natural antioxidant. The peroxidase activity is two to four times higher in buffalo milk than in cow's milk (Chantalakhana & Falvey, 1999). Buffalo milk appears to be whiter than cow's milk because it lacks the yellow pigment carotene, a precursor of vitamin A. But buffalo milk contains even more vitamin A than cow's milk.

In Pakistan, buffaloes not only fulfill the protein requirements of the human population by milk and meat, but also have a great share in providing the traction power for various agricultural purposes. In Pakistan we have the best breeds of buffaloes (Nili Ravi and Kundi) but they are not producing according to their potential. The ultimate reasons for low production of buffaloes in Pakistan are late age of maturity, long calving interval and silent heat (Anzar *et al*., 2003). All these problems can be solved by employing efficient managemental protocols and modern reproductive biotechniques. Despite of all these problems, there is room for improvement. Due to their versatile qualities they are rightly called as Black Gold of Pakistan.

Artificial insemination (AI) in buffalo

Artificial insemination (AI) is the manual placement of semen in the reproductive tract of the female by a method other than natural mating. It is one of a group of technologies commonly known as "assisted reproduction technologies" (ART), whereby offspring are generated by facilitating the meeting of spermatozoa and oocytes (Foote, 2003). In this regard, the production potential of livestock can be augmented by genetic improvement

using one of the modern ways of breed improvement, e.g., AI. The quality of frozen–thawed semen is one of the most influential factors affecting the probability of pregnancy (Saacke, 1984). Use of AI with frozen–thawed semen has been reported on a limited scale in buffalo, because of poor freezability and fertility of buffalo spermatozoa in comparison with cattle spermatozoa (Muer *et al*., 1988; Singh & Pant 2000; Andrabi *et al*., 2001; 2008; Ahmad *et al*., 2003; Senatore *et al*. 2004; Kumaresan *et al*., 2005).

Major constraints in maximizing the reproductive efficiency in this species are the delayed attainment of puberty by the female buffalo, long postpartum anoestrus, poor expression of oestrous behaviour and seasonality. Regarding AI, which is routine practice in cattle breeding, is limited in buffaloes because of the weakness of oestrous symptoms, bulls used in AI with unknown fertility and ultimately semen quality (Barile, 2005; Drost, 2007; Minervini *et al*., 2013). Therefore, it is mandatory to cryopreserved buffalo bull semen of high fertility outcomes for long-term storage of their genetic material for enhancement of pregnancy rate, milk and meat production.

Adaptation of buffalo to environmental temperatures

The ability to withstand the environmental conditions by buffalo in its rearing countries has widely been recognized. However, the buffalo is amazingly versatile; it does indeed have less physiological adaptation to extremes of environment change compared to different breeds of cattle. The body temperature of buffaloes is slightly lower than that of cattle, despite the fact that buffalo skin is usually black and heat-absorbent and only sparsely protected by hair. Moreover, buffaloes have fewer sweat glands than most other bovidae do, which, by poorly dispersing heat by sweating, makes them fairly sensitive to heat (Ligda, 1999; Nowak, 1999). If buffaloes were worked or driven excessively in the hot sun, their body temperature, pulse rate, respiratory rate and general distress levels would increase more quickly than those of cattle. Therefore, buffaloes usually cool down by wallowing in mud, rather than seeking shade. Wallowing in mud helps them to cool their body temperature because water in mud evaporates more slowly than does water on its own, thus extending the effectiveness of cooling when ambient temperatures and humidity are high (Ligda, 1999; Nowak, 1999; Shackleton & Harestad, 2003).

Season of semen collection

It has been reported that freezability of buffalo bull semen is also affected by the season of collections, including environmental factors like prevailing temperature, day length, relative humidity and rainfall of that specific season (Kapoor, 1973; Bhavsar *et al*., 1989, Sagdeo *et al*., 1991; Bhosrekar *et al*., 1992, Mandal *et al*., 2003; Koonjaenak *et al*., 2007a, b; Andrabi, 2009; Andrabi, 2014). Moreover, seasonality also affects semen quality and fertility of many species including bovine (Erb *et al*., 1942; Chandler *et al*., 1985, Parkinson, 1987, Rekwort *et al*., 1987, Hernandez *et al*., 1991, Mathevon *et al*., 1998; Snoj *et al*., 2013, Soderquist *et al*., 1996, 1997), ovine (D'Alessandro & Martemucci, 2003), caprine (Zarazaga *et al*., 2009) and equine (Pickett *et al*., 1975; Janett *et al*., 2003a, b).

For the first time in buffalo, Tuli & Mehar (1983) reported that semen collected, processed and cryopreserved from buffalo bull during winter exhibit better freezability in terms of post-thaw motility as compared to summer. Later on, seasonal variations in freezability of buffalo bull in terms of post–thaw motility and fertility were also reported (Bhavsar *et al*., 1989a; Bahga & Khokar 1991; Sagdeo *et al*., 1991). The results of these studies showed that buffalo bull semen samples cryopreserved during winter was provided with better post–thaw motility than summer. Regarding the influence of season on *in vivo* fertility of frozen–thawed buffalo bull semen, Heuer *et al*. (1987) reported that semen collected in winter yielded significantly higher conception rate than semen collected in summer (40.90 % vs 34.00 %). After that, Younis *et al*. (1998) studied the freezability of buffalo bull semen collected and processed during peak and low breeding seasons. They reported that post–thaw motility and livability of spermatozoa were significantly higher during peak breeding season as compared to low breeding season. Moreover, the proportions of sperm abnormalities and deleterious enzymatic activity in frozen–thawed buffalo bull semen were significantly lower during peak than low breeding season.

Koonjaenak *et al*. (2007a) studied the influence of season on quality of frozen–thawed buffalo spermatozoa and compared quality of post–thaw semen comprising three seasons of the year i.e., rainy (July–October), winter (November–February) and summer (March–June), with discrete ambient temperature, humidity and rainfall. They concluded that sperm post-thaw plasma membrane integrity and stability were significantly higher in ejaculates processed during winter than in samples processed during the other seasons of

the year. In another study, Koonjaenak *et al*. (2007c) investigated the frozen–thawed buffalo sperm nuclear DNA fragmentation by flow cytometry and head morphology comprising the same three seasons of the year i.e., rainy (July–October), winter (November–February) and summer (March–June). They found that the DNA fragmentation index (DFI) values varied statistically among seasons, being lower in the rainy season than in winter or summer. Recently, Tiwari *et al*. (2011) have studied the influence of season on semen quality of buffalo bull during winter and summer. It was noticed that the semen samples collected, processed and cryopreserved during winter were better in terms of ejaculate volume, initial motility and post-thaw motility. Similarly the discard ratio of neat and frozen-thawed semen was lowest during winter than summer.

Seasonal variation in the biochemical composition of seminal plasma and spermatozoa may exist like other farm animals (Cabrera *et al*., 2005; Argov *et al*., 2007; Koonjaenak *et al*., 2007a). Argov *et al*. (2007) have observed that cattle semen samples collected during summer are considered to be of better quality having modifications in lipid concentrations, fatty-acid composition and cholesterol concentrations. Moreover, some reports exist regarding the differences in chemical composition of buffalo seminal plasma and spermatozoa under different climatic conditions (Singh *et al*., 1969; Sidhu & Guraya, 1979).

However, the data given in these studies are insufficient to explain the variation in quality of spermatozoa during different seasons. Moreover, these studies have used subjective semen evaluation techniques, which are now considered as of limited scope in buffalo. Therefore, detailed studies needs to be carried out in riverine buffalo to ascertain the structural and functional parameters that might be influencing the quality of fresh and frozen-thawed spermatozoa during peak and low breeding seasons.

Semen quality parameters

The assessment of sperm quality is one of the major concerns in semen research and AI programs. Presently, no single precise laboratory assay is available to predict frozen-thawed semen fertility exactly and accurately (Graham *et al*., 1984). However, the combination of several assays may better predict fertility (Linford *et al*., 1976; Amann & Hammerstedt, 1993; Januskauskas *et al*., 2001; Braundmeier & Miller, 2001). The common viability related semen parameters used for the assessment of sperm quality and fertility includes

subjective motility, computer aided sperm motion analysis (CASA) motility parameters, motion kinematics, plasma membrane integrity, acrosome integrity (Linford *et al*., 1976; Budworth *et al*., 1987; 1988; Brito *et al*., 2003; Muino *et al*., 2008a, b; 2009). The semen quality variables like subjective motility, computer-assisted motility and velocity, track motility, and acrosomal integrity have been correlated with fertility in bulls (O' Connor *et al*., 1981; Kathiravan *et al*., 2008; 2011).

Sperm motility assessment and its relationship with fertility

Sperm motility is an important criterion to determine the quality of semen samples earlier than AI and in the laboratory to evaluate the effect of experimental procedures. Sperm motility is routinely estimated visually (subjectively) by the use of closed circuit television connected with phase contrast microscope (Graham *et al*., 1970). Motility is one of the key features in mammalian sperm to deliver the male's genetic material to the oocyte during fertilization. The movement of spermatozoa is delicately regulated for being its requirement during the different stages inside the female reproductive tract and their proximity to the oocyte (Knobil, 1981). The sperm tail is the main organelle linked to motility. One of the most outstanding characteristics of mammalian sperm is the fusion of mitochondria in a mitochondrial sheath, situated around the apical portion of the tail region (Eddy & O'Brien, 1988).

It has been observed that there is a direct correlation between motility and mitochondrial activity in humans (Troiano *et al*., 1998). In this regard, sperm subjective motility assessment has been significantly correlated with fertility in bulls (Linford *et al*. 1976; Galli *et al*., 1991; Correa *et al*., 1997; Zhang *et al*., 1998; Januskauskas *et al*., 2001). In contrast, post-thaw subjective motility assessment of spermatozoa was not significantly correlated with fertility in bulls (Saacke & White, 1972; Soderquist *et al*., 1991). The reasons for these contradictory observations may be that the visual estimation of sperm motility is highly a subjective assessment method, depends on observer's accuracy, semen handling techniques or cut off values used for estimation (Graham *et al*., 1980; Rodriguez–Martinez, 2003).

For the last few decades, various strategies have been applied for the development of objective, precise and accurate instrument with repeatable outcomes to appraise sperm motility parameters and motion kinematics, because the objective appraisal of motility is

based on massive movements of spermatozoa and their trajectories (Rothschild, 1948). Moreover, objective measurements provide individual velocities of those spermatozoa (Rothschild, 1953). The details of sperm motion characteristics can provide insights into the physiology of cells, the mechanisms of their transport and fertilization (Yanagimachi, 1994).

Computer–aided sperm motion analysis

Computer–aided sperm motion analysis (CASA) allows an objective classification of a given population of spermatozoa by using digital images of each sperm cell's track; CASA is able to analyze, by processing algorithms, the motion properties of spermatozoa. The commonly reported parameters analyzed by CASA are motility parameters, velocity distribution, motion kinematics, and subpopulations of motility based on various motion kinematics. These CASA parameters have been modeled and refined mathematically to best describe the motion parameters of each spermatozoon as it travels through a microscopic field (Boyer *et al*., 1989; Muino *et al*., 2008a, b; 2009). The accuracy, precision and ultimately the reliability of CASA have been demonstrated under various experimental conditions (Comhaire *et al*., 1992; Davis *et al*., 1992; Davis & Kats, 1993; Holt *et al*., 1994; Farrell *et al*., 1995; Rijsselaere *et al*., 2003).

Sperm motility measurement with CASA

Motility assessment by CASA is one of the most important parameters used for sperm quality evaluation in both raw and cryopreserved semen and provides important information on the energy status of mammalian sperm (Quintero–Moreno *et al*., 2003; 2004; 2007; Muino *et al*., 2008a, b; 2009). The motility functions play an important role once spermatozoa reach the female reproductive tract (Jansen 1978; Jansen & Bajpai 1982; Farrell *et al*., 1998; Mortimer, 1997; Hoflack *et al*., 2007). Spermatozoa with low or hazy movement are improbable to reach the oviduct, and it is reasonable to assume that the more the spermatozoa with progressive motility, the higher will be chance to reach the ampulla of the oviduct (Muino *et al*., 2008a). CASA system has been used in studies exploring sperm motility measurements of many species such as rats (Moore & Akhondi, 1996), bulls (O'Conner *et al*., 1981; Muino *et al*., 2008a, b; 2009), boars (Holt *et al*., 1997), rabbits (Farrell *et al*., 1993), turkeys (Bakst & Cecil, 1992), humans (MacLeod & Irvine, 1995) and buffalo (Anzar & Graham, 1995; Iqbal *et al*., 2015; 2016; Andrabi *et al*., 2016).

Sperm motion kinematics measurement with CASA

The measurement of motion kinematics has been considered as an indicator of functionality of spermatozoa in bovine (Budworth *et al*., 1988; Anzar *et al*., 1991; Farrell *et al*., 1998; Kathiravan *et al*., 2008; 2011), equine (Jasco *et al*., 1988) and canine (Ellington *et al*., 1993). The straight line velocity of spermatozoa is an indication of flagellar motion and thrust and has been correlated with fertilization rates (Katz *et al*., 1989; Suarez *et al*., 1992; Leidel *et al*., 1993; Liu *et al*., 1991; Wainer *et al*., 1996). The average path velocity is generally considered an indicator of the efficiency of flagellation and forward progression of the spermatozoon and is important for negotiating the female reproductive tract. The beat cross frequency (BCF) is a useful value in the estimation of gross changes in the flagellar beat pattern and depends on the frame rate of the machine (Mortimer & Swan, 1999).

Subpopulations of motile spermatozoa based on motion kinematics

It has been found that four subpopulations of motile spermatozoa coexist in most of the mammalian ejaculates (Holt, 1996; Abaigar *et al*., 1999; Quintero–Moreno *et al*., 2003; 2007; Cremades *et al*., 2005; Nunez–Martinez *et al*., 2006; Rivera *et al*., 2006) including bovines ejaculates. These subpopulations are identified based on motion kinematics of motile spermatozoa. This variation in sperm population based on motion kinematics exhibit the differences in their morphology, motility, viability and ultimately fertility (Rodriguez–Martinez & Barth, 2007). It has been reported that semen samples having higher proportions of rapid and progressively motile spermatozoa are more resistant to cryopreservation and are linked with fertility (Gaddum-Rosse, 1981; Olds-Clarke, 1986; Shalgi *et al*., 1992; Scott, 2000; Quintero–Moreno *et al*., 2003; Muino *et al*., 2008a; 2009; Martínez–Pastor *et al*., 2011).

Plasma membrane integrity relationship with fertility

The evaluation of plasma membrane integrity is of particular importance due to its involvement in metabolic exchanges with the surrounding medium (Andrabi *et al*., 2016). The hypo–osmotic swelling test (HOST) was developed by Jeyendran *et al*. (1984) to evaluate sperm membrane functional integrity. This test is based on the semi permeability of the intact cell membrane, which allows the sperm to swell under hypo–osmotic condition (Buckett *et al*., 1997; Brito *et al*., 2003). It has been suggested that the ability of

spermatozoa to swell in the presence of hypo–osmotic medium reflects normal water transport across the sperm membrane, which is a sign of normal membrane integrity and functional activity (Jeyendran *et al*., 1984; Revell & Mrode, 1994; Rasul *et al*., 2000; Tartagni *et al*., 2002). Sperm plasma membrane is essential to establish a barrier between intracellular and extra cellular environments and is important to sustain osmotic equilibrium and cellular homeostasis. Damages in this structure lead to a cellular instability caused by homeostasis loss, resulting in cellular death. Therefore, plasma membrane integrity exerts a crucial role on sperm survival in the female reproductive tract and its fertilizing ability (Parks & Graham, 1992).

Chan *et al*. (1991) developed supra–vital plasma membrane integrity test (SV–PMI) which is based on eosin staining in the presence of hypo-osmotic swelling (HOS) solution in case of structurally damaged membrane and vice versa. This test measures the structural and functional integrity of spermatozoa simultaneously. Structural and functional intactness of sperm plasma membrane is of fundamental importance in assessing the fertilizing capacity of semen samples (Chan *et al*., 1991; Cabrita *et al*., 1999; Selvaraju *et al*., 2008), because only a spermatozoa with structurally and functionally intact plasma membrane can undergo a series of complex changes inside the female reproductive tract and acquire the ability to fertilize an oocyte (Parks & Graham, 1992; Yanagimachi, 1994; Sharma *et al*., 2005).

It has been reported that the sperm plasma membrane integrity has a good correlation with fertility and morphology in human (Jeyendran *et al*., 1984; Van Der Ven., 1986; Gehring, 1987) and sperm motility in bull (Correa & Zavos, 1994). A significant correlation has been observed between plasma membrane integrity and mucus penetration test (Gehring, 1987) and *in vitro* fertilizing rate (IVF) rate in boar and bull (Pintado *et al*., 2000). A good correlation existed between ability of spermatozoa to undergo capacitation, penetration of oocyte and intact plasma membrane (Revell & Mrode, 1994). Wood *et al*. (1986) demonstrated that the combination of sperm motility and proportion of plasma membrane integrity provide the best prediction of fertility. For this reason, the assessment of structural and functional integrity of plasma membrane is one of the parameters to take into account for semen quality evaluation.

Acrosome integrity relationship with fertility

The acrosome is an organelle that develops over the anterior half of the head in the spermatozoa of many animals. It is a cap–like structure derived from the Golgi apparatus. The acrosome contains digestive enzymes known as hyaluronidase and acrosin which are fundamental for the breakdown of outer membrane of the egg, known as zona pellucid; to achieve fertilization, while the flagellum contains the energy sources and machinery to generate the motility necessary for the sperm to reach the egg. These functions are essential for delivery of the genetic material contained in the sperm nucleus to the cytoplasm of the egg, where combination of the haploid male and female pronuclei occurs to produce the zygote and initiate development (Knobil, 1981; Thomas *et al*., 1997).

Sperm acrosome integrity is vital for the acrosomal reaction leading to fertilization and it has been shown to be one of the useful parameters to predict the fertilization potential of buffalo bull spermatozoa (Kumar *et al.,* 2012). The acrosome integrity is a valuable test used to judge sperm viability, motility and fertilizing ability of frozen thawed semen in bovine (De Leeuw *et al*. 1991; Pandey *et al*., 2004). Sperm viability and acrosome integrity have been assessed with Trypan blue/ Giemsa staining simultaneously in cattle (Brito *et al*., 2003; Tartaglione & Titta., 2004) and equine (Kovacs & Foote, 1992). Tartaglione & Ritta, (2004) have combined sperm plasma membrane and acrosome integrity tests in order to obtained information regarding their predictive values in terms of IVF rate. Stepwise regression analyses yielded that a combination of supra–vital plasma membrane integrity test (SV–HOST) provides the greatest proportion of variation in fertilization rates (78%). Moreover, SV–HOST and Trypan blue/ Giemsa staining could be used for the prognosis of the potential fertility of bovine semen samples used for IVF (Brito *et al*., 2003).

Kjoestad *et al*. (1993) observed that the fertility of frozen bull semen to be more closely related to acrosome integrity than to motility with a low correlation between semen motility and acrosome integrity. The combined predictive value of both these parameters had a significant effect on fertility, which would further emphasize the advantage of assessing both parameters to establish a reliable basis for post thaw estimation of semen quality. Recently, Serafini *et al*. (2014) have test the reliability of Trypan blue/ Giemsa staining to evaluate sperm viability/ acrosomal integrity in stallion and showed that Trypan blue/Giemsa staining can be an accurate method and could be regarded as a useful

tool to judge the sperm fertilizing ability. However, there is not a single study regarding the assessment of sperm viability and acrosome integrity with Trypan blue/ Giemsa staining in buffalo as well as their relationships with *in vivo* fertility during peak and low breeding seasons.

Mitochondrial transmembrane potential and its relationship with fertility

Mitochondria en–sheathes the mid–piece of spermatozoa and delivers adenosine triphosphate (ATP) to the exoneme where it is utilized for flagellar propulsion and motility for a longer period of time (Bartoov *et al*., 1980). These organelles are required for efficient energy metabolism, production of membrane lipids, cell growth and are the primary sites of cellular life or death i.e., apoptosis. Sperm mitochondrial transmembrane potential can be determined by using the fluorescent probe "5,5,6,6–tetrachloro–1,1,3,3–tetraethylbenzimidazolyl carbocyanine iodide" (JC–1, Garner *et al*., 1997; Thomas *et al*., 1998; Finkel, 2001). The JC–1 is a fluorochromes and is able to identify sperm mitochondria exhibiting low membrane potentials by the emission of green fluorescence (range, 510–520 nm) and mitochondria exhibiting relatively high membrane potentials by the emission of orange fluorescence (range, 590–630 nm). This discrimination occurs because JC–1 forms J–aggregates when spermatozoa are provided with high mitochondrial transmembrane potential (Reers *et al*., 1991; Garner & Thomas, 1999).

Sperm mitochondrial transmembrane potential is considered to be one of the most sensitive test for semen quality assessment and its qualification for *in vivo* or *in vitro* fertility in cattle (Garner *et al*., 1997), human (Baumber *et al*., 2000, Curtis *et al*., 2000; Kasai *et al*., 2002; Marchetti *et al*., 2002; 2004; Wang *et al*., 2003) boar (Pena *et al*., 2003) and buffalo (Selvaraju *et al*., 2008). High mitochondrial transmembrane potential is an indicator of the energetic state of mitochondria and determines functionally intact mitochondria that have been mainly related to sperm motility (Marchetti *et al*., 2002). Mitochondria provide the energy needed for the tail to maintain its movement (Garner *et al*., 1986) and plays an important part in penetration of the cervix by sperm (Garner *et al*., 1986; Windsor, 1997). The reduction in mitochondrial membrane potential leads to an early stage of apoptosis, DNA fragmentation, and depletion in ATP, increase in membrane permeability and reduction in sperm motility (Garner *et al*., 1997; Marchetti *et al*., 2002; Guillaume *et al*., 2004).

In an elegant study, Garner *et al.*, (1997) established a strong association between spermatozoa exhibiting progressive forward motility and high mitochondrial membrane potential in cryopreserved bovine spermatozoa. They reported that bull with a relatively low percentage of motile spermatozoa exhibited a lower percentage of spermatozoa with high mitochondrial transmembrane potential. Moreover, the cleavage rate had significant correlations with the mitochondrial membrane potential and plasmalemma integrity in frozen–thawed Surti buffalo bull semen (Selvaraju *et al.*, 2008). Furthermore, a strong association has been observed between spermatozoa with intact plasma membrane integrity and high mitochondrial transmembrane potential in bovine (Graham & Moce, 2005; Bollwein *et al.*, 2008). Review of literature revealed that there is not a single published report regarding the use of mitochondrial transmembrane potential for buffalo bull spermatozoa as a biomarker of fertility. Hence, assessment of mitochondrial transmembrane potential may be considered as an important parameter in buffalo bull semen analysis for future use as biomarker of fertility**.**

DNA fragmentation/integrity

The appraisal of sperm DNA feature is one of the major concerns in semen research and AI programs. Numerous techniques have been developed to scrutinize DNA integrity of spermatozoa, such as the sperm chromatin structure assay (SCSA, Evenson *et al.*, 1980), sperm chromatin dispersion assay (SCDA, Fernandez *et al.*, 2003), single cell gel electrophoresis method (Comet assay, Singh *et al.*, 1989) and acridine orange assay (Tejada *et al.*, 1984). SCSA characterizes abnormal chromatin structure as an increased susceptibility of sperm DNA to acid-induced denaturation *in situ* and is used to evaluate sperm DNA quality in human (Evenson *et al.*, 1980), bulls (Ballachey *et al.*, 1987; 1988; Januskauskas *et al.*, 2001; Januskauskas *et al.*, 2003; Boe-hansen *et al.*, 2005; Waterhouse *et al.*, 2006; Fortes *et al.*, 2012), stallions (Love & Kenney, 1998) and boars (Evenson *et al.*, 1994). The SCDA is based on the principal that spermatozoa with non-fragmented DNA provide the extended halos of DNA dispersion as measured with fluorescent microscopy (Fernandez *et al.*, 2003).

Spermatozoa with high levels of DNA strand breaks have increased comet tail intensity (Hughes *et al.*, 1996), comet tail length (Singh & Stephens, 1998), higher olive moment (Olive *et al.*, 1993) and more DNA in the tail region (Boe-hansen *et al.*, 2005). The neutral assay measures only double-strand breaks (Singh *et al.*, 1989; Singh & Stephens, 1998).

The neutral comet assay allowed DNA to dissociate from other macromolecules and freely migrate through the gel (Singh & Stephens, 1998). In the comet assays, sperm DNA breaks migrate away from the head region to form "comets" following electrophoresis, whereas intact DNA remains in the original head position (Shaman & Ward, 2006).

Evenson *et al*. (2002) and Baumgartner *et al*. (2009) proposed that the neutral comet assay identifies double stranded DNA (dsDNA) breaks and closely associated single stranded DNA (ssDNA) breaks, whereas the alkaline Comet assay identifies only ssDNA breaks. Sperm from fertile men had less ssDNA and dsDNA breaks compared to sub fertile men when using neutral and alkaline Comet assays (Ribas-Maynou *et al*., 2012). In bulls, the neutral Comet assay detected more DNA breaks (i.e., higher tail moment) in non sex-sorted sperm, as compared to sex-sorted sperm (Boe-Hansen *et al*., 2005). Acridine orange assay measure the DNA integrity by quantifying the metachromatic shift of acridine orange fluorescence from green (native DNA) to red (denatured DNA, Tejada *et al*., 1984).

Sperm DNA injury leads to ssDNA or dsDNA breaks due to oxidative or enzymatic damage (Aitken *et al*., 2013). The ssDNA breaks may impair fertilization (Simon & Lewis, 2011; Ribas-Maynou *et al*., 2012) whereas dsDNA breaks have also been reported to interfere with embryonic development and implantation (Lewis & Aitken, 2005) and are associated with recurrent miscarriages (Duran *et al*., 1998). Furthermore, Serafini *et al*. (2015) have reported that the comets obtained in neutral comet assay were elongated as compared to the rounder-shaped comets observed in alkaline comet assay, suggesting that sperm DNA remains in a more compact form in the alkaline comet, resulting in fewer actual comet shapes.

Sperm DNA integrity have been significantly related to male fertility in many species (Ballachey *et al*., 1988; Love & Kenney, 1998; Bungum *et al*., 2004). A wide variation in DNA integrity/fragmentation parameters have been observed among ejaculates of bulls with lower fertility potential (Bochenek *et al*., 2001). These tests evaluate different aspects of the sperm DNA structure in different species. There is not a single study regarding the use of acridine orange assay and neutral comet assay for use as biomarker of fertility for water buffalo bull spermatozoa during low and peak breeding seasons.

Sperm morphology and its relationship with fertility

Appraisal of sperm morphology is a major component of routine semen quality assessment. Evaluation of sperm morphology in buffalo bulls is usually done using phase contrast light microscopy to examine wet smears of buffered formalin solution–fixed spermatozoa (Gopalakrishna & Rao, 1978; Jainudeen *et al*., 1982), or light microscopy for smears stained either with eosin and nigrosin (Ahmad *et al*., 1987; Nordin *et al*., 1990, Saacke & Almquist, 1964; Saacke & Marshall, 1968; Foote *et al*., 1992; Suzuki *et al*., 1997).

The sperm abnormalities can be classified into primary (major) and secondary (minor) (Blom, 1950), The primary abnormalities occur due to problems at the level of developing spermatogonia, whereas secondary abnormalities occur in the transit of sperm through the epididymis or during the ejaculation process (Blom, 1983; Ott, 1986; Ahmad *et al*., 1987). Moreover, mishandling of the ejaculate i.e., excessive agitation, over–heating to rapid cooling, mixture of water, urine or antiseptic in the semen during processing are also responsible to cause abnormalities (Hossain *et al*., 1990).

Spermatozoa with abnormal morphology have direct influence on fertility in bovine. Experiments revealed that the proportions of morphologically abnormal spermatozoa in the semen are negatively correlated with fertility in bovine (Soderquist *et al*., 1991; Shamsuddin *et al*., 1993) and caprine (Chandler *et al*., 1988), whereas morphologically abnormal spermatozoa are unable to fertilize the oocytes (Shamsuddin & Rodriguez-Martinez, 1994). Studies have shown that the presence of higher abnormalities of spermatozoa is associated with reduced fertility in ruminants (Sarder, 2004). Reddy *et al.* (1975) have reported significant negative correlation between abnormal spermatozoa and conception rate in buffalo. Moreover, the morphological characteristics of spermatozoa are influenced by several factors including the genetic make-up and physiological stage of the animal, nutrition, season, climatic factors, and disease (Dowsett & Knott, 1996; Barth & Oko, 1989).

Estimating fertility with artificial insemination

When estimating fertility with AI it must be considered that a female not becoming pregnant after a single service tells little about the true fertility of the male (Muller, 2000). This is true because there are many factors causing a zygote not to develop into a fetus

detectable by rectal palpation or ultrasonography (Amann, 2005). Some causes of embryo death are associated with the fertilizing spermatozoon (Saacke *et al*., 2000), but most are associated with the ovum, environment within the female reproductive tract, physiology of the female, oestrus detection or timing of AI (Amann, 2005). Consequently, failure of pregnancy does not allow a conclusion if male, female or both were responsible for the failure. Pregnancy, on the other hand, is evidence that the female provided a satisfactory ovum and environment (Muller, 2000), and that a few sperm displayed the full repertoire of the attributes (Amann & Hammerstedt, 1993) necessary to survive in the oviduct and fertilize an ovum (Amann, 2005).

Another important factor impairing our ability to accurately estimate fertility is the number of spermatozoa present in the AI dose (Rodriquez–Martinez, 2003). There is a statistically significant relationship between the total number of viable spermatozoa inseminated (but not their percentage in the dose) and the fertility post AI, depending upon the fertility level of each sire (Shannon & Vishwanath 1995; Den Daas *et al*., 1998). It means that the addition of more spermatozoa per AI dose from sub-fertile bull will not result in better fertility (Pace *et al*., 1981; Den Daas *et al*., 1998). For this reason, it is suggested that development of a dose response curve for each sire in the AI dose that is required for maximum fertility is usually between 5 and 20 million spermatozoa (Rodriguez–Martinez, 2003). Additionally, the fertility rate must be corrected for several factors, such as inseminator, category of female considered, season and geographical area etc., to maximize the influence of external factors (Rodriguez–Martinez, 2003).

The most critical aspect of predicting the fertilization potential of semen samples is to have specific, precise, accurate and highly repeatable semen quality tests. Predicting the fertilising potential of semen samples on the basis of semen quality characteristics and analyzing their relationships with fertility needs high quality fertility data for comparison with standardized laboratory test results. For successful implementation of AI, field fertility results of semen used are important and indispensable. It is generally established that large data sets of field fertility and ejaculate data are more apposite to analyze effects of semen quality characteristics on field fertility. Variation in bull fertility is a common observation based on laboratory test as well as field fertility data (Parkinson & Whitfield, 1987; Totey *et al*., 1993; Yadav *et al*., 2001; Defoin *et al*., 2008; Oliveira *et al*., 2012). Moreover, bull fertility variation might be assigned to semen quality parameters. It is the

need of the day to design a semen quality test that possesses maximum capability to predict the fertilising potential of individual's bulls in a better way. In this regard, it is very mandatory that newly developed semen quality parameters should be validated for their relationship with field fertility.

This study provides an opportunity to apply various semen quality parameters to better understand the causes of male factor losses in the Pakistan's buffalo AI services. The techniques used in this study include CASA motility parameters, velocity distribution, motion kinematics, subpopulations of motile spermatozoa based on motion kinematics, targeted fluorochromes for sperm DNA fragmentation and integrity, viability/ mitochondrial transmembrane potential using acridine orange, propidium iodide and JC–1 respectively under epifluorescent microscope, supra-vital plasma membrane integrity, viability/ acrosome integrity and morphology using hypo–osmotic swelling test, Trypan blue/ Giemsa staining, wet mount and *in vivo* fertility during low and peak breeding seasons. These techniques were applied to identify the best biomarker in order to reduce male factor losses in buffalo AI services for improvement in fertility rate.

Objectives

This study was designed to investigate more thoroughly the semen quality parameters of buffalo bull spermatozoa to establish the best strategy for future prediction of fertility during low and peak breeding seasons with following objectives,

1. Semen quality parameters as fertility predictors of water buffalo bull spermatozoa during low–breeding season
2. Use of post–thaw semen quality parameters to predict fertility of water buffalo bull during peak breeding season
3. Seasonal influences on structural–functional parameters and *in vivo* fertility of buffalo bull spermatozoa
4. Response of semen quality parameters of buffalo bull spermatozoa to hydrogen peroxide (H_2O_2)

ABSTRACT

Background: Artificial insemination (AI) is useful for genetic improvement by the propagation of elite male germplasm. The present study was carried out to assess various post–thaw semen quality parameters for the prediction of fertility in buffalo bull during low-breeding season.

Materials and Methods: Semen (30 ejaculate) was collected from five adult buffalo bulls with artificial vagina (42 ℃). Sperm motility parameters, velocity distribution, motion kinematics, and subpopulations were analyzed by computer–aided sperm motion analyzer (CASA). Moreover, sperm subjective motility, supra–vital plasma membrane integrity, viability/acrosome integrity, viability/mitochondrial transmembrane potential, DNA fragmentation/integrity, and morphology were analyzed by phase–contrast microscope, supravital hypoosmotic swelling test, Trypan blue/Giemsa staining, propidium iodide/"5,5,6,6'–tetrachloro–1,1,3,3'–tetraethylbenzimidazolyl carbocyanine iodide" (JC–1) fluorochromes, neutral comet assay/acridine orange assay and wet mount technique, respectively. Outcome of 528 inseminations (at least 100 inseminations per bull) was analyzed for the *in vivo* fertility during low-breeding season.

Results: Pearson's correlation coefficients revealed that sperm progressive motility (PM, %), rapid velocity (RV, %), average path velocity (VAP, µm/s), straight line velocity (VSL, µm/s) and rapid subpopulation 1 (most rapid, and progressive) of motile spermatozoa (%) were significantly correlated with *in vivo* fertility during low breeding season ($r = 0.64$, $P < 0.01$; $r = 0.57$, $P < 0.01$; $r = 0.52$, $P < 0.01$; $r = 0.56$, $P < 0.01$ and $r = 0.73$, $P < 0.001$). Medium velocity (MV, %), curvilinear velocity (VCL, µm/s), amplitude of lateral head displacement (ALH, µm), beat cross frequency (BCF, Hz), straightness (STR, %), linearity (LIN, %), hyperactivated subpopulation 2 (%), low subpopulation 3 (%) and poor subpopulation 4 (%) were not correlated with *in vivo* fertility during low breeding season ($P > 0.05$). Moreover, supra–vital plasma membrane integrity (SV–PMI, %) and viable spermatozoa with intact acrosome (V/IACR, %) were significantly correlated with *in vivo* fertility ($r = 0.74$, $P < 0.001$ and $r = 0.88$, $P < 0.001$); whereas nonviable spermatozoa with damaged acrosome or low-mitochondrial transmembrane potential (NV/DACR or NV/LP, %) and comet length (CL, µm) of neutral comet assay were negatively associated with *in vivo* fertility during low breeding season ($r = –0.79$, $r = 0.75$, $P < 0.001$, and $r = –0.60$, $P < 0.05$, respectively). Percent subjective motility was not

correlated with *in vivo* fertility ($P > 0.05$) during low breeding season. Tail length (TL, μm), Tail DNA (TDNA, %), tail moment (TMom) and olive moment (OM) parameters of neutral comet assay were negatively correlated with *in vivo* fertility, but the results were non–significant ($P > 0.05$). Morphological parameters i.e., head abnormalities (%), tail abnormalities (%) and mid-piece abnormalities (%) were not correlated ($P > 0.05$) with *in vivo* fertility during low breeding season. Multiple regression analysis reported that combination of semen quality parameters as predictor of fertility were better (R^2 adjusted = 81.30%, $P < 0.001$) as compared with single parameter (R^2 adjusted = 50.20%, $P < 0.007$).

Conclusion: It is concluded that assessment of CASA parameters and some other sperm structural and functional parameters i.e., integrity of plasma membrane and acrosome, and transmembrane potential of mitochondria were able to predict the *in vivo* fertility of water buffalo bull during low-breeding season.

INTRODUCTION

Artificial insemination (AI) in water buffalo is useful for genetic improvement by the propagation of elite male germplasm (Anzar *et al*., 2003; Sohail *et al.,* 2013; Iqbal *et al.,* 2015; 2016). Just a single ejaculate can provide enough AI doses to uplift the genetic potential of a herd. The fertility in the field through AI of the frozen–thawed buffalo semen is low as compared to fresh ejaculates (Akhter *et al*., 2007; Andrabi, 2009). Because of this reason, accurate evaluation of the frozen–thawed semen is of significance for the AI process, since it can provide insights upon the fertilizing potential of the cryopreserved spermatozoa (Chatterjee *et al*., 2001; Rodriguez–Martinez, 2003). Sperm has a highly complex structure, with each component performing its specific function. Therefore, a single test or combination of tests to evaluate the structural cum functional integrity of frozen–thawed spermatozoa and to predict its fertilizing capacity in the field is required (Rodriguez–Martinez, 2003).

Subjective assessment of sperm motility is one of the most commonly used viability test, but manual analysis is markedly slow and prone to errors due to technician subjectivity (Rodriguez–Martinez, 2003). Moreover, it has been reported that subjective assessment of sperm motility has low predictive value (Broekhuijse *et al.,* 2011; Amann & Waberski, 2014; Sellem *et al*., 2015). Computer–aided sperm motion analysis (CASA) provides precise and consistent analysis of sperm motility parameters, velocity distribution and motion kinematics (Amann, 1989; Liu *et al*., 1991; Kathiravan *et al.,* 2008; 2011; Verstegen *et al.,* 2002; Freour, 2015). In a recent study, the prognostic value by the equation of CASA motility and motion characteristics in frozen–thawed samples accounted significantly to predict the *in vitro* or *in vivo* fertility of buffalo bull (Sohail *et al.,* 2013). For some time, mitochondrial transmembrane potential and acrosome integrity are in focus to assess the post–thaw semen quality of buffalo bull (Minervini *et al.,* 2013). Regarding sperm DNA integrity/fragmentation parameters in bovine, a wide variation has been observed among frozen–thawed samples with low and high fertilizing potential (Ballachey *et al.,* 1988; Karabinus *et al*., 1990; Aravindan *et al*., 1997; Bochenek *et al.,* 2001). Likewise, supra–vital hypo–osmotic swelling test is used to evaluate the structural and functional intactness of sperm plasmalemma in buffalo at post–thawing (Iqbal *et al.,* 2015; 2016). However, most of these tests are required to be exploited for the fertility prediction of frozen–thawed buffalo bull spermatozoa.

Fertility of cryopreserved water buffalo spermatozoa through AI is reported to be affected by seasonality, that is, ambient temperature, relative humidity, and day length in a particular season (Andrabi, 2014). Several studies (Tuli & Mehar, 1983; Heuer *et al.*, 1987; Bahga & Khokar, 1991; Koonjaenak *et al.,* 2007a, b, c) have reported lower cryo-damage and subsequently higher fertility of buffalo spermatozoa during the autumn/winter (peak breeding season) compared with that processed during the summer (low-breeding season). Post–thawing sperm motility was significantly higher in winter than in summer season (Tuli & Mehar, 1983; Bahga & Khokar, 1991). Likewise at post–thaw, plasma membrane integrity was higher in winter than summer (Koonjaenak *et al.,* 2007a). Regarding the post–thaw sperm DNA fragmentation index in swamp buffalo, its values varied significantly among seasons, being lower in humid months than winter (Koonjaenak *et al.,* 2007c), whereas Nandre *et al*. (2011) found a significant difference in post–thaw sperm DNA damage during winter as compared to summer in buffalo. A higher fertility rate was observed in frozen–thawed semen samples of buffalo bull during peak than low breeding season (Heuer *et al*., 1987; Younis *et al*., 1991). Hence, this highlights that accurate evaluation of structural cum functional integrity of cryopreserved spermatozoa and to predict its fertilizing capacity is critical for the success of AI (Park *et al*., 2012) in summer (Koonjaenak *et al.,* 2007b). Therefore, the development of association between semen quality parameters and *in vivo* fertility and accurate prediction of bull fertility may provide new insights during low breeding season.

The various post–thaw semen quality tests that can be used for accurate evaluation include (1) computer-aided sperm analysis (CASA) motility parameters, velocity distribution, and motion kinematics, (2) mitochondrial transmembrane potential (% of alive spermatozoa), (3) acrosome integrity (% of alive spermatozoa), (4) DNA damage (by acridine orange and neutral comet assays), and (5) supravital hypoosmotic swelling. However, most of these tests are required to be exploited as fertility predictors of cryopreserved buffalo bull spermatozoa during low-breeding season. The present study was carried out to assess various post–thaw semen quality parameters for the prediction of fertility in buffalo bull during low-breeding season.

MATERIALS AND METHODS

Chemicals

All the chemicals used in this study were purchased from Merck, Darmstadt, Germany.

Extender preparation

The stock tris citric acid (TCA) extender consisted of 7.80 g citric acid, 15.00 g tris–(hydroxymethyl) aminomethane, 1.00 g fructose, 35 mL glycerol, 0.05 g streptomycin sulphate and 100 mL hen egg yolk in distilled water to make a total volume of 500 mL. The final pH of extender was adjusted at 7.0. The extender was kept at -20 until use (Andrabi *et al*., 2008).

Selection of animals

Present study was conducted at Livestock Research Station (LRS), National Agricultural Research Centre (NARC), Park Road Islamabad, Pakistan (located at 33.43 °N 73.04 °E having elevation 507 meters). Five mature donor Nili-Ravi buffalo bulls of similar age (5–6 years) and body weight (500–600 kg) were used. Bulls were individually housed and maintained under uniform feeding and managemental conditions. Good quality seasonal green fodder was provided with 10 % body weight of bulls. Fixed amount of concentrate (3 kg/day) was offered to each bull and water *ad libitum*.

Semen collection, extension, cryopreservation and thawing

Semen was collected from five adult Nili–Ravi buffalo bull during May–June (low breeding season). The bulls were kept at LRS, NARC, Islamabad, under nearly identical nutritional and managemental conditions. Prior to semen collection, each bull was subjected to teasing by permitting one false mount and then restrained for a short period of time to increase semen quality and quantity (Branton *et al*., 1951). Semen was collected from each bull with artificial vagina (AV) at 42 °C for three times (n = 3 replicates and n = 2 ejaculates per bull on each collection day). After collection semen samples were transferred to the laboratory immediately. Sperm progressive motility was assessed using phase contrast microscope (x 400) connected with closed circuit monitor, and sperm concentration was measured by using the specific spectrophotometer (SDM 5, Minitube, Germany) at 546 nm. Qualifying ejaculates having > 60 % sperm motility and > 0.5 x 10^9

sperm/mL concentration from each bull were diluted in Tris–citric acid egg yolk glycerol (TCA) extender. Semen from each bull was processed and cryopreserved individually. For cryopreservation, semen of each bull was extended in TCA extender at 37 °C, cooled to 4 °C in 2 hr, and equilibrated for 4 hr in cold cabinet unit (4 °C). Extended semen was then packed in polyvinyl French straws (0.54 mL, IMV, France) and frozen in a programmable cell freezer (Kryo–550, Planer, Middlesex, UK). Finally, semen straws were plunged into liquid nitrogen (LN_2; –196 °C). The straws were stored in liquid nitrogen tank for at least 24 hr before post–thaw analyses. Thawing of semen straws was carried out for 30 sec at 37 °C in water bath (Andrabi *et al*., 2008, Figure 1).

Computer–aided sperm motion analysis (CASA)

Frozen–thawed semen samples were maintained at 37 °C for 5 min before evaluation. After thorough mixing, semen sample (7 µL) was placed on a prewarmed slide and cover slipped (Katz & Davis 1987, Nöthling *et al*., 2012). The sample loaded slide was fitted in a portable Mini Therm stage (37 ºC) of microscope (x 100) connected to a computer having CASA software (CEROS, version 12.3, Hamilton Thorne Biosciences, USA) and following sperm motility parameters, velocity distribution and motion kinematics were recorded:

1. Sperm motility parameters; total Motility (TM, %), progressive motility (PM, %)
2. Sperm velocity distribution; rapid velocity (%) and medium velocity (%)
3. Sperm kinematic parameters (Table 1, Figure 2); average path velocity (VAP, µm/s), curvilinear velocity (VCL, µm/s), straight line velocity (VSL, µm/s), amplitude of lateral head displacement (ALH, µm), beat cross frequency (BCF, Hz), straightness (STR, ratio of VSL/VAP, %) and linearity (LIN, ratio of VSL/VCL, %).

Identification of motile sperm subpopulations based on CASA kinematics (%)

Four subpopulations of motile spermatozoa were defined in each sample according to Muino *et al*. (2008a, b; 2009) with slight adjustments. The four distinguishable sperm subpopulations in each sample were calculated by using CASA generated frequency distribution values (bar charts) of kinematics. Finally, the average percentages of spermatozoa showing (1) VAP > 80 µm/s, VSL > 90 µm/s, VCL > 120 µm/s, ALH > 4–6 µm, BCF > 42 Hz, STR > 90 % and LIN > 80 % were classified as subpopulation 1 (rapid; most rapid and progressive), (2) VAP > 60–80 µm/s, VSL 30–60 µm/s, VCL 90–120,

ALH > 6 μm, BCF 30–36 Hz, STR < 60 % and LIN 30–50 % were classified as subpopulation 2 (Hyperactivated; highly active but non–progressive), (3) VAP 20–60 μm/s, VSL 60–90 μm/s, VCL 60–90 μm/s, ALH 2–4 μm, BCF 36–42 Hz, , STR 70–90 % and LIN 50–80 % were classified as subpopulation 3 (low; low velocity but relatively high progressiveness) and (4) VAP 10–20 μm/s, VSL 0–30 μm/s, VCL 20–60 μm/s, ALH 0–2 μm, BCF < 30 Hz, STR 60–70 % and LIN < 30 % were classified as subpopulation 4 (poor; poorly motile and non–progressive Table 2).

Sperm subjective motility assessment (%)

Subjective motility of frozen–thawed semen samples was assessed under phase–contrast microscope (Olympus BX20, x 400) to the nearest 5 %, and taking the average of these readings for the final motility estimate in 4–5 microscopic fields.

Sperm viability and plasma membrane integrity assay (%)

At post–thawing, sperm viability and plasma membrane integrity (structural and functional) was evaluated with supra–vital plasma membrane integrity (SV–PMI). The HOST solution (osmolarity 190, mOsmol/kg) composed of sodium citrate (0.735 g) and fructose (1.351 g) in 100 mL distilled water. Aliquots of frozen–thawed semen samples (50 μL) were mixed with HOST solution (500 μL) and incubated at 37 ºC for 40 min. After incubation, 5 μL of semen was mixed with 5 μL of Eosin (0.5 % in 2.92 % tri–sodium citrate dihydrate) on a prewarmed glass slide. A cover slip was placed on the mixture and analyzed under microscope (phase–contrast, x 400). Two hundred spermatozoa per sample were counted for estimation of structural and functional integrity of plasma membrane. Spermatozoa displaying clear heads and swollen tails were designated as structurally and functionally viable, whereas, sperm with pink heads and un–swollen tails were classified as nonviable (Chan *et al*., 1991).

Sperm viability and acrosome integrity assay (%)

Sperm viability and acrosome integrity was assessed according to Kovacs & Foote, (1992) and Serafini *et al*., (2014). Five μL of semen and Trypan blue (0.4 % in distilled water) were placed on a slide, mixed thoroughly and dragged across the slide with the edge of another slide, air–dried in vertical position and put into a fixative consisting of 86 mL 1N HCl, 14 mL of 37 % formaldehyde and 0.20 g Neutral Red for 2 min, rinsed with distilled

water followed by application of Giemsa stain (7.50 %) for 2.5 hr at 37 ºC. The slides were rinsed with distilled water, air–dried in vertical position, mounted with DPX and coverslipped (24 x 50 mm). Two hundred spermatozoa per sample were categorized as viable spermatozoa with intact acrosome (white head region and purple acrosomal region, V/IACR, %), viable spermatozoa with damaged acrosome (white head region and pale lavender acrosomal region, V/DACR, %), nonviable spermatozoa with intact acrosome (blue head region and purple acrosomal region, NV/IACR, %), and nonviable spermatozoa with damage acrosome (blue head region and pale lavender acrosomal region, NV/DACR, %) under phase contrast microscope (x 400).

Sperm viability and mitochondrial transmembrane potential assay (%)

Viability and mitochondrial transmembrane potential of frozen–thawed buffalo spermatozoa was evaluated with PI (propidium iodide, 1 mg/mL in PBS) and 5,5',6,6'–tetrachloro–1,1',3,3'–tetraethylbenzimidazolyl carbocyanine iodide (JC–1, 0.15 mmoL in DMSO) according to Guthrie & Welch, (2006). Semen (100 µL, 2.5 x 10^6 sperm) was incubated with 10 µL PI and 20 µL JC–1 for 10 min at 37 ºC in complete darkness. After incubation, 10 µL of sample was placed on a glass slide and coverslipped and observed under epifluorescence microscope (x 400, Nikon AFX–1 Optiphot, 590/630 nm excitation/barrier filter). Two hundred spermatozoa per sample were counted for V/HP (%, viable with high mitochondrial potential, unstained head with bright orange mitochondria), NV/HP (%, nonviable with high mitochondrial potential, stained head with bright orange mitochondria), V/LP (%, viable with low mitochondrial potential, unstained head with green mitochondria), and NV/LP (%, nonviable with low mitochondrial potential, stained head with green mitochondria).

Sperm morphology assay (%)

To assess sperm morphology in frozen–thawed samples, a drop of semen was fixed in 1% formal citrate and examined under oil immersion (x 1000). Two hundred spermatozoa were counted to determine the percentages of head, mid–piece and tail abnormalities.

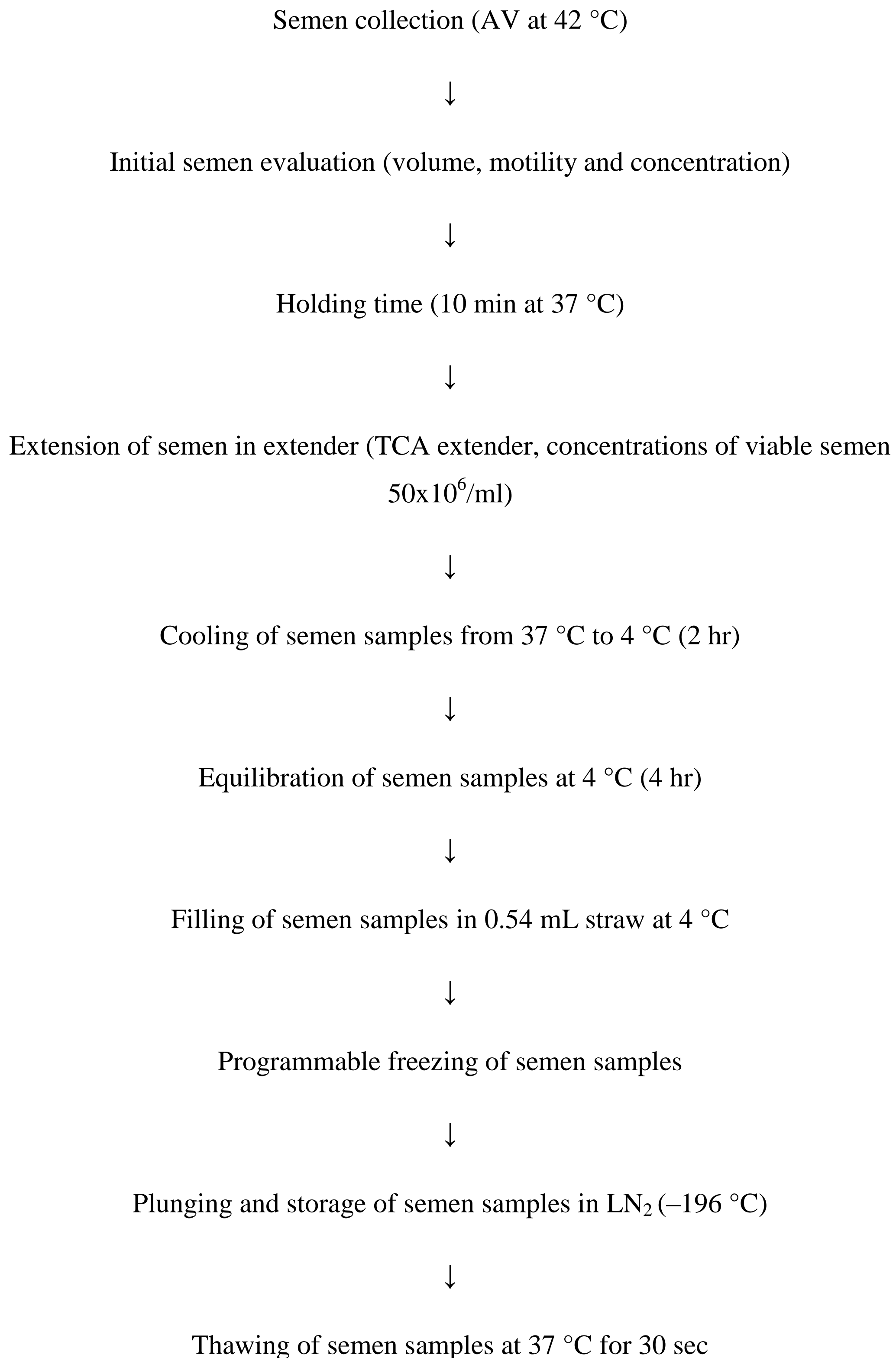

Figure 1. Semen Cryopreservation protocol for buffalo bull spermatozoa

Table 1. Analysis setup of CASA for estimation of sperm motility and kinematic parameters

Frames acquired	30
Frame rate	60 Hz
Minimum cell size	5 Pixels
Minimum static contrast	30
Motile, VAP cutoff	20 µm/s
Progressive motile	VAP 80 µm/s, STR 80 %
Rapid (velocity)	VAP > 80 µm/s
Medium (velocity)	VAP > 20 µm/s < 80 µm/s
Slow (velocity)	VAP < 20 µm/s > 10 µm/s
Magnification	1.89
Temperature, set	37 ºC

VAP = Average Path Velocity, STR = Straightness

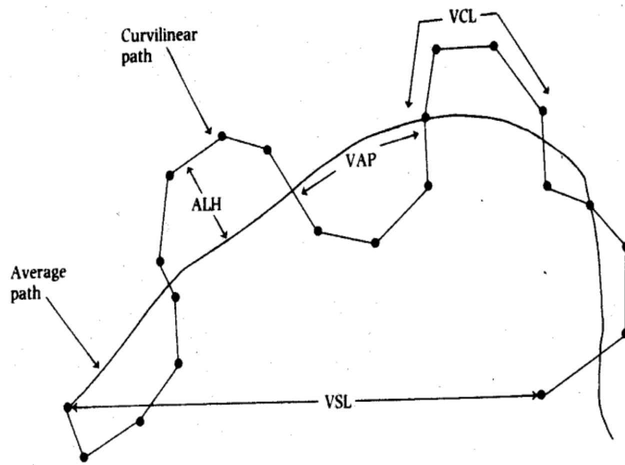

Figure 2. Schematic representation of the sperm kinematic parameters as measured by the computer–aided sperm motion analysis. VSL = straight line velocity, VAP = average path velocity, and VCL = curvilinear velocity, ALH = amplitude of lateral head displacement.

Table 2. Sperm motility kinematics for the four subpopulations identified in frozen–thawed semen samples of buffalo bull

Sperm motility kinematics	Sperm subpopulations			
	Rapid (1)	Hyperactivated (2)	Low (3)	Poor (4)
VAP (µm/s)	> 80	60–80	20–60	10–20
VSL (µm/s)	> 90	30–60	60–90	0–30
VCL (µm/s)	> 120	90–120	60–90	20–60
ALH (µm)	4–6	> 6	2–4	0–2
BCF (Hz)	> 42	30–36	36–42	< 30
STR (%)	> 90	< 60	70–90	60–70
LIN (%)	> 80	30–50	50–80	< 30

Sperm DNA fragmentation assay (neutral comet assay)

The sperm DNA fragmentation status in each of the frozen–thawed semen sample was determined by neutral comet assay (Donnelly *et al.,* 2000; Boe–Hansen *et al.,* 2005). Frosted microscopic slides were covered with 100 µL of 1% normal melting point agarose at 45 ºC and coverslipped. The slides were then placed over a pre–chilled tray and left at 4 ºC for 30 min to solidify the agarose. After removing the coverslip, a second layer containing 20 µL of semen (1 x 10^5 spermatozoa/mL) and 65 µL of 1% low melting point agarose at 37 ºC was made on top of the first layer. The cells were then lysed in cold lysis buffer (pH, 10.3) containing 2.5 M NaCl, 100 mmoL EDTA, 10 mmoL Tris Base (pH, 10.3), 1% (v/v) Triton X–100, 10 mmoL DTT (Dithiothreitol) and 10 µg/L Proteinase K for 1 hr at room temperature followed by 3.5 hr at 37 ºC. Slides were placed facing anode in the electrophoresis tray containing neutral electrophoresis buffer (54 g/L Tris base, 27.5 g/L boric acid, 0.5 M EDTA, pH 8.0). Electrophoresis was performed for 20 min at 25V (0.714 V/cm). When electrophoresis was completed the slides were dried overnight at 5 °C, rehydrated with distilled water for 10 min, stained with Acridine orange and observed under epifluorescence microscope (x 400, Nikon AFX–1 Optiphot, 590/630 nm excitation/barrier filter). Digital images were captured for subsequent analyses and scoring Figure 3 and Table 3) with TRITEK software. The parameters analyzed were comet length

(CL, µm), head DNA (HDNA, %), tail length (TL, µm), tail DNA (TDNA, %), tail moment (TMom) and olive moment (OM).

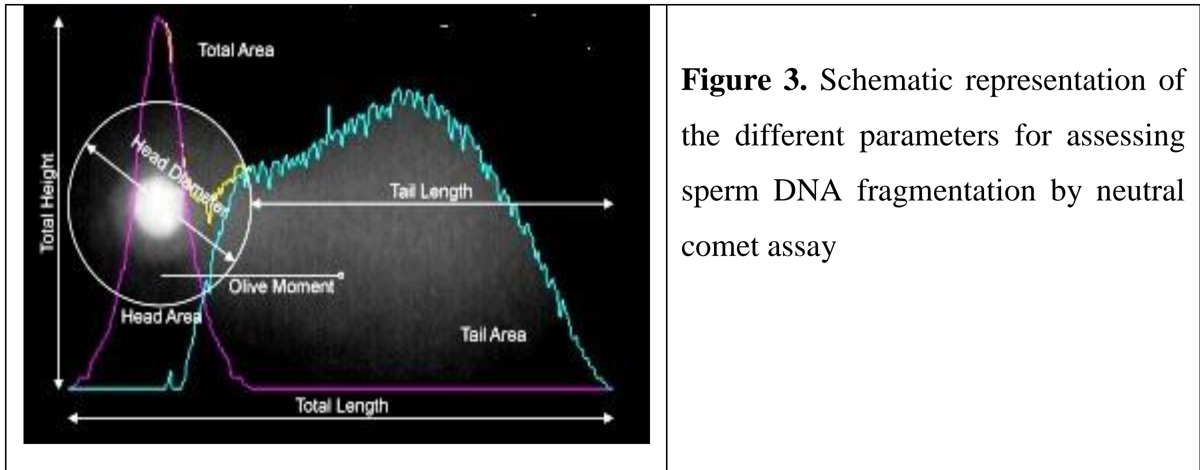

Figure 3. Schematic representation of the different parameters for assessing sperm DNA fragmentation by neutral comet assay

Table 3. Parameters for assessing sperm DNA fragmentation by NCA (neutral comet assay)

Comet length (CL) or Total length (µm)	The distance between the right and left edges of the comet
Head DNA (HDNA, %)	The proportion of total DNA present in the head
Tail length (TL, µm)	The distance of DNA migration from the body of the nuclear core
Tail DNA (TDNA, %)	The proportion of total DNA present in the tail
Tail moment (TMom)	Product of DNA in the tail and mean distance of DNA migration in the tail
Olive moment (OM)	Product of DNA in the tail and mean distance of DNA migration in the tail and head

Sperm DNA integrity assay (%, acridine orange assay)

DNA integrity of water buffalo bull spermatozoa was determined according to Martins *et al*. (2007) with slight modifications i.e., fixing of semen smear in Carnoys solution (fixative, methanol and glacial acetic acid, ratio 3:1) for 2 hr at room temperature (25 ºC)

instead of 24 hr. Two slides per bull were prepared by making a smear of 20 µL and dried. The slides were then air dried and finally kept in Tampon solution (buffer having 15 mmol/L $Na_2HPO_4.7H_2O$ and 80 mmol/L citric acid; having pH 2.5 at 75 °C) for 10 min to check the chromatin stability. Slides were stained with acridine orange (0.2 mg ml/L in distilled water). The images of 200 randomly selected nuclei per sample were analyzed for intact and damaged DNA with epifluorescence microscope (x 400, Nikon, Optiphot; 490/550 nm excitation/barrier filter). Green fluorescence was considered as an intact DNA (%, double stranded), whereas red fluorescence was taken as denatured DNA (%, single stranded).

In vivo **fertility estimation (%)**

A total of 528 buffaloes in their 2nd or 3rd lactation with clinically normal reproductive tract and showing signs of true estrus were inseminated maintained by farmers in the vicinity of Islamabad in summer of 2014. Estrus detection was based on observations like mucus discharge and decrease in milk production. All the experimental inseminations were performed approximately 24 hrs after onset of heat. Semen straws of 0.54 mL were used for AI in buffaloes. The artificially bred animals were examined for pregnancy through rectal palpation at least 60 days post–insemination.

Statistical analyses

Cryopreserved semen from five buffalo bulls were used for the laboratory tests and fertility estimation. Pearson's correlation coefficients were determined to provide a linear association between semen quality parameters and *in vivo* fertility. Multiple regression analysis was used to establish the prognostic values of semen quality parameters for *in vivo* fertility as a dependent variable. The level of significance was $P < 0.05$. Minitab (MINITAB® Release 12.22, 1998) statistical package was used for data analyses.

RESULTS

Pearson's correlation coefficients between CASA parameters and *in vivo* fertility of water buffalo bull spermatozoa during low-breeding season

The data on Pearson's correlation coefficients between CASA motility parameters, velocity distribution, and motion kinematics, subpopulations of motile spermatozoa, and *in vivo* fertility of water buffalo bull during low-breeding season are presented in Table 4. The results showed that sperm PM, RV, VAP, VSL, and rapid subpopulation 1 were significantly correlated with *in vivo* fertility during low-breeding season ($r = 0.64$, $P < 0.01$; $r = 0.57$, $P < 0.01$; $r = 0.52$, $P < 0.01$; $r = 0.56$, $P < 0.01$; $r = 0.73$, $P < 0.001$). Medium velocity and ALH were negatively correlated with *in vivo* fertility, but the results were non–significant ($P > 0.05$). Moreover, VCL, BCF, STR, LIN, hyperactivated subpopulation 2, low subpopulation 3 and poor subpopulation 4 were not correlated with *in vivo* fertility (Table 4).

Pearson's correlation coefficients between sperm functional parameters and *in vivo* fertility of water buffalo bull during low-breeding season

The data on Pearson's correlation between sperm functional parameters and *in vivo* fertility of water buffalo bull during low-breeding season are presented in Table 5. Sperm SV–PMI and V/IACR were significantly correlated with *in vivo* fertility ($r = 0.74$; $P < 0.001$ and $r = 0.88$; $P < 0.001$), whereas NV/DACR or NV/LP were negatively associated with *in vivo* fertility ($r = 0.79$; $P < 0.001$ and $r = 0.75$; $P < 0.001$) during low-breeding season. CL parameter of neutral comet assay was negatively associated with *in vivo* fertility ($r = –0.60$, $P < 0.05$). Percent subjective motility was not correlated with *in vivo* fertility ($P > 0.05$). TL, TDNA, TMom and OM were negatively correlated with *in vivo* fertility, but the results were non–significant ($P > 0.05$, Table 5).

Predictive model for frozen–thawed semen samples to estimate *in vivo* fertility of buffalo bull during low breeding season

Data on multiple regression models based on semen quality parameters to predict the *in vivo* fertility of buffalo bull during low-breeding season ate presented in Table 6. Various predictive models explained 46.7%–81.3% of the variation in fertility of frozen–thawed

buffalo spermatozoa. The prognostic value to predict the *in vivo* fertility by the equation of VAP, VSL, VCL, rapid subpopulation 1, SV–PMI, V/IACR, and V/HP accounted for 81.30% (P < 0.001) during low-breeding season (Table 6).

Table 4. Correlations (r–values) between post–thaw CASA parameters and *in vivo* fertility of buffalo bull during low breeding season (n = 5 bulls, n = 3 replicates).

Semen quality parameters	Mean value	r–value	P–value
TM (%)	63.87±3.16	0.413	0.135
PM (%)	24.78±2.12	0.642	0.010
RV (%)	29.10±2.07	0.570	0.014
MV (%)	36.24±4.56	–0.283	0.312
VAP (µm/s)	71.97±1.29	0.520	0.018
VSL (µm/s)	61.37±1.27	0.560	0.014
VCL (µm/s)	110.00±2.56	0.303	0.272
ALH (µm)	05.83±0.10	–0.142	0.602
BCF (Hz)	32.36±0.66	0.153	0.591
STR (%)	84.08±0.60	0.230	0.410
LIN (%)	55.62±0.97	0.301	0.283
Rapid subpopulation (%)	18.57±1.21	0.732	0.002
Hyperactivated subpopulation (%)	15.57±1.04	0.314	0.262
Low subpopulation (%)	22.03±2.81	0.280	0.313
Poor subpopulation (%)	07.53±0.84	0.252	0.365

TM= Total motility, PM= Progressive motility, RV = Rapid velocity, MV= Medium velocity, VAP = Average path velocity, VSL = Straight line velocity, VCL = Curvilinear velocity, ALH = Amplitude of lateral head displacement, BCF = Beat cross frequency, STR = Straightness, LIN = Linearity

Table 5. Correlations (r–values) between post–thaw sperm functional parameters and *in vivo* fertility of buffalo bull during low breeding season (n = 5 bulls, n = 3 replicates).

Semen quality parameters	Mean value	r–value	P–value
SM (%)	48.67±1.33	0.430	0.113
SV–PMI (%)	25.27±0.95	0.741	0.001
V/IACR (%)	32.27±1.16	0.883	0.001
V/DACR (%)	15.33±0.51	0.070	0.403
NV/IACR (%)	15.90±0.66	0.332	0.225
NV/DACR (%)	36.50±1.62	–0.790	0.001
V/HP (%)	26.31±0.95	0.490	0.065
NV/HP (%)	07.67±0.88	0.232	0.412
V/LP (%)	30.77±2.67	0.401	0.062
NV/LP (%)	35.25±1.24	–0.753	0.001
CL (μm)	343.06±3.55	–0.600	0.034
HDNA (%)	81.80±0.54	0.142	0.082
TL (μm)	87.40±1.07	–0.203	0.080
TDNA (%)	18.20±0.54	–0.144	0.090
TMom	31.85±1.34	–0.303	0.074
OM	20.61±0.47	–0.402	0.071
DNA integrity (%)	86.63±0.47	0.411	0.080
Head abnormalities (%)	05.93±0.18	0.174	0.550
Mid–piece abnormalities (%)	04.33±0.27	–0.060	0.442
Tail abnormalities (%)	01.90±0.20	–0.083	0.600

SM= Subjective motility, SV–PMI = Supra–vital plasma membrane integrity, V/IACR = Viable with intact acrosome, V/DACR = Viable with damaged acrosome, NV/IACR = Nonviable with intact acrosome, NV/DACR = Nonviable with damaged acrosome, V/HP = Viable with high mitochondrial transmembrane potential, NV/LP = Nonviable with low mitochondrial transmembrane potential, CL = Comet length, HDNA = Head DNA, TL = Tail length, TDNA = Tail DNA, TMom = Tail moment, OM = Olive moment

Table 6. Prognostic values to predict the *in vivo* fertility rate (FR, %) by the predictive equation based on post–thaw semen quality parameters in buffalo bull during low breeding season (n = 5 bulls, n = 3 replicates).

Response	Variables in predictive equations	R^2 (adjusted)	P–value
FR	VAP, VSL,VCL, Rapid subpopulation 1, SV–PMI, V/IACR, V/HP	81.30%	0.001
FR	VAP, VSL, VCL, SV–PMI, V/IACR	79.00 %	0.002
FR	VAP, VSL, VCL, SV–PMI, V/IACR, V/HP	76.5 %	0.002
FR	PM, VAP, VSL, Rapid subpopulation 1	70.30 %	0.002
FR	PM, VAP, VSL, VCL, Rapid subpopulation 1	68.40 %	0.006
FR	TM, PM, VAP, VSL, Rapid subpopulation 1	67.80 %	0.007
FR	VAP, VSL, Rapid subpopulation 1	67.80 %	0.002
FR	Rapid subpopulation 1, SV–PMI, VSL, V/IACR	60.20 %	0.003
FR	PM, VAP, Rapid subpopulation 1	58.60 %	0.005
FR	Rapid subpopulation 1, SV–PMI	55.20 %	0.003
FR	VSL, Rapid subpopulation 1	50.50 %	0.006
FR	Rapid subpopulation 1	50.20 %	0.007
FR	VAP, Rapid subpopulation 1	46.70 %	0.009

TM = Total motility, PM = Progressive Motility, VAP = Average Path Velocity, VSL = Straight Line Velocity, VCL = Curvilinear Velocity, ALH = Amplitude of Lateral Head Displacement, BCF = Beat Cross Frequency, V/HP = Viable with high mitochondrial transmembrane potential, SV–PMI = Supravital–plasma membrane integrity, V/IACR = Viable with intact acrosome

DISCUSSION

The present study was carried out to assess various post–thaw semen quality parameters for the prediction of fertility in buffalo bull during low-breeding season. To the best of our knowledge, this is the first comprehensive study of its kind in water buffalo bull in Pakistan with ultimate goal of improving the outcome of AI in low-breeding season.

Sperm CASA motility is generally considered to be one of the most significant features linked with the fertilizing potential (Mortimer, 1994; Verstegen *et al*., 2003; Kathiravan *et al*., 2008). Results of the present study reported that CASA progressive motility of buffalo bull spermatozoa was positively associated with *in vivo* fertility during low-breeding season. Previously, CASA progressive motility has been positively correlated with in vitro fertility in buffalo (Koonjaenak *et al*., 2007a) and bovine (Farrell *et al*., 1998; Kathiravan *et al*., 2008). Moreover, previous studies suggest that sperm progressive motility can be used to estimate the fertility of frozen–thawed swamp buffalo spermatozoa during breeding seasons (Koonjaenak *et al*., 2007a).

CASA provides precise and useful information on various sperm motion characteristics that are helpful to predict fertility status of semen samples (Macleod & Irvine, 1995; Kathiravan *et al*., 2008; 2011). Furthermore, sperm motion kinematics has been associated with *in vivo* fertility in bovine (Farrell *et al*., 1998). In the present study, sperm motion kinematics i.e., average path and VSLs have established positive association with *in vivo* fertility during low-breeding season. These results are in agreement with the findings of Farrell *et al*. (1998), who reported that spermatozoa with higher velocity are more potent for fertilization in bovine. Moreover, sperm motion kinematics is related to several aspects of sperm function including penetration to cervical mucus, zona pellucida, and IVF outcome (Mortimer, 2000; Wang *et al*., 1991). Januskauskas *et al*. (2001) found no relationship between sperm motion kinematics and *in vivo* fertility in bovine during the breeding season. This difference between results might be due to CASA settings for calculating sperm motility parameters and kinematics.

The presence of four subpopulations of motile spermatozoa based on motion kinematics has been defined in bovine (Muino *et al*., 2008a; 2009). Rapid subpopulation 1 represented the most rapid and progressively motile spermatozoa and is considered essential for the fertilization process. Whereas, subpopulations 2, 3, and 4 of spermatozoa

show motility pattern with less–thrusting power (Farrell *et al*., 1998; Nunez–Martinez *et al*., 2006). In the present study, four subpopulations of motile spermatozoa were defined in frozen-thawed semen samples of buffalo. Our results reported that percentages of rapid subpopulation 1 (most rapid and progressive) of motile spermatozoa were significantly associated with *in vivo* fertility during low-breeding season. These results are comparable with the findings in bovine (Muino *et al*., 2008a; 2009) and equine (Quintero–Moreno *et al*., 2003) during their respective breeding seasons.

Subjective motility estimation of spermatozoa is the most commonly used semen quality test. In this study, percent subjective motility of buffalo bull spermatozoa was not correlated with *in vivo* fertility during low-breeding season. However, Younis *et al*. (1999) have reported a highly significant relationship between frozen–thawed sperm subjective motility and *in vivo* fertility of buffalo bull during low-breeding season. This difference between the studies might be due to the cut off values for assessing the post–thaw subjective motility and/or number of spermatozoa in the AI dose.

Plasma membrane integrity is considered to be useful for predicting the fertilizing ability of semen sample of buffalo bull (Andrabi *et al*., 2016). Supra–vital HOST evaluates the structural and functional integrity of the sperm plasma membrane. Results of the present study revealed that viable spermatozoa with intact plasmalemma were positively associated with *in vivo* fertility during low-breeding season. Previous studies have shown relationship between sperm membrane integrity and fertilizing ability in Swamp buffalo (Koonjaenak *et al*., 2007a) and bovine (Januskauskas *et al*., 2001) during their respective breeding seasons. Whereas Zhang *et al*. (1999) found no association between plasmalemma integrity and fertility in bovine during the breeding season.

The presence of normal acrosome on a spermatozoon is essential at the time of fertilization. Moreover, spermatozoa with an intact acrosome are fundamental for oocyte penetration and improved the chances of fertilization (Saacke *et al*., 1972). Results of the present study reported that viable spermatozoa with intact acrosome were significantly associated with *in vivo* fertility during low-breeding season. These results are in accordance with that of Minervini *et al*. (2013) in buffaloes that optimum fertility of bull depends on viable spermatozoa with intact acrosome.

Sperm mitochondrial transmembrane potential is considered as one of the most sensitive tests for semen quality assessment and qualification of field fertility (Hallap *et al.,* 2005, Selvaraju *et al*., 2008). In this study, viable spermatozoa with higher mitochondrial transmembrane potential have established positive relationship with *in vivo* fertility during low-breeding season. These findings are comparable with that of Selvaraju *et al*. (2008) in buffalo bull and human (Kasai *et al*., 2002; Marchetti *et al*., 2002).

Normal sperm genetic material is required for successful fertilization, as well as for further embryo and fetal development that will result in a healthy offspring (Saacke, 1998; Andrabi, 2007). In this study, neutral comet assay parameter, that is, comet length was negatively associated with *in vivo* fertility during low-breeding season. Similarly, Koonjaenak *et al*. (2007a) reported that higher DNA fragmentation of buffalo spermatozoa is linked with low-fertilization potential. Interestingly in present study, sperm DNA integrity evaluated with acridine orange assay did not correlate with *in vivo* fertility during low-breeding season. The discrepancy can be attributed to the sensitivity of the assay compared with neutral comet assay (Andrabi, 2007).

Evaluation of sperm morphology is a major component of routine semen quality assessment. The percentage of spermatozoa with normal morphology in a semen sample is related to its viability (Barth, 1989). In the present study, sperm morphology of frozen–thawed samples was evaluated in terms of head, mid–piece, and tail abnormalities. The results reported that sperm abnormalities were not correlated with *in vivo* fertility during low-breeding season. Conversely, a negative relationship has been documented between morphologically abnormal spermatozoa and fertility in buffaloes during low- and peak-breeding season (Younis *et al*., 1999). This deviation could be due to the fact that optimum cryopreservation process does not necessarily increase the sperm abnormalities particularly the tail abnormalities (Holt, 1997), which may affect the in vivo fertility of buffalo bull spermatozoa.

It has been reported that the analysis of single parameter usually does not give a high predictive value of fertility (Januskauskas *et al*., 2001). Therefore, several predictive equations were generated based on various semen quality parameters and *in vivo* fertility data during low breeding season at post-thawing. In this study, the prognostic value to predict the *in vivo* fertility by the equation of VAP, VSL, VCL, subpopulation 1, SV–PMI, V/IACR, and V/HP accounted for 81.30 % ($P < 0.001$). Farrell *et al*. (1998) reported that

combinations of PM, VSL, ALH, and BCF provided higher predictive value for fertility (R^2 = 0.89). It was reported earlier, that sperm motion characteristics and PM could be used to predict the fertility of bull (Kathiravan *et al*., 2008). In a nut shell, we observed that predicting the fertilizing potential of buffalo bull semen was better when number of parameters were combined during low-breeding season.

Conclusion

It is concluded that assessment of CASA parameters and sperm structural and functional parameters i.e., integrity of plasma membrane and acrosome, and transmembrane potential of mitochondria were able to predict the *in vivo* fertility of water buffalo bull during low-breeding season. It is expected that application of these findings will improve the outcome of AI in water buffalo during low-breeding season.

ABSTRACT

Background: Quality of frozen semen is one of the most influential factors to establish a reasonable conception rate in the farm animals. The present study was designed to predict the fertility of water buffalo bull by using post–thaw semen quality parameters during peak breeding season.

Materials and Methods: Semen (30 ejaculate) was collected from five adult buffalo bulls with artificial vagina (42 ºC). Sperm motility parameters, velocity distribution, motion kinematics, and subpopulations were analyzed by computer–aided sperm motion analyzer (CASA). Moreover, sperm subjective motility (SM, %), supra–vital plasma membrane integrity (SV–PMI, %), viability/acrosome integrity (V/IACR, %), viability/mitochondrial transmembrane potential, DNA fragmentation/integrity, and morphology were analyzed by phase–contrast microscope, supravital hypoosmotic swelling test, Trypan blue/Giemsa staining, propidium iodide/"5,5,6,6'–tetrachloro–1,1,3,3'–tetraethylbenzimidazolyl carbocyanine iodide" (JC–1) fluorochromes, neutral comet assay/acridine orange assay and wet mount technique, respectively. Outcome of 514 inseminations (at least 100 inseminations per bull) was analyzed for *in vivo* fertility during peak breeding season.

Results: Pearson's correlation coefficients showed sperm CASA progressive motility (PM, %), rapid velocity (RV, %), average path velocity (VAP, µm/s), straight line velocity (VSL, µm/s) and straightness (STR, %) of buffalo bull were significantly correlated with *in vivo* fertility during peak breeding season ($r = 0.81$, $P < 0.01$; $r = 0.85$, $P < 0.01$; $r = 0.64$, $P < 0.05$; $r = 0.73$, $P < 0.05$ and $r = 0.57$, $P < 0.05$, respectively). Rapid subpopulation 1 (%) was positively correlated with *in vivo* fertility ($r= 0.65$, $P < 0.05$), whereas sperm total motility (TM, %), medium velocity (MV, %) curvilinear velocity (VCL, µm/s), beat cross frequency (BCF, Hz), linearity (LIN, %), hyperactivated subpopulation 2 (%), low subpopulation 3 (%) and poor subpopulation 4 (%) were not correlated with *in vivo* fertility ($P > 0.05$) of buffalo bull during peak breeding season. Moreover, sperm SM, SV–PMI, V/IACR and viability/ high mitochondrial transmembrane potential (V/HP, %) were positively correlated *in vivo* fertility during peak breeding season ($r = 0.79$; $P < 0.01$, $r = 0.88$, $P < 0.01$, $r = 0.84$, $P < 0.01$ and $r = 0.81$, $P < 0.01$, respectively). However, tail length (TL, µm) and nonviable with damaged acrosome (NV/DACR, %) were negatively correlated with *in vivo* fertility ($r = –0.70$, $P < 0.05$ and $r = –0.81$, $P < 0.01$, respectively). Neutral comet assay parameters like comet length (CL,

μm), head DNA (HDNA, %), tail moment (TMom) and OM were not correlated ($P > 0.05$) with *in vivo* fertility during peak breeding season. Sperm morphology parameters like head, mid–piece and tail abnormalities were not significantly correlated with *in vivo* fertility ($P > 0.05$) of buffalo bull during peak breeding season. Multiple regression analyses showed that the best predictive equation (R^2 adjusted=83.50 %, $P < 0.000$) of fertility included five parameters i.e., PM, RV, VAP, VSL and SV–PMI during peak breeding season. The best single predicator of fertility for frozen–thawed buffalo semen was CASA PM (R^2 adjusted=43.10 %, $P < 0.04$).

Conclusion: In conclusion, fertility of buffalo bull can be predicted through some of the post–thaw *in vitro* semen quality tests during peak breeding season.

INRODUCTION

Artificial insemination (AI) is one of the major reproductive techniques of the 20th century through which rapid genetic improvement has been achieved in the developed countries. Quality of frozen semen is one of the most influential factors to establish a reasonable conception rate in the farm animals. Although male factors have been long recognized, it is only recently that scientific advances have allowed insight into specific causes and effects of male factors as a cause of significant loss in fertility. However, exploitation of these advances has largely yet to occur in buffalo AI services (Andrabi, 2009).

To achieve an optimal fertility of elite bulls through AI in buffalo is mostly dependent on precise and accurate assessment of semen quality. In this regard, computer–assisted sperm analysis (CASA) provides an objective classification of a given population of spermatozoa and is considered as one of the most reliable methods to evaluate semen quality (Comhaire *et al*., 1992; Den Daas *et al*., 1998; Mortimer, 2000; Verstegen *et al.,* 2002; Rijsselaere *et al*., 2003; 2005; Amann & Katz 2004; Rodriguez-Martinez, 2007; Marquez & Suarez, 2007).

Sperm mitochondrial transmembrane potential is indispensable for the fertilization process, and the damaged sperm DNA can lead to early embryonic or fetal death (Januskauskas *et al*., 2005; Bollwein *et al*., 2008; Guthrie *et al.,* 2011). Sperm mitochondrial transmembrane potential can be evaluated by using the fluorescent probe "5,5',6,6'–tetrachloro–1,1',3,3'–tetraethylbenzimidazolyl carbocyanine iodide" (JC–1) (Garner *et al*., 1994; Garner & Thomas 1999; Finkel, 2001). Sperm DNA damage can be detected all the time with accuracy by using targeted fluorochromes (propidium iodide–PI or acridine orange–AO) i.e., sperm DNA fragmentation and integrity through Neutral Comet and Acridine Orange assays, respectively (Andrabi, 2007). Semen quality parameters like mitochondrial transmembrane potential, viability, plasma membrane and acrosome integrity of bovine spermatozoa have been correlated with non–return rates or *in vitro* fertilization rates (Oura & Toshimori, 1990; Thomas *et al.,* 1997; Rodriguez–Martinez, 2003; Tartaglione & Ritta, 2004; Selvaraju *et al.,* 2008; Christensen *et al.,* 2011). Moreover, it has been suggested that a combination of *in vitro* semen quality assays were better predictor of *in vivo* fertility compared to single test in bovine (Amann & Hammerstedt, 1993; Zhang *et al*., 1999; Braundmeier & Miller, 2001; Philips *et al*., 2004;

Brito *et al*., 2003; Kastelic & Thundathil, 2008). However, exploitation of these advances in semen quality assessment has largely yet to be occurred in buffalo AI services (Andrabi, 2009).

Buffalo, as potential producer of milk and meat, contributes considerably to the agricultural economy of many countries. One of the major constraints to maximize the production of this species remains the poor field fertility rate due to bulls in AI service with unknown fertility that ultimately diminish overall fertility rate (Barile, 2005; Borghese & Mazzi 2005; Minervini *et al*., 2013).

Review of literature reveals that there is not a single published report that envisages the use of modern andrological techniques for predicting fertility of buffalo bull semen. Therefore, the present study was designed to use various semen quality parameters including CASA motility parameters, velocity distribution, motion kinematics, subpopulations of motile spermatozoa, supra–vital plasma membrane integrity, viability/acrosome integrity, viability/mitochondrial transmembrane potential, neutral comet assay/ acridine orange assay for DNA fragmentation/ integrity to predict the fertility of frozen–thawed semen of water buffalo during peak breeding season.

MATERIALS AND METHODS

Chemicals

All the chemicals used in this study were purchased from Merck, Darmstadt, Germany.

Semen collection, evaluation, extension and cryopreservation

Semen was collected from five mature water buffalo bull maintained at Livestock Research Station, NARC, Islamabad, Pakistan with artificial vagina (42 °C) from September–November (peak breeding season). After collection, semen volume (mL) was assessed from graduated falcon tube, sperm progressive motility (%) was assessed using phase contrast microscope (Olympus BX20, x 400) connected with closed circuit monitor, and sperm concentration (1 x 10^9) was measured by using the specific spectrophotometer with a wavelength of 546 nm. Qualifying ejaculates (n = 5 bulls, n = 3 replicates, n = 2 ejaculates per bull on each collection day, n = overall 30 ejaculates) having > 60% sperm progressive motility, > 1 ml volume per ejaculate and > 0.5 x 10^9 sperm/mL concentration from each bull were diluted in Tris–citric acid egg yolk glycerol extender (TCA) at 37 °C, cooled to 4 °C in 2 hr, and equilibrated for 4 hr in cold cabinet unit (4 °C). Extended semen was then packed in polyvinyl French straws (0.54 mL, IMV, France) and frozen in a programmable cell freezer (Kryo–550, Planer, Middlesex, UK). Finally, semen straws were plunged into liquid nitrogen (–196 °C). The straws were stored in liquid nitrogen tanks for at least 24 hr before post–thaw analyses (Andrabi *et al*., 2008).

Computer–aided sperm motion analysis (CASA)

Fresh and frozen–thawed semen samples were evaluated immediately after dilution and thawing. After thorough mixing, semen sample (7 µl) was placed on a prewarmed slide and coverslipped. The sample loaded slide was fitted in a portable Mini Therm stage (37 ºC) of microscope (x 100) connected to a computer having CASA software (CEROS, Hamilton Thorne Biosciences, version 12.3; USA). Motility parameters, velocity distribution, motion kinematics and subpopulations were analyzed for at least 200 spermatozoa per sample by using “Standard” settings of CEROS CASA.

Identification of motile sperm subpopulations based on CASA kinematics (%)

Four subpopulations of motile spermatozoa in each sample were calculated by using CASA generated frequency distribution values (bar charts) of kinematics according to Muino *et al*. (2008 a, b, 2009) with slight adjustments (Ahmed *et al*., 2016b).

Sperm subjective motility assessment (SM, %)

Subjective motility of fresh and frozen–thawed semen samples was assessed under phase–contrast microscope (Olympus BX20, x 400) to the nearest 5 % by viewing the sperm activity in 4–5 fields.

Sperm viability and plasma membrane integrity assay (%)

Sperm supra–vital plasma membrane integrity of fresh and frozen–thawed semen samples was assessed using hypo–osmotic swelling test according to Chan *et al*. (1991). Two hundred spermatozoa were observed in at least five different fields. Clear heads and swollen tails were considered as structurally and biochemically active sperm with intact membrane, while pink heads and un–swollen tails were considered as dead spermatozoa.

Sperm viability and acrosome integrity assay (%)

Sperm viability and acrosome integrity of fresh and frozen–thawed semen samples was assessed according to Kovacs & Foote (1992) with slight modifications like use of Giemsa stain (7.50 %) for 2.5 hr at 37 ºC instead of room temperature. Two hundred spermatozoa per sample were categorized as viable spermatozoa with intact acrosome (V/IACR, %), viable spermatozoa with damage acrosome (V/DACR, %), nonviable spermatozoa with intact acrosome (NV/IACR, %), and nonviable spermatozoa with damage acrosome (NV/DACR, %) under phase contrast microscope (x 400).

Sperm viability and mitochondrial transmembrane potential assay

Viability and mitochondrial transmembrane potential of fresh and frozen–thawed buffalo spermatozoa was evaluated with propidium iodide (PI, 1 mg/mL in PBS) and "5,5',6,6'–tetrachloro–1,1',3,3'–tetraethylbenzimidazolyl carbocyanine iodide" (JC-1, 0.15 mmoL in DMSO) as described by Ahmed *et al*. (2016b) in buffalo. Two hundred spermatozoa per sample were counted for V/HP (%, viable with high mitochondrial potential, unstained

head with bright orange mitochondria), NV/HP (%, nonviable with high mitochondrial potential, stained head with bright orange mitochondria), V/LP (%, viable with low mitochondrial potential, unstained head with green mitochondria), and NV/LP (%, nonviable with low mitochondrial potential, stained head with green mitochondria under epifluorescence microscope (Nikon AFX–1 Optiphot, x 400).

Sperm DNA fragmentation assay (Neutral comet assay)

The sperm DNA fragmentation status of fresh and frozen–thawed semen samples was determined by neutral comet assay (Donnelly *et al.,* 2000; Boe–Hansen *et al.,* 2005; Ahmed *et al*., 2016b). Digital images were captured for subsequent analyses and scoring with TRITEK software. The parameters analyzed were comet length (CL, µm), head DNA (HDNA, %), tail length (TL, µm), tail DNA (TDNA, %), tail moment (TMom) and olive moment (OM) under epifluorescence microscope (Nikon AFX–1 Optiphot, x 400).

Sperm DNA integrity assay (%, Acridine orange assay)

DNA integrity of fresh and frozen–thawed semen samples was determined according to Martins *et al*. (2007) with slight modifications i.e., fixing of semen smear in Carnoys solution for 2 hr at room temperature (25 ℃) instead of 24 hr. The images of 200 randomly selected nuclei per sample were analyzed for intact and damaged DNA under the epifluorescence microscope (Nikon AFX–1 Optiphot, x 400). Green fluorescence was considered as an intact DNA (%, double stranded), whereas red fluorescence was taken as denatured DNA (%, single stranded).

***In vivo* fertility estimation (%)**

A total of 514 buffaloes in their 2nd or 3rd lactation with clinically normal reproductive tract and showing signs of true estrus were inseminated maintained by farmers in the vicinity of Islamabad in in the winter of 2015. Estrus detection was based on observations like mucus discharge and decrease in milk production. All the experimental inseminations were performed approximately 24 hrs after onset of heat. Semen straws of 0.54 mL were used for AI in buffaloes. The artificially bred animals were examined for pregnancy through rectal palpation at least 60 days post–insemination.

Statistical analyses

Pearson's correlation coefficients were used to provide a linear association between semen quality parameters and *in vivo* fertility during peak breeding season. Step forward multiple regression analyses were used to determine the prognostic values of semen quality parameters for *in vivo* fertility as a dependent variable. The level of significance was $P < 0.05$. Minitab (MINITAB® Release 12.22, 1998) statistical package was used for data analyses.

RESULTS

Pearson's correlation coefficients of CASA parameters in frozen-thawed semen with *in vivo* fertility during peak breeding season

The data on Pearson's correlation coefficients between CASA motility parameters, velocity distribution, and motion kinematics, subpopulations of motile spermatozoa and *in vivo* fertility of buffalo bull during peak breeding season are presented in Table 7. Sperm CASA PM, RV, VAP, VSL and STR of buffalo bull during peak breeding season were significantly correlated with *in vivo* fertility (r = 0.81, P < 0.01; r = 0.85, P < 0.01; r = 0.64, P < 0.05; r = 0.73, P < 0.05 and r = 0.57, P < 0.05, respectively; Table 7). Rapid subpopulation was positively correlated with *in vivo* fertility (r= 0.65, P < 0.01), whereas sperm TM, MV, VCL, BCF, LIN Hyperactivated subpopulation 2 (%), low subpopulation 3 (%) and poor subpopulation 4 (%) were not correlated with *in vivo* fertility (P > 0.05) of buffalo bull during peak breeding season (P > 0.05, Table 7).

Pearson's correlation coefficients of functional parameters in frozen-thawed semen with *in vivo* fertility during peak breeding season

The data on Pearson's correlation coefficients between functional parameters in frozen-thawed semen with *in vivo* fertility during peak breeding season are presented in Table 8. Sperm SM, SV–PMI, V/IACR and V/HP were positively correlated *in vivo* fertility (r = 0.79; P < 0.01, r = 0.88, P < 0.01, r = 0.84, P < 0.01 and r = 0.81, P < 0.01, respectively; Table 8). However, TL, OM and NV/DACR were negatively correlated with *in vivo* fertility (r = –0.70, P < 0.05, r = –0.55, P < 0.05 and r = –0.81, P < 0.01, respectively; Table 8). Neutral comet assay parameters like CL, HDNA, TMom and OM were correlated with *in vivo* fertility, but the results were non-significant (P > 0.05). Sperm morphology parameters like head, mid-piece and tail abnormalities were not significantly correlated with *in vivo* fertility of buffalo bull during peak breeding season (Table 8).

Predictive model for frozen–thawed semen samples to estimate *in vivo* fertility of buffalo bull during peak breeding season

The data on step forward multiple regression analyses and *in vivo* fertility of buffalo bull spermatozoa during peak breeding season are presented in Table 9. The best predictive

equation (R^2 adjusted=83.50 %, $P < 0.000$; Table 9) of fertility included CASA PM, RV, VAP, VSL and SV–PMI. The best single predicator of fertility for frozen–thawed buffalo semen was CASA progressive motility (R^2 adjusted=43.10 %, $P < 0.04$, Table 9).

Table 7. Correlation coefficients (r–values) between post–thaw semen quality parameters and *in vivo* fertility rate (FR, %) of water buffalo bull spermatozoa during peak breeding season

Semen quality parameters	Mean vaue	r-value	P-value
TM (%)	77.10±3.04	0.45	0.07
PM (%)	26.27±2.97	0.81	0.01
RV (%)	32.79±3.90	0.85	0.01
MV (%)	44.38±6.34	0.18	0.52
VAP (µm/s)	75.36±2.84	0.64	0.04
VSL (µm/s)	64.48±2.25	0.73	0.04
VCL (µm/s)	115.73±4.78	0.40	0.07
ALH (µm)	05.41±0.18	0.11	0.68
BCF (Hz)	33.58±0.67	0.40	0.13
STR (%)	84.52±0.79	0.57	0.03
LIN (%)	56.48±0.89	0.42	0.11
Rapid subpopulation (%)	25.09±1.92	0.65	0.01
Hyperactivated subpopulation (%)	15.08±0.90	0.42	0.08
Low subpopulation (%)	19.72±1.07	0.33	0.26
Poor subpopulation (%)	09.21±0.83	0.01	0.67

TM= Total motility, PM= Progressive motility, RV = Rapid velocity, MV= Medium velocity, VAP = Average path velocity, VSL = Straight line velocity, VCL = Curvilinear velocity, ALH = Amplitude of lateral head displacement, BCF = Beat cross frequency, STR = Straightness, LIN = Linearity

Table 8. Correlations (r–values) between post–thaw sperm quality parameters and *in vivo* fertility of buffalo bull during peak breeding season

Semen quality parameters	Mean vaue	r-value	P-value
SM (%)	53.00±1.60	0.79	0.01
SV-PMI (%)	29.67±1.09	0.88	0.01
V/IACR (%)	37.40±1.70	0.84	0.01
V/DACR (%)	16.53±0.66	0.12	0.66
NV/IACR (%)	16.33±0.67	0.26	0.35
NV/DACR (%)	29.73±1.63	–0.81	0.01
V/HP (%)	32.31±0.96	0.81	0.01
NV/HP (%)	5.64±1.34	0.29	0.28
V/LP (%)	31.72±2.34	0.33	0.09
NV/LP (%)	30.33±0.96	–0.82	0.01
CL (µm)	222.97±2.67	–0.39	0.14
HDNA (%)	87.65±0.49	0.27	0.33
TL (µm)	78.48±1.19	–0.70	0.04
TDNA (%)	12.35±0.49	–0.27	0.33
TMom	18.24±0.62	–0.42	0.12
OM	17.27±0.39	–0.55	0.06
DNA integrity (%)	91.73±0.44	0.40	0.06
Head abnormalities (%)	03.70±0.17	–0.05	0.85
Mid–piece abnormalities (%)	01.13±0.11	–0.19	0.50
Tail abnormalities (%)	03.33±0.22	0.01	0.80

SM = Subjective motility, SV–PMI = Supra–vital plasma membrane integrity, V/IACR = Viable with intact acrosome, V/DACR = Viable with damaged acrosome, NV/IACR = Nonviable with intact acrosome, NV/DACR = Nonviable with damaged acrosome, V/HP = Viable with high mitochondrial transmembrane potential, NV/LP = Nonviable with low mitochondrial transmembrane potential, CL = Comet length, HDNA = Head DNA, TL = Tail length, TDNA = Tail DNA, TMom = Tail moment, OM = Olive moment

Table 9. Prognostic values to predict the *in vivo* fertility rate (FR, %) by the predictive equation based on post–thaw semen quality parameters during peak breeding season

Predictive equations	R^2 (adjusted)	P–value
FR%= 3.8–0.25 PM+0.05 RV+0.23 VAP+0.70 VSL+0.83 SV–PMI	83.50 %	0.000
FR%= 6.70+1.90 V+HP+5.72 PM+8.70 VSL	78.80 %	0.001
FR%= 2.70+1.59 RV–2.03 VAP+2.96 VSL	71.30 %	0.001
FR%= 8.80+0.445 VCL–2.02 VAP+2.79 VSL	72.00 %	0.002
FR%= 1.10+0.50 PM+1.82 VAP–0.74 VCL	68.80 %	0.002
FR %= 8.5+0.40 TM–0.45 VAP+1.87 VSL–0.44 VCL+1.99 ALH	76.30 %	0.002
FR%= 24–0.41 CL–2.04 TL+1.47 TM+0.86 OM	60.00 %	0.004
FR%= 10.80+0.42 TM–0.95 VSL–5.72 ALH	67.10 %	0.005
FR %= 20.2+0.56 PM–1.10 VAP+1.75 VSL	61.50 %	0.005
FR %= 4.6+0.43 TM–1.64 VAP+2.88 VSL–0.25 VCL+2.22 ALH–0.47 BCF	74.30 %	0.005
FR %= 10.6+0.41 TM+0.08 PM–1.92 VAP+2.65 VSL	70.20 %	0.005
FR %= 18.3+0.31 TM+0.27 PM+0.78 VSL–5.19 ALH	67.30 %	0.005
FR %= 5.6+0.51 PM+2.97 VAP–0.81 VSL–0.99 VCL	66.30 %	0.040
FR%= 11.6+0.83 PM	43.10 %	0.040

TM = Total motility, PM = Progressive Motility, VAP = Average Path Velocity, VSL = Straight Line Velocity, VCL= Curvilinear Velocity, ALH = Amplitude of Lateral Head Displacement, BCF = Beat Cross Frequency, CL = Comet length, TL = Tail length, OM = Olive moment, V/HP = Viable with high mitochondrial transmembrane potential, SV–PMI = Supra–vital plasma membrane integrity

DISCUSSION

The present study was designed to predict the fertility of water buffalo bull by using post–thaw semen quality parameters during peak breeding season. To the best of our knowledge this is the first comprehensive study in buffalo.

Spermatozoa with forward movement have greater capability to reach the oocyte. In the present study, significant positive relationship was established between CASA progressive motility and *in vivo* fertility. Similar results have been reported in bovine (Farrell *et al.,* 1998) and human (Fetterholf & Rogers, 1990; Liu *et al.,* 1991). In a comprehensive study by Kasimanickam *et al.* (2007), the competitive bull fertility was found to be positively correlated with progressive motility. It is put forward that CASA progressive motility may predict the fertility of buffalo bull during peak breeding season.

The measurement of kinematics has been considered as an indicator of functionality of spermatozoa (Budworth *et al.,* 1988). Significant positive correlation obtained in this study between VAP or VSL and *in vivo* fertility is comparable with the previous report in bovine (Kathiravan *et al.,* 2008). These results indirectly point out that spermatozoa with progressive motility can cover longer distance in a short period of time resulting in higher conception rate (Farrell *et al.,* 1998; Verstegen *et al.,* 2002). These results suggested that semen samples of buffalo bull with higher values of motion characteristics i.e., VAP and VSL might show higher fertility during peak breeding season.

The presence of four subpopulations of motile spermatozoa based on motion kinematics has been defined in bovine (Muino *et al*., 2008a, b; 2009) and buffalo (Ahmed *et al*., 2016b). In the present study, four subpopulations of motile spermatozoa were defined in frozen–thawed semen samples of buffalo. Our results reported that percentages of rapid subpopulation 1 (most rapid and progressive) of motile spermatozoa were significantly associated with *in vivo* fertility during peak breeding season. These results are comparable with the findings in bovine (Muino *et al*., 2008a, b; 2009) and equine (Quintero–Moreno *et al*., 2003) during their respective breeding seasons.

High sperm mitochondrial transmembrane potential is linked with increased motility and fertilizing capacity (Garner *et al.,* 1997; Martinez-Pastor *et al*., 2011). In the present study, viable spermatozoa with high mitochondrial transmembrane potential were significantly associated with field fertility. The results of the present study are in agreement with the

findings in buffalo (Selvaraju *et al.,* 2008; Minervini *et al.,* 2013) human (Kasai *et al.,* 2002; Marchetti *et al.,* 2002) and ram (Windsor *et al.,* 1997). Therefore, it can be stated that spermatozoa with higher mitochondrial transmembrane potential have the energy to reach and fertilize the oocyte in female reproductive tract.

The ability of a single spermatozoon to achieve fertilization depends on several cellular characteristics. Only viable spermatozoa are able to interact with the oocyte and pursue fertilization in cattle (Zhang *et al*., 1998) and buffalo (Ahmed *et al*., 2016b). Results of the present investigation showed that subjective motility of buffalo bull spermatozoa was positively correlated with *in vivo* fertility during peak breeding season. Significant correlations between subjective motility assessment and field fertility have been found by some authors in bovine (Lindford *et al*., 1976; Kjaestad *et al*., 1993, Correa *et al*., 1997; Zhang *et al*., 1999; Januskauskas *et al*., 2000), whereas other authors failed to found significant positive association between subjective motility assessment and fertility in bovine and have suggested that sperm subjective assessment of motility is markedly slow and error prone and depends on subjectivity of observer (Graham *et al*., 1980; Soderquist, 1991; Januskauskas *et al*., 1996). These differences between results might be due to number of sample evaluated, expertise of observer, species or even season.

The assessment of plasma membrane integrity is of particular importance due to its involvement in metabolic exchanges with the surrounding medium. In this study, significant positive relationship between sperm plasma membrane integrity and field fertility was observed. These results are comparable with previous studies in bovine semen (Oura & Toshimori, 1990; Correa *et al.,* 1997; Brito *et al.,* 2003; Tartaglione & Ritta, 2004; Celeghini *et al.,* 2007; Kasimanickam *et al.,* 2007). It is therefore, hypothesized that plasma membrane intactness may be helpful in predicting the fertility of buffalo bull during peak breeding season.

Intact acrosome is essential for the acrosome reaction and is required at the proper time to facilitate fertilization process (Thomas *et al.,* 1997; Oura & Toshimori, 1990). In current study, percentages of viable spermatozoa with intact acrosome were positively correlated with field fertility. These results are in accordance with the findings of Zhang *et al.* (1999) and Minervini *et al.* (2013) in bovine and buffalo, respectively. It can be concluded that fertility potential of buffalo bull depends on the percentage of viable spermatozoa with intact acrosome.

Normal sperm genetic material is required for successful fertilization, as well as for further embryo and fetal development that will result in a healthy offspring (Sailer *et al*., 1995; Agarwal & Said, 2003). According to our results, neutral comet assay parameters were negatively correlated with field fertility. In a similar study, Boe–Hansen *et al.* (2005) have reported that ejaculate with higher DNA damage exhibit higher tail length and olive moment. The negative association between neutral comet assay parameters and *in vivo* fertility confirms the findings of earlier studies in human and bovine (Chan *et al*., 2001; Kasimanickam *et al.,* 2007). It is put forward that compromised sperm DNA fragmentation may affect the fertilizing potential of buffalo bull.

It was observed that the assessment of a single parameter does not provide high predictive value of fertility. Therefore, several regression equations were obtained by combining semen quality parameters for the prediction of fertility. In our study, the best predictive equation (R^2 adjusted=83.50 %, $P < 0.000$) of fertility for frozen–thawed buffalo semen included PM, RV, VAP, VSL and SV–PMI. Moreover, most of the predictive equations did depend upon the CASA parameters. Interestingly, the best single predictive equation was also derived from the CASA progressive motility. Farrell *et al.* (1998) demonstrated that multiple combinations of CASA variables had higher correlations with bull fertility than single CASA parameters; for instance, the single sperm parameter (total motility) was weakly correlated with field fertility ($r^2 = 0.34$), while the combination of progressive motility, ALH and BCF was strongly correlated ($r^2 = 0.83$) and the combination of progressive motility, ALH, BCF and VSL was even more strongly correlated ($r^2 = 0.89$). Similarly, Elia *et al.* (2010) concluded that differences between the sperm motility parameters were the necessary parameter for the evaluation of sperm fertilization ability. It was reported earlier, that among the sperm motion characteristics; VCL, VSL and VAP, and PM could be used to predict the fertility of bull spermatozoa (Kathiravan *et al.,* 2011). We found that CASA motility parameters and kinematics were critical for the assessment of semen quality to predict fertility of buffalo bull during peak breeding season.

Conclusion

It is concluded that the fertility of buffalo bull can be predicted through some of the post–thaw *in vitro* semen quality tests during peak breeding season. This will assist to use semen from high fertility buffalo bull for improvement in field pregnancy rate.

ABSTRACT

Background: Fertility of cryopreserved water buffalo spermatozoa through AI is reported to be affected by seasonality. In this study, we investigated the seasonal influences on various sperm quality parameters in fresh and frozen-thawed buffalo bull semen. Moreover, effect of seasons on *in vivo* fertility of frozen–thawed spermatozoa was evaluated.

Materials and Methods: Semen was collected from five mature buffalo bull with artificial vagina (AV, 42 °C) from May–July (low breeding season) and September–November (peak breeding season). Twelve ejaculates were collected per bull; overall sixty ejaculates were collected per season (n = 60 ejaculates and n = 2 seasons). Sperm motility parameters, velocity distribution, motion kinematics, and subpopulations were analyzed by computer–aided sperm motion analyzer (CASA). Moreover, sperm subjective motility (SM. %), supra–vital plasma membrane integrity (SV–PMI, %), viability/acrosome integrity (V/IACR, %), viability/mitochondrial transmembrane potential, DNA fragmentation/integrity, and morphology parameters were analyzed by phase–contrast microscope, supra–vital hypoosmotic swelling test, Trypan blue/Giemsa staining, propidium iodide/"5,5,6,6'–tetrachloro–1,1,3,3'–tetraethylbenzimidazolyl carbocyanine iodide" (JC–1) fluorochromes, neutral comet assay/acridine orange assay and wet mount technique, respectively. For *in vivo* fertility, data of at least 100 inseminations per bull per season was collected in a fertility trial carried out during low breeding season.

Results: Analyses of variance (ANOVA) showed that sperm total motility (TM, %) progressive motility (PM, %), average path velocity (VAP, μm/s), straight line velocity (VSL, μm/s), straightness (STR, %), linearity (LIN, %;) and subpopulation 1 of motile spermatozoa (%) in fresh semen samples of buffalo bull were significantly higher ($P < 0.05$) during peak than low breeding season. Similarly, sperm SV–PMI, V/IACR, viable with high mitochondrial transmembrane potential (V/HP, %) and DNA integrity (%) in fresh semen of buffalo bull were significantly higher ($P < 0.05$) during peak than low breeding season. Moreover, percent head abnormalities, DNA fragmentation indices i.e., comet length (CL, μm), tail length (TL, μm), tail DNA (TDNA, %), tail moment (TMom) and olive moment (OM) in fresh semen samples of buffalo bull were significantly lower ($P < 0.05$) during peak than low breeding season. At post–thawing, sperm TM and subpopulation 1 were significantly higher ($P < 0.05$) during peak than low breeding

season. Similarly, sperm SV–PMI, V/IACR and V/HP in frozen-thawed semen samples of buffalo bull were significantly higher ($P < 0.05$) during peak than low breeding season. Percent head and tail abnormalities, DNA fragmentation indices i.e., CL, TL, TDNA, TMom and OM in frozen-thawed semen samples of buffalo bull were significantly lower ($P < 0.05$) during peak than low breeding season. *In vivo* fertility of frozen–thawed spermatozoa processed during peak breeding season was significantly higher (58.98 % vs. 52.49 %, $P < 0.05$) than semen cryopreserved during low breeding season.

Conclusion: In conclusion, structural and functional parameters and *in vivo* fertility of buffalo spermatozoa were found to be superior during peak than low breeding season. It is therefore, suggested that in order to increase fertility rate in buffalo, semen should be collected and preserved during cooler months and used for artificial insemination throughout the year.

INTRODUCTION

The water buffalo (*Bubalus bubalis*) is classified as riverine and swamp type, and has an important role in the agricultural economy of many developing countries by providing milk, meat and draught power. The world population of water buffalo is estimated to be 199 million (Food & Agricultural Organization, 2012) with more than 96% of the population is located in Asia. In recent decades, water buffalo farming has also expanded widely in Mediterranean areas and in Latin America (Andrabi, 2014). The production potential of water buffalo can be increased by using one of the modern ways of breed improvement like artificial insemination (AI). Moreover, the quality of semen is one of the most important factors that can affect the outcome of AI (Saacke, 1984; Andrabi, 2009).

Buffalo bulls are capable of breeding throughout the year, but some seasonal fluctuation in reproductive functions is evident in most of the buffalo rearing countries. Several studies have reported better quality and conception rate of spermatozoa harvested during the autumn/winter (peak breeding season) compared with those collected and processed during the summer (dry or wet, low breeding season) in riverine buffalo (Tuli & Singh, 1983; Heuer *et al*., 1987; Bhavsar *et al*., 1989a, b; Sagdeo *et al*., 1991; Bahga & Khokar, 1991; Younis *et al*., 1998; 1999). Regarding *in vivo* fertility of frozen-thawed semen, Heuer *et al*. (1987) reported that semen collected during peak breeding season produced significantly higher conception rate than semen collected during low breeding in riverine buffalo (40.9 vs 34.0%).

Most of the above referred studies (Tuli & Singh, 1983; Heuer *et al*., 1987; Bhavsar *et al*., 1989a, b; Sagdeo *et al*., 1991; Bahga & Khokar, 1991; Younis *et al*., 1998; 1999) in river type buffalo do explain partly the causes of reduced quality of semen collected/ processed during peak and low breeding seasons. However, the data given in these studies are insufficient to explain the variation in quality of spermatozoa during the different seasons. Moreover, these studies have used subjective semen evaluation techniques, which are now considered as of limited scope in buffalo (Ahmed *et al*., 2016a, b). Therefore, detailed studies should be carried out in riverine buffalo to ascertain the structural and functional parameters that might be influencing the quality of fresh and frozen-thawed spermatozoa during peak and low breeding seasons.

The present study was undertaken to evaluate various semen quality parameters including CASA motilities, velocity distribution, kinematics, subpopulations, viability/ plasma membrane integrity or acrosome integrity or mitochondrial transmembrane potential, DNA integrity and fragmentation indices in fresh and frozen–thawed riverine buffalo semen during peak and low breeding seasons. Moreover, effect of seasons on *in vivo* fertility of frozen-thawed riverine buffalo spermatozoa was evaluated.

MATERIALS AND METHODS

Chemicals

All the chemicals used in this study were purchased from Merck, Darmstadt, Germany.

Semen collection, evaluation, extension and cryopreservation

Semen was collected from five mature water buffalo bull maintained at Livestock Research Station, NARC, Islamabad, Pakistan with artificial vagina (42 °C) from May–July (low breeding season) and September–November (peak breeding season). Twelve ejaculates were collected per bull; overall sixty ejaculates were collected per season (n = 60 ejaculates and n = 2 seasons). After collection, semen volume (mL) was assessed from graduated falcon tube, sperm progressive motility (%) was assessed using phase contrast microscope (Olympus BX20, x 400) connected with closed circuit monitor, and sperm concentration (1 x 10^9) was measured by using the specific spectrophotometer with a wavelength of 546 nm. Qualifying ejaculates (n = 120) having > 60 % visual motility, > 1 mL volume and > 0.5 x 10^9 sperm/mL concentration from each bull were diluted in Tris–citric acid egg yolk glycerol extender (TCA) at 37 °C, cooled to 4 °C in 2 hr, and equilibrated for 4 hr in cold cabinet unit (4 °C). Extended semen was then packed in polyvinyl French straws (0.54 mL, IMV, France) and frozen in a programmable cell freezer (Kryo–550, Planer, Middlesex, UK). Finally, semen straws were plunged into liquid nitrogen (–196 °C). The straws were stored in liquid nitrogen tanks for at least 24 hr before post–thaw analyses (Andrabi *et al.*, 2008).

Computer–aided sperm motion analysis (CASA)

Fresh and frozen–thawed semen samples were evaluated immediately after dilution and thawing. After thorough mixing, semen sample (7 µl) was placed on a prewarmed slide and coverslipped. The sample loaded slide was fitted in a portable Mini Therm stage (37 ºC) of microscope (x 10) connected to a computer having CASA software (CEROS, Hamilton Thorne Biosciences, version 12.3; USA). Total motility (TM, %), progressive motility (PM, %), rapid velocity (RV, %), average path velocity (VAP, µm/s), curvilinear velocity (VCL, µm/s), straight line velocity (VSL, µm/s), amplitude of lateral head displacement (ALH, µm), beat cross frequency (BCF, Hz), straightness (STR, %; ratio of

VSL/VAP) and Linearity (LIN, %; ratio of VSL/VCL) were analyzed for at least 200 spermatozoa per sample by using "Standard" settings of CEROS CASA.

Identification of motile sperm subpopulations based on CASA kinematics (%)

Four subpopulations of motile spermatozoa in each sample were calculated by using CASA generated frequency distribution values (bar charts) of kinematics according to Muino *et al*. (2008, 2009) with slight adjustments Ahmed *et al*. (2016b).

Sperm subjective motility assessment (SM, %)

Subjective motility of fresh and frozen–thawed semen samples was assessed under phase–contrast microscope (Olympus BX20, x 400) to the nearest 5 % by viewing the sperm activity in 4–5 fields.

Sperm viability and plasma membrane integrity assay (%)

Sperm supra–vital plasma membrane integrity of fresh and frozen–thawed semen samples was assessed using hypo–osmotic swelling test according to Chan *et al*. (1991). Two hundred spermatozoa were observed in at least five different fields under phase contrast microscope, (Olympus BX20, x 400). Clear heads and swollen tails were considered as structurally and biochemically active sperm with intact membrane, while pink heads and un–swollen tails were considered as dead spermatozoa (Chan *et al*., 1991; Tartaglione & Rirra, 2004).

Sperm viability and acrosome integrity assay (%)

Sperm viability and acrosome integrity of fresh and frozen–thawed semen samples was assessed according to Kovacs & Foote (1992) with slight modifications like use of Giemsa stain (7.50 %) for 2.5 hr at 37 ºC instead of room temperature. Two hundred spermatozoa per sample were categorized as viable spermatozoa with intact acrosome (V/IACR, %), viable spermatozoa with damage acrosome (V/DACR, %), nonviable spermatozoa with intact acrosome (NV/IACR, %), and nonviable spermatozoa with damage acrosome (NV/DACR, %) under phase contrast microscope (x 400).

Sperm viability and mitochondrial transmembrane potential assay

Viability and mitochondrial transmembrane potential of fresh and frozen–thawed buffalo spermatozoa was evaluated with propidium iodide (1 mg/mL in PBS) and "5,5',6,6'–tetrachloro–1,1',3,3'–tetraethylbenzimidazolyl carbocyanine iodide" (JC-1, 0.15 mmoL in DMSO) as described by Ahmed *et al*. (2016a, b) in buffalo. Two hundred spermatozoa per sample were categorized as viable with high mitochondrial potential, unstained head with bright orange mitochondria, V/HP, %), and nonviable with low mitochondrial potential, stained head with green mitochondria, NV/LP, %) under epifluorescence microscope (Nikon AFX–1 Optiphot, 400 x).

Sperm DNA fragmentation assay (Neutral comet assay)

The sperm DNA fragmentation status of fresh and frozen–thawed semen samples was determined by neutral comet assay (Ahmed *et al*., 2016a). Digital images were captured for subsequent analyses and scoring with TRITEK software. The parameters analyzed were comet length (CL, µm), head DNA (HDNA, %), tail length (TL, µm), tail DNA (TDNA, %), tail moment (TMom) and olive moment (OM).

Sperm DNA integrity assay (%, Acridine orange assay)

DNA integrity of fresh and frozen–thawed semen samples was determined according to Martins *et al*. (2007) with slight modifications i.e., fixing of semen smear in Carnoys solution for 2 hr at room temperature (25 ºC) instead of 24 hr. The images of 200 randomly selected nuclei per sample were analyzed for intact and damaged DNA under the epifluorescence microscope. Green fluorescence was considered as an intact DNA (%, double stranded), whereas red fluorescence was taken as denatured DNA (%, single stranded).

Artificial insemination and pregnancy diagnosis

A total of 1081 buffaloes in their 2nd or 3rd lactation and with good body score were inseminated with frozen–thawed semen (doses processed during peak breeding season, n = 544; doses processed during low breeding season, n = 537) during the months of May–July (low breeding season). The oestrus detection of buffaloes was judged through mucus discharge and decline in milk yield. All the inseminations were performed 24 h after the

start of heat. The artificially bred animals were examined for pregnancy through rectal palpation at least 60 days post-insemination (Iqbal *et al*., 2015; 2016).

Statistical analyses

The data were analyzed using the general linear model procedure within the ANOVA module in Minitab (Release 17.3.1; Minitab, Inc., Pine Hall Road, State College, PA, USA) to calculate differences between seasons and/or bulls at fresh and post–thawing. No interaction was observed between season and bull with GLM procedure, so bull data were pooled. Prior to analysis, all percentage data were normalized with an arcsine transformation. When F–ratio was significant ($P < 0.05$), Tukey's pairwise comparison test was used to further differentiate between the mean. The *in vivo* fertility data were analyzed by Chi-square test.

RESULTS

Evaluation of sperm quality parameters in fresh semen during peak and low breeding seasons

Data on ejaculate volume, concentration of spermatozoa per ml, SM, CASA motility parameters, velocity distribution, motion kinematics and subpopulations of motile spermatozoa in fresh semen of buffalo bull during peak and low breeding seasons are presented in Table 10 and Figure 4. Ejaculate volume and concentration of spermatozoa per mL, in fresh semen samples of buffalo bull were significantly higher ($P < 0.05$) during peak than low breeding season (Table 10). Percent TM, PM, VAP, VSL, STR, LIN and subpopulation 1 of motile spermatozoa, in fresh semen samples of buffalo bull were significantly higher ($P < 0.05$) during peak than low breeding season (Table 10 and Figure 4). CASA velocity distribution parameter i.e., RV and motion kinematics VCL, ALH and BCF were similar ($P < 0.05$) during peak and low breeding seasons (Table 10).

Data on SV–PMI, viability/acrosome integrity, viability/mitochondrial transmembrane potential, neutral comet assay parameters, DNA integrity and morphology parameters in fresh semen samples of buffalo bull are presented in Table 11 and Figure 4. Sperm SV–PMI, V/IACR, V/HP and DNA integrity were significantly higher ($P < 0.05$) during peak than low breeding season (Table 11 and Figure 5), whereas, percent head abnormalities, CL, TL, TDNA, TMom and OM were significantly lower ($P < 0.05$) in fresh semen samples of buffalo bull during peak than low breeding season (Table 11). Tail and mid–piece abnormalities were similar ($P < 0.05$) during peak and low breeding seasons (Table 11).

Evaluation of sperm quality parameters in frozen-thawed semen during peak and low breeding seasons

Data on SM, CASA motility parameters, velocity distribution, motion kinematics and subpopulations of motile spermatozoa in frozen–thawed semen of buffalo bull during peak and low breeding seasons are presented in Table 12 and Figure 5. Sperm TM and subpopulation 1 in frozen–thawed semen samples of buffalo bull were significantly higher ($P < 0.05$) during peak than low breeding season, whereas SM, PM, RV, VAP, VSL, VCL, ALH, BCF, STR, LIN, subpopulation 2, 3 and 4 were similar ($P > 0.05$) during peak and low breeding seasons (Table 12).

Data on SV–PMI, viability/acrosome integrity, viability/mitochondrial transmembrane potential, neutral comet assay parameters, DNA integrity and morphology parameters in frozen–thawed semen samples of buffalo bull are presented in Table 13 and Figure 5. Sperm SV–PMI, V/IACR, V/HP and DNA integrity in frozen-thawed semen samples of buffalo bull were significantly higher ($P < 0.05$) during peak than low breeding season. Moreover, percent head and tail abnormalities, CL, TL, TDNA, TMom and OM in frozen–thawed semen samples of buffalo bull were significantly lower ($P < 0.05$) during peak than low breeding season (Table 13).

Seasonal influences on *in vivo* fertility of frozen-thawed buffalo spermatozoa

In vivo fertility of frozen-thawed buffalo spermatozoa processed during peak breeding season was significantly higher ($P < 0.05$) than semen cryopreserved during low breeding season in a fertility trial carried out during low breeding season (58.95 % vs. 52.49 %, Table 14 and Figure 6a, b).

Table 10. Evaluation of volume, concentration, motility parameters, velocity distribution, motion kinematics and subpopulations in fresh semen of buffalo during peak and low breeding seasons (n = 5 bulls and n = 60 ejaculates/season)

Variables	**Breeding Seasons**		**P–Value**
	Peak	**Low**	
Volume (mL)	03.33±0.15[a]	02.69±0.11[b]	0.020
Concentration (1 x 10^9)	01.36±0.05[a]	01.08±0.05[b]	0.038
SM (%)	70.67±1.18	67.67±0.83	0.047
TM (%)	96.51±0.88[a]	89.33±1.45[b]	0.001
PM (%)	70.26±1.17[a]	61.42±1.93[b]	0.001
RV (%)	81.01±1.73	77.38±2.74	0.271
VAP (µm/s)	133.08±4.70[a]	110.85±2.58[b]	0.001
VSL (µm/s)	123.67±4.82[a]	96.52±1.87[b]	0.004
VCL (µm/s)	165.47±7.13	154.69±5.08	0.228
ALH (µm)	04.46±0.27	05.07±0.23	0.097
BCF (Hz)	38.94±0.39	39.17±0.85	0.860
STR (%)	89.99±0.60[a]	86.27±0.73[b]	0.040
LIN (%)	74.13±1.18[a]	64.42±1.47[a]	0.030
Rapid subpopulation (%)	56.95±1.84[a]	45.02±1.29[a]	0.004
Hyperactivated subpopulation (%)	09.15±0.76[b]	15.02±0.59[a]	0.044
Low subpopulation (%)	17.14±0.52[b]	20.91±0.51[a]	0.035
Poor subpopulation (%)	04.78±0.42	05.94±0.42	0.739

Means with different superscripts in a row differed significantly ($P < 0.05$).

SM = Subjective motility, TM = Total motility, PM = Progressive motility, RV = Rapid velocity, VAP = Average path velocity, VSL = Straight line velocity, VCL = Curvilinear velocity, ALH = Amplitude of lateral head displacement, BCF = Beat cross frequency, STR = Straightness, LIN = Linearity

Table 11. Evaluation of supra–vital plasma membrane integrity, viability/ acrosome integrity, viability/ mitochondrial transmembrane potential, DNA fragmentation indices, DNA integrity and morphology in fresh semen of buffalo bull during peak and low breeding seasons (n = 5 bulls and n = 60 ejaculates/season)

Variables	Breeding seasons		P–Value
	Peak	Low	
SV–PMI (%)	81.40±0.66[a]	77.07±0.79[b]	0.040
V/IACR (%)	58.20±0.93[a]	53.50±0.80[b]	0.032
V/DACR (%)	13.33±0.49	12.60±0.40	0.259
NV/IACR (%)	14.07±4.73	18.20±0.67	0.080
NV/DACR (%)	14.40±0.80	15.70±0.75	0.248
V/HP (%)	74.77±1.03[a]	70.13±0.89[b]	0.020
NV/LP (%)	10.33±1.09	11.73±0.80	0.310
CL (µm)	142.31±1.46[a]	158.19±4.18[b]	0.001
HDNA (%)	88.57±0.47[b]	83.56±0.75[a]	0.001
TL (µm)	68.56±0.83[b]	75.92±0.72[a]	0.001
TDNA (%)	12.28±0.46[b]	16.48±0.74[a]	0.001
TMom	12.90±0.67[b]	24.74±1.20[a]	0.001
OM	12.83±0.54[b]	15.45±0.48[a]	0.001
DNA integrity (%)	94.27±0.43[a]	89.80±0.55[b]	0.001
Head abnormalities (%)	02.93±0.83[b]	04.07±0.23[a]	0.006
Tail abnormalities (%)	02.13±0.15	02.13±0.19	0.870
Mid–piece abnormalities (%)	01.30±0.14	01.43±0.13	0.582

Means with different superscripts in a row differed significantly ($P < 0.05$).

SV–PMI = Supra-vital plasma membrane integrity, V/IACR = Viable with intact acrosome, V/DACR = Viable with damaged acrosome, NV/IACR = Nonviable with intact acrosome, NV/DACR = Nonviable with damaged acrosome, V/HP = Viable with high mitochondrial transmembrane potential, NV/LP = Nonviable with low mitochondrial transmembrane potential, CL = Comet length, HDNA = Head DNA, TL = Tail length, TDNA = Tail DNA, TMom = Tail moment, OM = Olive moment

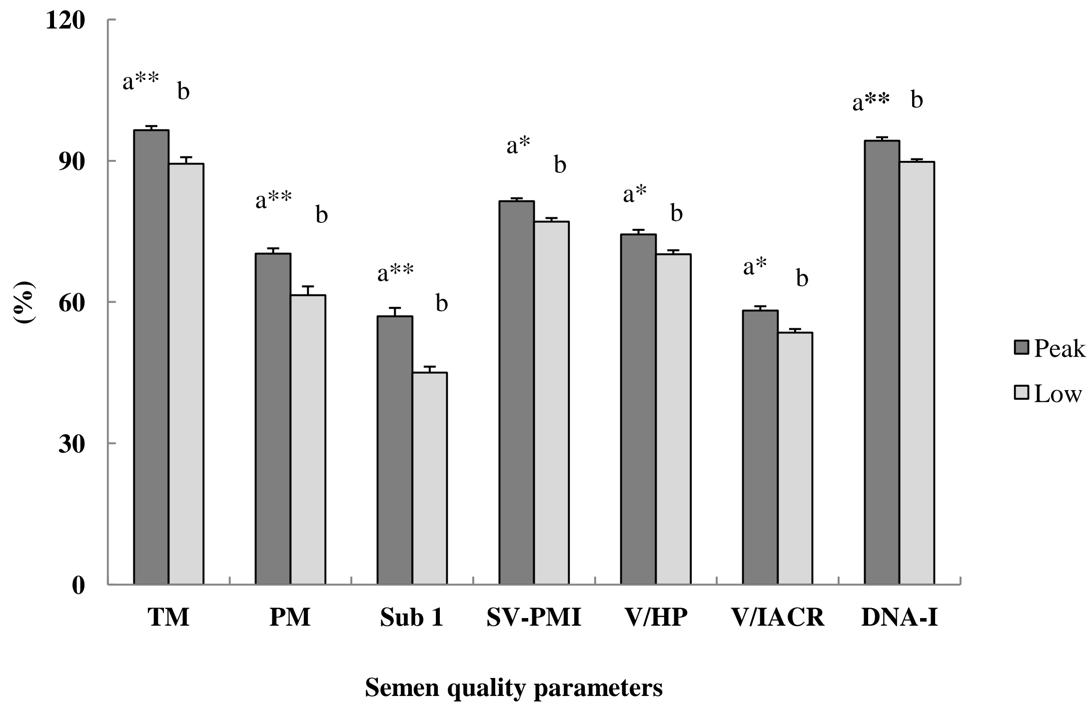

Figure 4. Evaluation of TM (total motility, %), PM (progressive motility, %), Sub 1 (subpopulation 1, %), SV–PMI (supra–vital plasma membrane integrity, %), V/HP (Viable with high mitochondrial transmembrane potential, %), V/IACR (viable with intact acrosome, %) and DNA–1 (DNA integrity, %) in fresh semen of buffalo bull during peak and low breeding seasons (n = 5 bulls and n = 60 ejaculates/season). Asterisks (* or **) shows a significance level between the two seasons. Bars with different alphabets with in the same variable showed significant differences ($P < 0.05$) due to season. Values are expressed as Mean±S.E.M.

Table 12. Evaluation of motility parameters, velocity distribution, motion kinematics and subpopulations in frozen–thawed buffalo semen during peak and low breeding seasons (n = 5 bulls and n = 60 ejaculates/season)

Variables	Breeding seasons		P–Value
	Peak	Low	
SM (%)	53.00±1.60	48.67±1.33	0.047
TM (%)	77.10±3.04[a]	63.87±3.16[b]	0.042
PM (%)	26.27±2.97	24.78±2.12	0.685
RV (%)	32.79±3.90	29.10±2.07	0.410
VAP (µm/s)	75.36±2.84	71.97±1.29	0.286
VSL (µm/s)	64.48±2.25	61.37±1.27	0.240
VCL (µm/s)	115.73±4.78	110.00±2.56	0.299
ALH (µm)	05.41±0.18	05.83±0.10	0.050
BCF (Hz)	33.58±0.67	32.36±0.66	0.737
STR (%)	84.52±0.79	84.08±0.60	0.658
LIN (%)	56.48±0.89	55.62±0.97	0.518
Rapid subpopulation (%)	25.09±1.92[a]	18.57±1.21[b]	0.013
Hyperactivated subpopulation (%)	15.08±0.90	15.57±1.04	0.853
Low subpopulation (%)	19.72±1.07	22.03±2.81	0.657
Poor subpopulation (%)	09.21±0.83	07.53±0.84	0.359

Means with different superscripts in a row differed significantly ($P < 0.05$).

SM = Subjective motility, TM = Total motility, PM = Progressive motility, RV = Rapid velocity, VAP = Average path velocity, VSL = Straight line velocity, VCL = Curvilinear velocity, ALH = Amplitude of lateral head displacement, BCF = Beat cross frequency, STR = Straightness, LIN = Linearity

Table 13. Evaluation of supra–vital plasma membrane integrity, viability/ acrosome integrity, viability/ mitochondrial transmembrane potential, DNA fragmentation indices, DNA integrity and morphology in frozen–thawed buffalo semen during peak and low breeding seasons (n = 5 bulls and n = 60 ejaculates/season)

Variables	Breeding Seasons		P–Value
	Peak	Low	
SV–PMI (%)	29.67±1.09^{a}	25.27±0.95^{b}	0.005
V/IACR (%)	37.40±1.70^{a}	32.27±1.16^{b}	0.019
V/DACR (%)	16.53±0.66	15.33±0.51	0.163
NV/IACR (%)	16.33±0.67	15.90±0.66	0.649
NV/DACR (%)	29.73±1.63^{a}	36.50±1.62^{b}	0.006
V/HP (%)	32.31±0.96^{a}	26.31±0.95^{b}	0.034
NV/LP (%)	30.33±0.96^{b}	35.25±1.24^{a}	0.037
CL (µm)	222.97±2.67^{a}	343.06±3.55^{b}	0.001
HDNA (%)	87.65±0.49^{a}	81.80±0.54^{b}	0.001
TL (µm)	78.48±1.19^{a}	87.40±1.07^{b}	0.001
TDNA (%)	12.35±0.49^{a}	18.20±0.54^{b}	0.010
TMom	18.24±0.62^{a}	31.85±1.34^{b}	0.001
OM	17.27±0.39^{a}	20.61±0.47^{b}	0.038
DNA integrity (%)	91.73±0.44^{a}	86.63±0.47^{b}	0.001
Head abnormalities (%)	03.70±0.17^{b}	05.93±0.18^{a}	0.008
Tail abnormalities (%)	03.13±0.22^{b}	04.33±0.27^{a}	0.034
Mid–piece abnormalities (%)	01.33±0.11	01.90±0.20	0.530

Means with different superscripts in a row differed significantly ($P < 0.05$).

SV–PMI = Supra–vital plasma membrane integrity, V/IACR = Viable with intact acrosome, V/DACR = Viable with damaged acrosome, NV/IACR = Nonviable with intact acrosome, NV/DACR = Nonviable with damaged acrosome, V/HP = Viable with high mitochondrial transmembrane potential, NV/LP = Nonviable with low mitochondrial transmembrane potential, CL = Comet length, HDNA = Head DNA, TL = Tail length, TDNA = Tail DNA, TMom = Tail moment, OM = Olive moment.

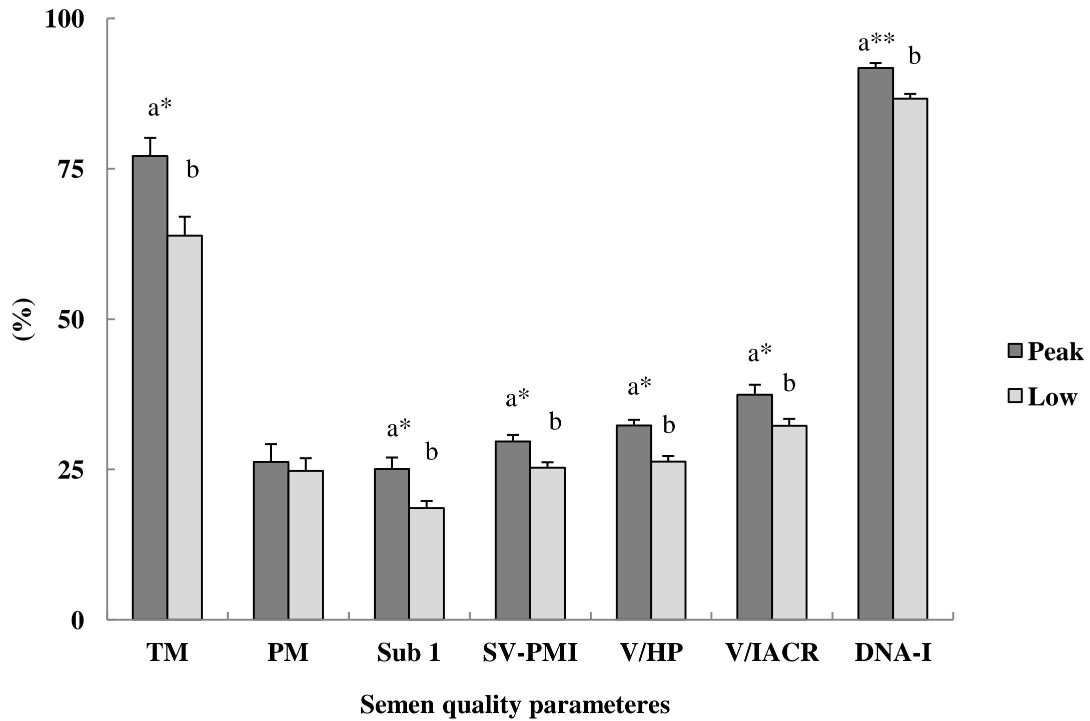

Figure 5. Evaluation of TM (total motility, %), PM (progressive motility, %), Sub 1 (subpopulation 1, %), SV–PMI (supra–vital plasma membrane integrity, %), V/HP (Viable with high mitochondrial transmembrane potential, %), V/IACR (viable with intact acrosome, %) and DNA–1 (DNA integrity, %) in frozen–thawed semen of buffalo bull during peak and low breeding seasons (n = 5 bulls and n = 60 ejaculates/season). Asterisks (* or **) shows a significance level between the two seasons. Bars with different alphabets with in the same variable showed significant differences (P < 0.05) due to season. Values are expressed as Mean±S.E.M.

Table 14. Comparison between *in vivo* fertility of frozen-thawed buffalo spermatozoa processed during peak breeding season and low breeding season in a fertility trial conducted during low breeding season

Semen processing season	Bull #	Inseminations performed (n)	Pregnancies achieved (n)	Pregnancy rate (%)
Peak breeding	B1	120	72	60.00
	B2	106	63	59.43
	B3	104	60	57.69
	B4	112	66	58.93
	B5	102	60	58.82
Total	**5**	**544**	**321**	**58.98**^{a}
Low breeding	B1	105	53	50.48
	B2	108	58	53.70
	B3	104	54	51.92
	B4	109	57	52.29
	B5	111	60	54.05
Total	**5**	**537**	**282**	**52.49**^{b}

Values lacking common superscripts in a column differ significantly ($P < 0.05$).

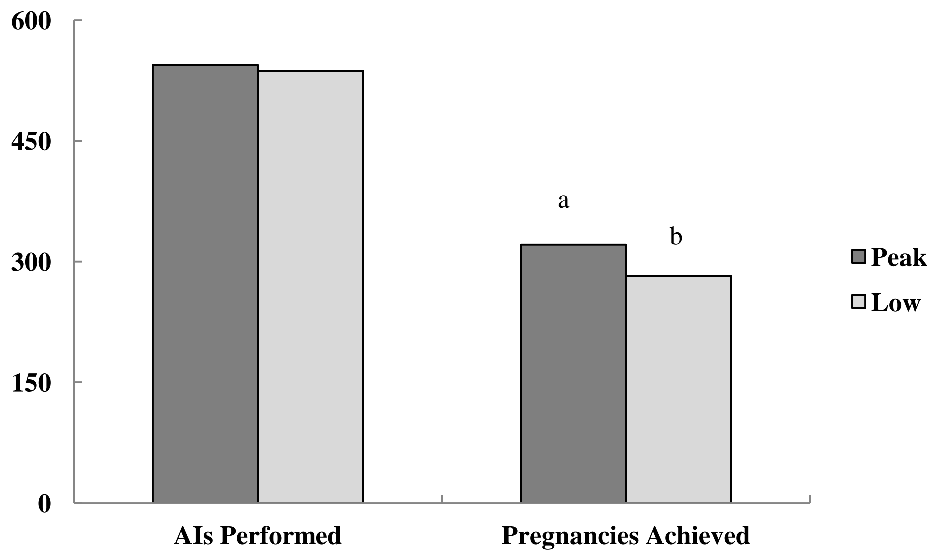

Figure 6a. Comparison between the AIs performed and pregnancy achieved based on frozen–thawed buffalo spermatozoa processed during peak and low breeding seasons in a fertility trial conducted during low breeding season.

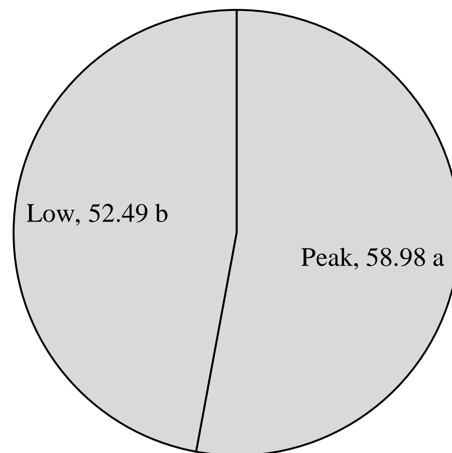

Figure 6b. Comparison between the *in vivo* fertility of frozen–thawed buffalo spermatozoa processed during peak breeding season and low breeding season in a fertility trial conducted during low breeding season. Different alphabet inside the pie chart shows a significant difference ($P < 0.05$) in overall fertility rate between the two seasons.

DISCUSSION

The present study was undertaken to investigate various sperm quality parameters in fresh and frozen–thawed water buffalo semen during peak and low breeding seasons. To the best of our knowledge this is the first comprehensive study that provides new insight regarding the stage (fresh and frozen-thawed) and seasonal influences (peak and low) on water buffalo bull spermatozoa.

The evaluation of fresh semen is essential for achieving an optimum reproductive efficiency in buffalo (Nandre *et al*., 2011). In the present study, semen volume and concentration of spermatozoa per ml volume were significantly higher during peak than low breeding season. Our results are in agreement with the previous studies conducted in Murrah buffalo (Tuli & Singh 1983), Surti buffalo (Bhosrekar *et al*., 1992) and Nili–Ravi buffalo bull (Zafar *et al*., 1988). However, our results contradict with the earlier reports in Egyptian (Oloufa *et al*., 1959) and Thai swamp (Koonjaenak *et al*., 2007a) buffalo bull, due to the differences between breeds or number of samples evaluated and/or geo–climatic conditions (Mandal *et al*., 2000).

In the present study, mean values of sperm TM, PM and subpopulation 1 (rapid and progressively motile) in fresh semen of buffalo bull were significantly higher during peak than low breeding season. In similar studies, Bhosrekar *et al.* (1992b) in Surti buffalo and Ravimurugan *et al.* (2003) in Murrah buffalo bulls have found better semen quality in terms of initial sperm motility. Moreover, higher sperm motility in buffalo bull have been reported during winter than summer season (Tuli & Singh, 1983; Tiwari *et al*., 2011), yet these studies performed only subjective analyses which were of limited scope in terms of fertility prediction (Ahmed *et al*., 2016a). In the current study, sperm motion kinematics including VAP, VSL, STR and LIN in fresh semen samples of buffalo bull were significantly higher during peak than low breeding season. Similarly, sperm quality parameters like SV–PMI, V/IACR, and V/HP in fresh semen were significantly higher during peak than low breeding season. All this highlights the variation in structural and functional integrity parameters of buffalo spermatozoa during peak and low breeding seasons. It is therefore, suggested that improvement in outcome of AI can be brought by using the semen collected during peak breeding season. Alternatively, the discard ratio of

ejaculates collected during low breeding season would be high to meet the minimum threshold values for semen cryopreservation.

Normal sperm genetic material is required for successful fertilization, as well as for further embryo and fetal development that will result in a healthy offspring (Andrabi, 2007). In the present study, percent head abnormalities in fresh semen samples of buffalo bull were significantly lower during peak than low breeding season. Similarly, DNA fragmentation indices like CL, TL, TDNA, TMom and OM were significantly lower in fresh semen during peak than low breeding season. Furthermore, the DNA integrity analyzed with acridine orange assay was significantly higher during peak than low breeding season. Similarly, Nandre *et al*. (2011) have found lower DNA damage in buffalo spermatozoa during peak than low breeding season. It is suggested that compromised DNA fragmentation indices may influence the fertilization potential of buffalo bull spermatozoa during low breeding season.

Evaluation of seasonal influences on the structures and functions of frozen–thawed spermatozoa is pivotal for obtaining optimum fertility rates (Wang *et al*., 2015; Patel & Dhami, 2016). This study found that sperm TM and subpopulation 1 were significantly higher during peak than low breeding season. These results suggesting better semen quality of buffalo bull during peak than low breeding season. High ambient temperature during summer seems to affect sperm motility in both riverine and swamp buffalo bulls (Kapoor, 1973; Sukhato *et al.*, 1988; Bahga & Khokar, 1991) leading to the assumption that the proportion of live spermatozoa is lowest during summer (Kapoor, 1973). Our results regarding no seasonal influence on sperm PM and motion kinematics are in consonance with reports of other researchers (Mandal *et al*., 2003) in Murrah buffalo and Koonjaenak *et al*. (2007b) in Thai swamp buffalo who reported similar pattern of sperm PM and motion kinematics during winter and summer seasons. Moreover, we found significantly higher sperm SV–PMI, V/IACR and V/HP during peak than low breeding season. These results are comparable with the previous studies in Murrah (Mandal *et al*., 2003), Thai swamp (Koonjaenak *et al*., 2007b) and Egyptian buffaloes (Alam *et al*., 2015), who have reported better sperm plasma membrane functionality during winter than summer season. Similarly, Farooq *et al*. (2013) have found better semen quality of Cholistani bull in terms of viability and acrosome integrity during winter than summer. It is put forward

that semen samples with higher SV–PMI, V/IACR and V/HP have more probability to reach and fertilize an oocyte in female reproductive tract (Ahmed *et al*., 2016 a).

In the present study, head and tail abnormalities in frozen–thawed buffalo bull spermatozoa were significantly lower during peak than low breeding season. These results are in accordance with the previous studies conducted on Malaya swamp (Jainudeen *et al*., 1982), riverine buffaloes (Bhattacharya *et al*, 1978; Gupta *et al*., 1978; Ahmad *et al*., 1987; Alam *et al*., 2015) and Cholistani cattle (Farooq *et al*., 2013). It is suggested that the higher level of sperm abnormalities during low breeding season (hot summer season) are due to heat stress experienced by bulls, as elevated environmental temperature impairs testicular functions (Chacon *et al*., 2002; Garcia *et al*. 2010; Barros *et al*., 2015). It is also reported that spermatozoa with abnormal tails cannot move properly or penetrate through cervical mucous and the corona radiate (Ahmad *et al*., 2003). Therefore, our finding that season influences the damage to frozen–thawed buffalo sperm tail is of enormous significance. It is suggested that the structural and functional parameters of frozen–thawed spermatozoa are compromised in low than peak breeding season.

As narrated above, normal sperm genetic material is required for successful fertilization, embryo and fetal development that will result in a healthy offspring (Andrabi, 2007). In the present study, higher DNA integrity was observed in frozen–thawed spermatozoa during peak than low breeding season. Moreover, DNA fragmentation indices like CL, TL, TDNA, TMom and OM were significantly lower during peak than low breeding season. Spermatozoa with high levels of DNA damage exhibited increased comet tail length (Singh & Stephens, 1998), olive moment (Olive *et al*., 1993) and more DNA in the tail region (Boe–Hansen *et al*., 2005). Similar to our results, Nandre *et al*. (2011) and Alam *et al*. (2015) have reported that season had greater influence on DNA integrity in Surti and Egyptian buffaloes, being higher in winter than summer. Previous studies conducted on spermatozoa of Thai swamp (Koonjaenak *et al*., 2007c), Nili–Ravi buffaloes (Ahmed *et al*., 2016a) and bovine (Januskauskas *et al*., 2003; Gillan *et al*., 2005; Madrid–Bury *et al*., 2005) have shown that the higher DNA fragmentation is linked either with *in vitro* or *in vivo* fertilizing potential. It is therefore, put forward that buffalo bull with higher DNA intactness possess better semen characteristics during peak breeding season.
In the present study, we found significantly higher *in vivo* fertility of frozen–thawed buffalo spermatozoa processed in peak compared to low breeding season in a fertility trial

carried out during low breeding season. Moreover, the seasonal differences in structural-functional parameters of buffalo spermatozoa were confirmed through this fertility trial. A similar trend in fertility was reported by Heuer *et al*. (1987) in water buffalo during low breeding season. It is therefore, suggested that the seasonality in buffalo bulls can be tackled by using the semen processed during peak breeding season for AI throughout the year.

Conclusion

This study concludes that semen collected during peak breeding season (September, October and November) demonstrated better initial quality i.e., viability/ plasma membrane integrity, acrosome and DNA integrities, thus are more suitable for cryopreservation than ejaculates collected during low breeding season (May, June and July). This was further confirmed by comparing the structural-functional parameters of frozen-thawed spermatozoa processed during peak and low breeding seasons. A field trial conducted during low breeding season concluded that *in vivo* fertility of frozen-thawed buffalo spermatozoa processed during peak breeding season had higher *in vivo* fertility compared to ejaculates processed during low breeding season.

ABSTRACT

Background: Hydrogen peroxide (H_2O_2) is a potent membrane–permeable oxidizing agent and is considered as one of the major reactive oxygen species (ROS) affecting the structural and functional integrity of mammalian spermatozoa. The present study was carried out to investigate the effects of H_2O_2 on semen quality parameters having the capability of prediction of fertility with ultimate aim to validate them for buffalo bull spermatozoa.

Materials and Methods: To study the effects of H_2O_2 on computer-aided sperm motion analysis (CASA) parameters and subjective motility (SM, %), supra–vital plasma membrane integrity (SV–PMI, %), viability/ mitochondrial transmembrane potential (V/HP, %) and viability/ acrosome integrity (V/IACR, %) of buffalo bull spermatozoa, qualifying ejaculates (motility > 60 %, concentrations, > 0.5 x 10^9/mL and volume > 1 mL) from five buffalo bull were diluted (25 x 10^6/mL) in PBS–0.1% BSA extender containing 10 µM H_2O_2, followed by measurement of semen quality parameters at different time intervals (0 min, 30 min, 60 min, 90 min and 150 min). Furthermore, for studying the effects of H_2O_2 on sperm DNA fragmentation indices, semen samples were diluted in PBS–0.1% BSA containing 0.0 mM, 25 mM, 50 mM, 75 mM and 100 mM H_2O_2 respectively, equilibrated for 1 hr to induce damage followed by neutral comet assay protocol.

Results: Analyses of variance showed that H_2O_2 at a dose of 10 µM significantly decreases ($P < 0.05$) sperm progressive motility (PM, %) rapid velocity (RV, %) average path velocity (VAP, µm/s), straight line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), SM, SV-PMI, V/HP and V/IACR in a time dependent manner as compared to control. Moreover, exposure of H_2O_2 increases ($P < 0.05$) DNA fragmentation, comet length, tail length, tail DNA, tail moment and olive moment in a dose dependent manner. Linear regression analysis showed that there was a strong relationship between decline in PM, RV, VAP, VSL, VCL and exposure of H_2O_2 (R^2= 0.966, $P < 0.01$, R^2= 0.988, $P < 0.01$, R^2= 0.961, $P < 0.01$, R^2= 0.956, $P < 0.01$ and R^2= 0.944, $P < 0.01$). Similarly, a strong relationship was observed between percent decline in SM, SV–PMI, V/HP, V/IACR and exposure of H_2O_2 (R^2= 0.961, $P < 0.01$, R^2= 0.919, $P < 0.01$, R^2= 0.977, $P < 0.01$ and R^2= 0.896, $P < 0.05$).

Conclusion: In conclusion, these results suggested that CASA and subjective parameters i.e., SM, SV-PMI, viability/ mitochondrial transmembrane potential, viability/ acrosome integrity and DNA fragmentation indices were negatively modulated by H_2O_2 in a time and dose dependent manner and may be used for monitoring the effectiveness of buffalo bull semen quality.

INTRODUCTION

Semen assessment tests such as concentration, subjective motility, morphology, plasma membrane integrity and acrosome integrity are often used to evaluate initial semen quality and functionality (Zhang *et al*., 1999; Brito *et al*., 2003). However, all these parameters are subjective in nature and can easily be influenced by the experience of the observer (Sellem *et al*., 2015). Only a few studies have investigated the predictive values of these semen quality parameters in bovine (Zhang *et al*., 1999; Brito *et al*., 2003; Sellem *et al*., 2015). Ahmed *et al*. (2016a, b) have observed that semen quality parameters which are subjective in nature are now considered as of limited scope in buffalo. In this regard, significant relationships have been reported between CASA progressive motility and fertility in bovine (Budworth *et al*., 1988; Farrell *et al*., 1998; Kathiravan *et al*., 2008) and buffalo (Ahmed *et al*., 2016a, b).

Hydrogen peroxide (H_2O_2) is a potent membrane–permeable oxidizing agent and is considered as one of the major reactive oxygen species (ROS) (Oehninger *et al*., 1995). It has dual effects on structural and functional integrity of mammalian spermatozoa (Oehninger *et al*., 1995; Hsu *et al*., 1999; Bilodeau *et al*., 2000; 2001). Lower concentrations of H_2O_2 added exogenously are believed to play a stimulatory role in sperm capacitation (Rivlin *et al*., 2004), hyperactivation (de Lamirade & Gagnon, 1994a, b), acrosome reaction (Griveau *et al*., 1995; Cocuzza *et al.,* 2007) and sperm–oocyte fusion (Aitken, 1995; Cocuzza *et al.,* 2007). However, at higher concentration, H_2O_2 behave as a genotoxic agent that is believed to deteriorate semen quality in terms of motility, plasma membrane integrity as well as acrosome integrity in a time and dose dependent manner in bovine (O'Flahery *et al*., 1999; Hu *et al*., 2010), human (Armstrong *et al*., 1999; Baumber *et al*., 2000; Bilodeau *et al*., 2001), fish (Dietrich *et al*., 2005), ram (Peris *et al*., 2007) and buffalo (Garg *et al*., 2009). It has been reported that the exposure of human spermatozoa to H_2O_2 is linked with a dramatic decrease in mitochondrial transmembrane potential (Armstrong *et al*., 1999), metabolic activity (Mammoto *et al*., 1996; Sharma *et al*., 2004) and assumed to cause protein and DNA damage (Beconi *et al*., 1993; Baumber *et al*., 2003; Dominguez–Rebolledo *et al*., 2010).

Perusal of literature shows that there is not a single study regarding the effect of H_2O_2 exposure on computer-aided sperm motion analysis, SV–PMI (viability/ plasma membrane integrity), viability/ mitochondrial transmembrane potential, viability/

acrosome integrity, and DNA fragmentation indices of buffalo bull spermatozoa. Therefore, the present study was carried out to investigate the effects of H_2O_2 on semen quality parameters having the capability of prediction of fertility with ultimate aim to validate them for buffalo bull spermatozoa.

MATERIALS AND METHODS

Experimental design

This study was performed as a 5 x 6 x 3 factorial design. The factors were number of bull versus number of semen quality parameters versus number of replicates.

Semen collection and dilution

Semen was collected from five mature water buffalo bull maintained at Livestock Research Station, NARC, Islamabad with artificial vagina maintained at 42 °C. After collection semen samples were transferred to the laboratory immediately for initial evaluation. Sperm progressive motility was assessed using phase contrast microscope (x 400, Olympus BX20) connected with closed circuit monitor, and sperm concentration was measured by using the specific spectrophotometer at a wavelength of 546 nm. Qualifying ejaculates having > 60% sperm motility, > 0.5 x 10^9 sperm/mL concentration and volume > 1 mL from each bull were diluted in PBS–0.1% BSA extender. Concentration of spermatozoa was fixed on extension at known values to carry out the selected semen quality assays at different time intervals.

Effect of H_2O_2 on CASA parameters of buffalo bull spermatozoa

In order to investigate the effect of H_2O_2 on CASA parameters of buffalo bull spermatozoa, semen samples were diluted (25 x 10^6/mL) in PBS–0.1% BSA extender containing 10 µM H_2O_2 followed by equilibration for 5 mins at 37 °C. After equilibration, semen samples (7 µL) were loaded on clean sterilized slide, coverslipped and CASA progressive motility (PM, %), rapid velocity (RV, %) and motion kinematics i.e., average path velocity (VAP, µm/s), straight line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), amplitude of lateral head displacement (ALH, µm), beat cross frequency(BCF, Hz), straightness (STR, %) and linearity (LIN, %) recorded at different time intervals (incubation times; 0 min, 30 min, 60 min, 90 min, 120 min, and 150 min).

Effect of H_2O_2 on subjective motility of buffalo bull spermatozoa

Effect of H_2O_2 on subjective motility of buffalo bull spermatozoa was assessed by diluting semen samples (25 x 10^6/mL) in PBS–0.1% BSA extender containing 10 µM H_2O_2 according to Garg *et al.*, (2009). Subjective motility of buffalo bull spermatozoa was

recorded by making a drop of semen on clean sterilized slide and coverslipped. Subjective motility was assessed in 4–5 fields under phase–contrast microscope at different time intervals (incubation times; 0 min, 30 min, 60 min, 90 min, 120 min, and 150 min).

Effect of H_2O_2 on supra–vital plasma membrane integrity of buffalo bull spermatozoa

Sperm supra–vital plasma membrane integrity of buffalo bull was assessed according to Chan *et al*. (1991). Semen samples were diluted in (25 x 10^6/mL) in PBS–0.1% BSA extender containing 10 µM H_2O_2 at different time intervals (incubation times; 0 min, 30 min, 60 min, 90 min, 120 min, and 150 min). Two hundred spermatozoa were observed in at least five different fields. Clear heads and swollen tails were considered as structurally and functionally active sperm with intact membrane, while pink heads and un–swollen tails were considered as dead spermatozoa.

Effect of H_2O_2 on viability/ mitochondrial transmembrane potential of buffalo bull spermatozoa

Effect of H_2O_2 on viability/ mitochondrial transmembrane potential of buffalo bull spermatozoa was studied by diluting semen samples (25 x 10^6/mL) in PBS–0.1% BSA extender containing 10 µM H_2O_2 according to Mahfouz *et al*. (2010). Semen sample (100 µL, sperm concentration; 2.5 x 10^6) was mixed with 20 µL of JC–1 (0.15 mmol/L in DMSO) and 10 µL of PI (1 mg/mL in PBS) and incubated at 37 °C for 10 min at different time intervals (incubation times; 0 min, 30 min, 60 min, 90 min, 120 min and 150 min). Two hundred spermatozoa were counted for two categories; viable with high mitochondrial transmembrane potential (white head region and bright orange mitochondrial region, V/HP, %) and nonviable with low mitochondrial transmembrane potential (red head region and green mitochondrial region, NV/LP, %) under epifluorescent microscopy (x 400, Nikon, Optiphot).

Effect of H_2O_2 on viability/ acrosome integrity of buffalo bull spermatozoa

Sperm viability and acrosome integrity of buffalo bull spermatozoa was evaluated according to Kovacs & Foote (1992) with slight modifications like use of Giemsa stain (7.50 %) for 2.5 hr at 37 ºC instead of room temperature. Semen samples were diluted in PBS–0.1% BSA extender containing 10 µM H_2O_2 Two hundred spermatozoa per sample

were categorized as viable spermatozoa with intact acrosome (white head region and purple acrosomal region, V/IACR, %) and nonviable spermatozoa with damaged acrosome (blue head region and pale lavender acrosomal region, NV/DACR, %) at different times interval (incubation times; 0 min, 30 min, 60 min, 90 min, 120 min, and 150 min) under phase contrast microscope (x 40).

Effects of H_2O_2 on DNA fragmentation parameters of buffalo bull spermatozoa

Effects of H_2O_2 on DNA fragmentation parameters of buffalo bull spermatozoa was evaluated according to Villani *et al*. (2010), Dietrich *et al*. (2005) by using different concentrations of H_2O_2 (0 mM, 25 mM, 50 mM, 75 mM and 100 mM). Semen samples were incubated at 37 ºC for 1 hr to induce damage by various concentrations of H_2O_2. Two slides per treatment were prepared for neutral comet assay protocol as described in chapter 1. Digital images were captured for subsequent analyses and scoring with TRITEK software. The parameters analyzed were comet length (CL, µm), head DNA (HDNA, %), tail length (TL, µm), tail DNA (TDNA, %), tail moment (TMom) and olive moment (OM).

Statistical analyses

All values were expressed as mean ± S.E. Linear regression analysis was performed to obtain linear association between exposure of H_2O_2 and semen quality parameters. The level of significance was $P < 0.05$. Minitab (MINITAB® Release 12.22, 1998) statistical package was used for data analysis. Tukey's pairwise comparison test was used for further differentiation between the means (Minitab).

RESULTS

Effect of H_2O_2 incubation times on CASA parameters of buffalo bull spermatozoa

Data on effect of H_2O_2 on CASA parameters are presented in Figures 7–11 and Table 15. The results showed that the exposure of H_2O_2 decreases sperm PM, RV, VAP, VSL and VCL in a time dependent manner ($P < 0.05$, Table 15). The results showed that there was a strong relationship between decline in PM, RV, VAP, VSL, VCL and incubation times of H_2O_2 ($R^2 = 0.966$, $P < 0.01$, $R^2 = 0.988$, $P < 0.01$, $R^2 = 0.961$, $P < 0.01$, $R^2 = 0.956$, $P < 0.01$, $R^2 = 0.944$, $P < 0.01$, respectively) (Figures 7–11).

Effect of H_2O_2 incubation times on subjective parameters of buffalo bull spermatozoa

Exposure of H_2O_2 decreases sperm SM, SV–PMI, V/HP and V/IACR in a time dependent manner ($P < 0.05$, Table 15). A strong relationship was noticed between percent decline in SM, SV–PMI, V/HP, V/IACR and incubation times of H_2O_2 ($R^2 = 0.961$, $P < 0.01$, $R^2 = 0.919$, $P < 0.01$, $R^2 = 0.977$, $P < 0.01$, $R^2 = 0.896$, $P < 0.05$, respectively) (Figure 12–15).

Relationship between CASA progressive motility and subjective parameters of buffalo bull spermatozoa

Data on relationship between CASA PM and subjective parameters (SM, SV–PMI, V/HP, and V/IACR) are presented in Figures 16–19. There was a strong relationship between percent decline in PM and SM, SV–PMI, V/HP, V/IACR at different incubation times of H_2O_2 ($R^2 = 0.944$, $P < 0.01$, $R^2 = 0.875$, $P < 0.05$, $R^2 = 0.941$, $P < 0.01$, $R^2 = 0.791$, $P < 0.05$, respectively).

Effects of H_2O_2 concentrations on DNA fragmentation parameters of buffalo bull spermatozoa

Effects of different concentrations of H_2O_2 on neutral comet assay parameters are presented in Table 16 and Figure 20. H_2O_2 exposure significantly increases ($P < 0.05$) CL, TL, TDNA, TMom and OM in a dose dependent manner as compared to control. Moreover, H_2O_2 exposure significantly increases DNA fragmentation in a dose dependent manner than control (Figure 20).

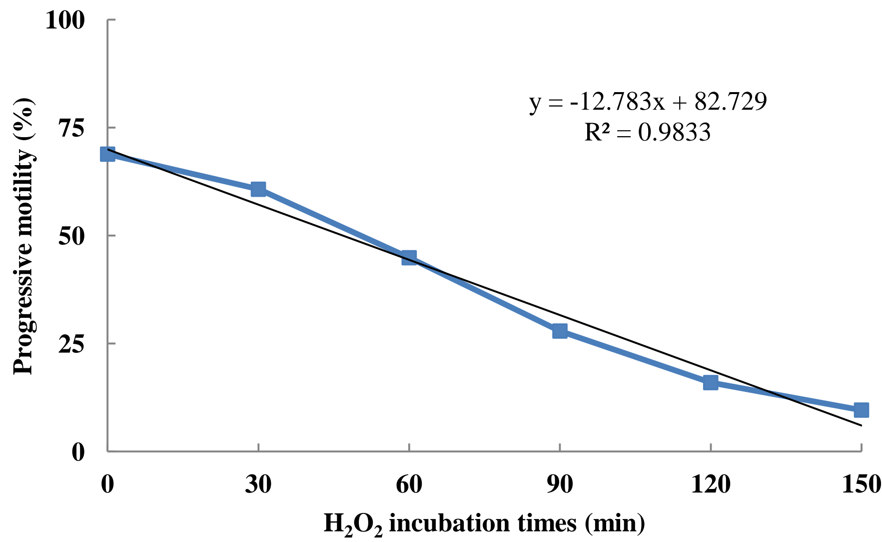

Figure 7. Relationship between percent decline in computer–aided sperm motion analysis (CASA) progressive motility and H_2O_2 incubation times of buffalo bull spermatozoa ($P < 0.01$).

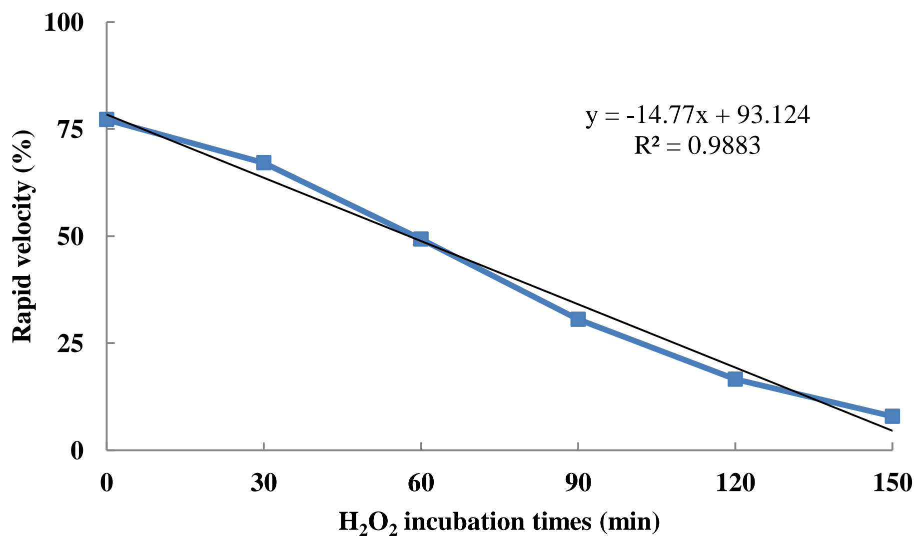

Figure 8. Relationship between percent decline in computer–aided sperm motion analysis (CASA) rapid velocity and H_2O_2 incubation times of buffalo bull spermatozoa ($P < 0.01$).

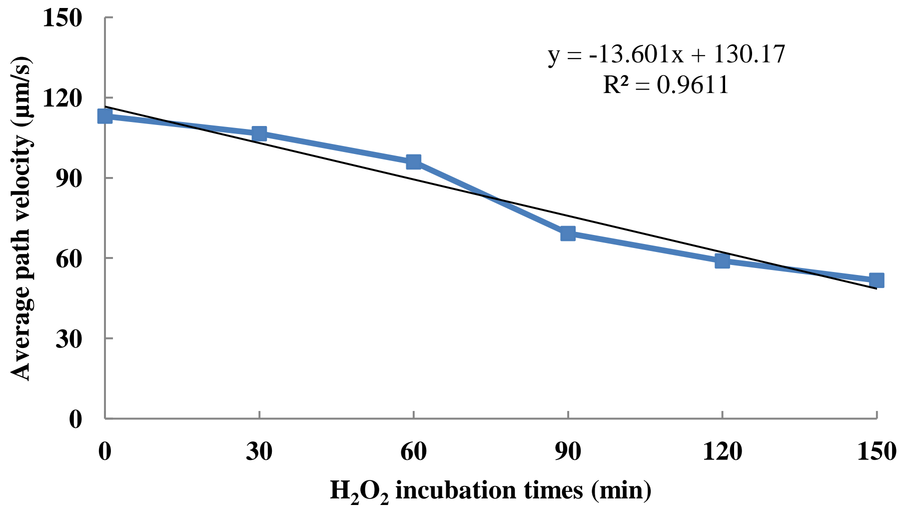

Figure 9. Relationship between decline in computer–aided sperm motion analysis (CASA) average path velocity (µm/s) and H_2O_2 incubation times of buffalo bull spermatozoa ($P < 0.01$).

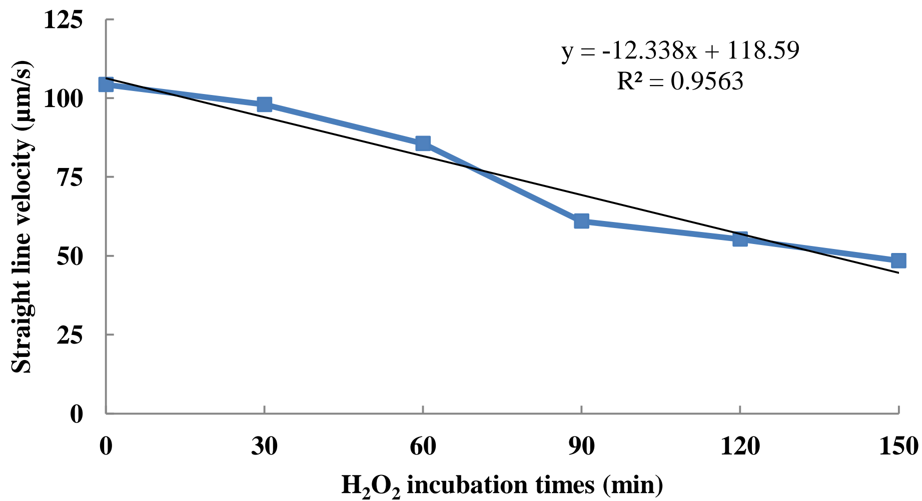

Figure 10. Relationship between decline in computer–aided sperm motion analysis (CASA) straight line velocity (µm/s) and H_2O_2 incubation times of buffalo bull spermatozoa ($P < 0.01$).

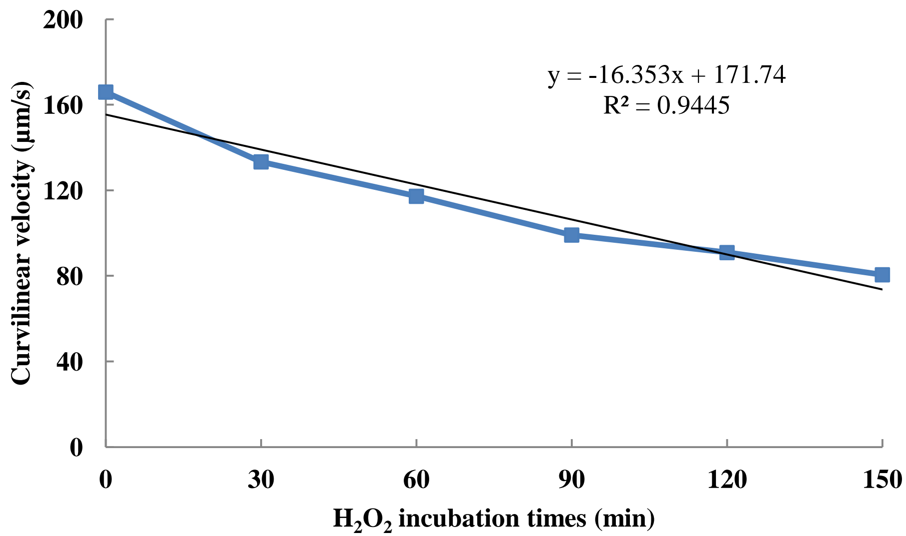

Figure 11. Relationship between decline in computer–aided sperm motion analysis (CASA) curvilinear velocity (µm/s) and H_2O_2 incubation times of buffalo bull spermatozoa ($P < 0.01$).

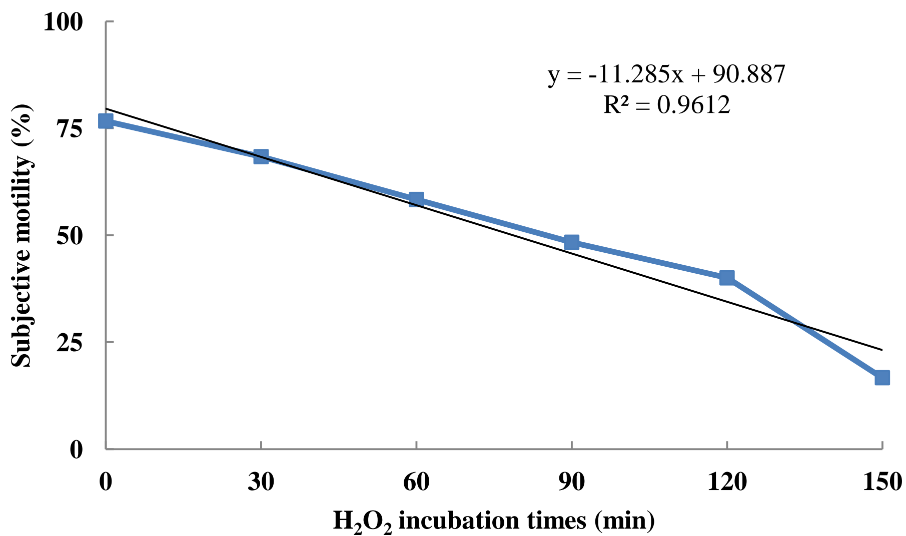

Figure 12. Relationship between percent decline in subjective motility and H_2O_2 incubation times of buffalo bull spermatozoa ($P < 0.01$).

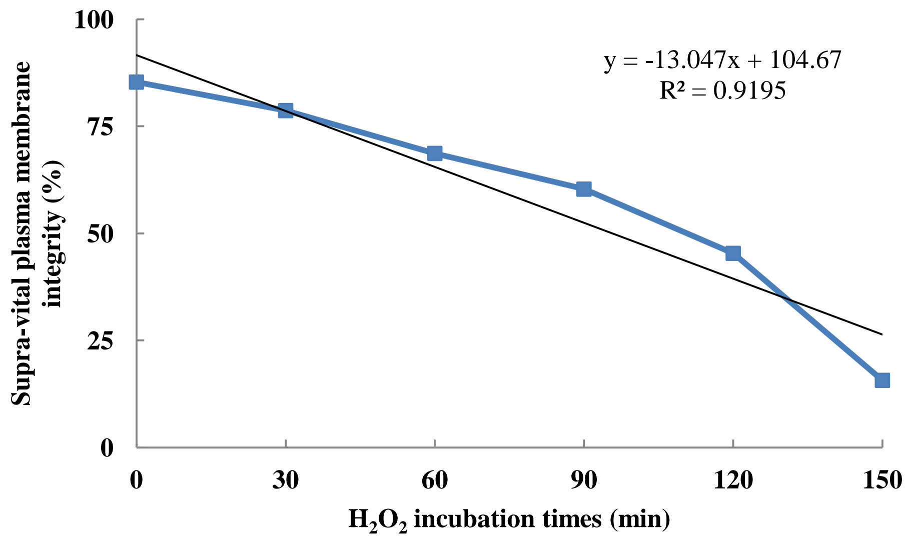

Figure 13. Relationship between percent decline in supra–vital plasma membrane integrity and H_2O_2 incubation times of buffalo bull spermatozoa ($P < 0.05$).

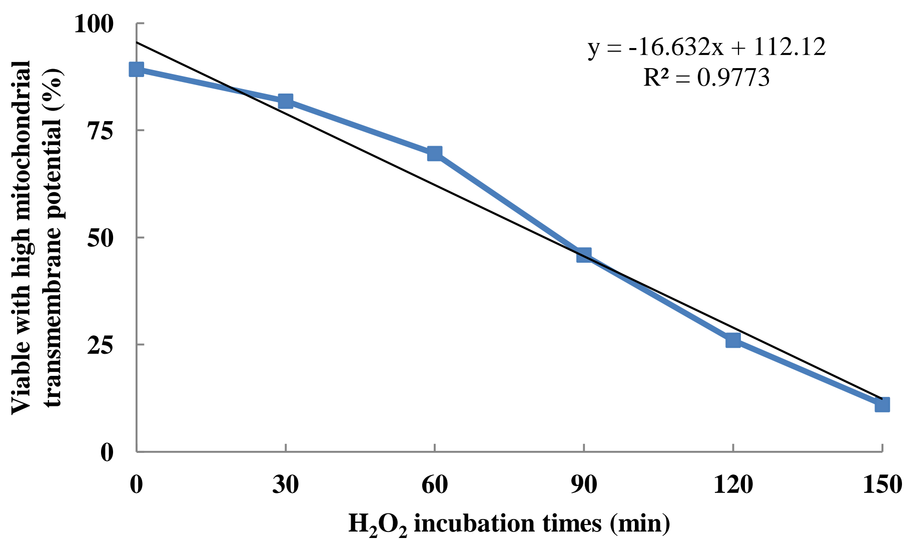

Figure 14. Relationship between percent decline in viable with high mitochondrial transmembrane potential and H_2O_2 incubation times of buffalo bull spermatozoa ($P < 0.01$).

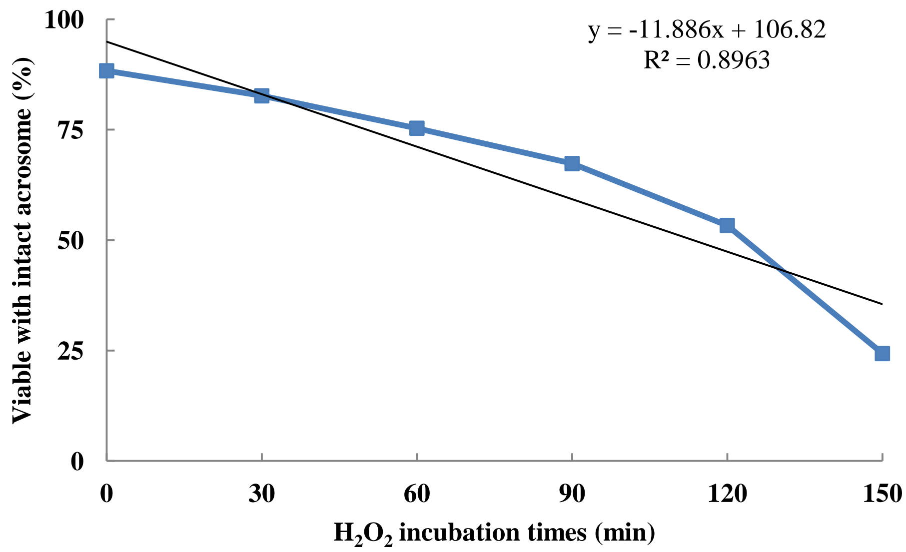

Figure 15. Relationship between percent decline in viable with intact acrosome and H_2O_2 incubation times of buffalo bull spermatozoa ($P < 0.05$).

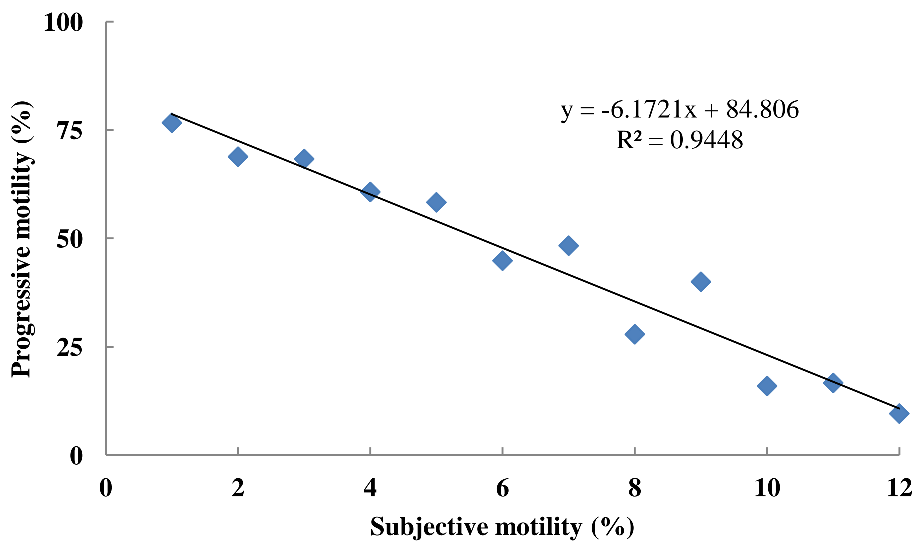

Figure 16. Relationship between percent decline in computer-aided sperm motion analysis (CASA) progressive motility and subjective motility of buffalo bull spermatozoa ($P < 0.01$).

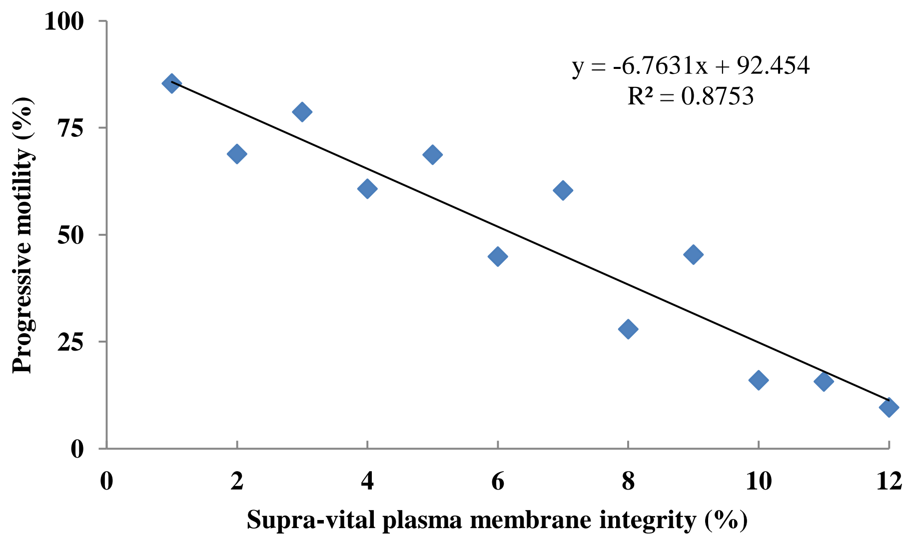

Figure 17. Relationship between percent decline in computer-aided sperm motion analysis (CASA) progressive motility and supra-vital plasma membrane integrity of buffalo bull spermatozoa ($P < 0.05$).

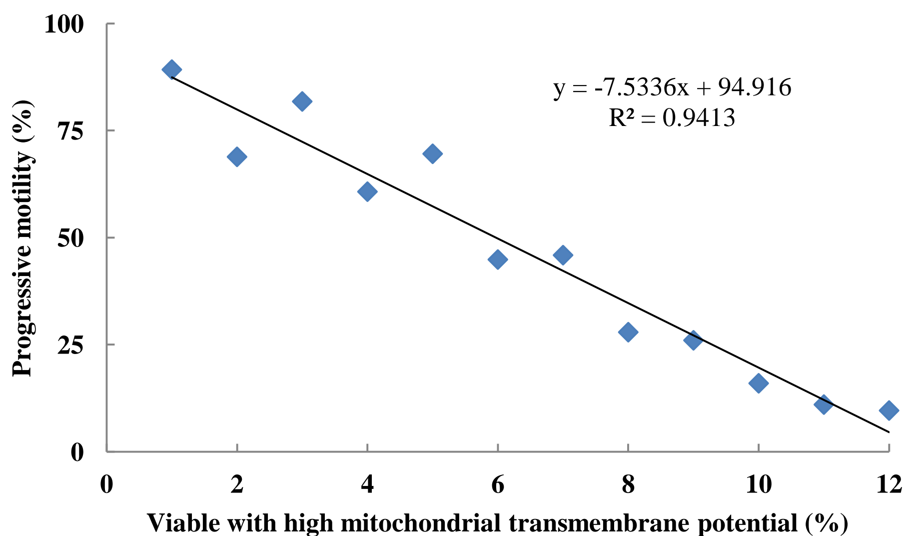

Figure 18. Relationship between percent decline in computer-aided sperm motion analysis (CASA) progressive motility and viable spermatozoa with high mitochondrial transmembrane potential of buffalo bull ($P < 0.01$).

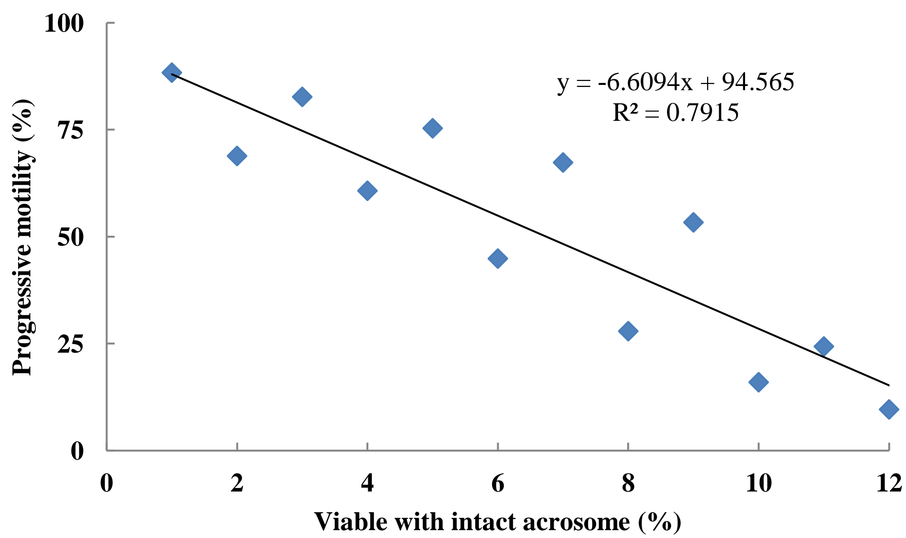

Figure 19. Relationship between percent decline in computer-aided sperm motion analysis (CASA) progressive motility and viable spermatozoa with intact acrosome of buffalo bull ($P < 0.05$).

Table 15. Effects of H_2O_2 exposure on CASA parameters, SM, SV–PMI, V/HP and V/IACR of buffalo bull spermatozoa at different time intervals (n = 5 bulls and n = 3 replicates)

CASA Parameters	H_2O_2 incubation times (min)					
	0	**30**	**60**	**90**	**120**	**150**
PM (%)	68.85±1.54[a]	60.72±3.27[a]	44.87±2.90[b]	27.90±3.17[c]	15.97±3.05[cd]	09.61±3.44[de]
RV (%)	77.20±2.28[a]	67.11±1.56[b]	49.28±0.75[c]	30.54±0.87[d]	16.56±1.13[e]	07.89±0.45[f]
VAP (μm/s)	113.06±4.26[a]	106.51±2.99[ab]	87.95±1.12[c]	72.23±1.22[cd]	60.99±4.36[de]	51.72±1.12[ef]
VSL (μm/s)	104.27±3.74[a]	97.93±1.89[ab]	81.58±1.10[c]	66.00±1.54[cd]	55.28±2.90[de]	46.42±2.94[ef]
VCL (μm/s)	152.94±10.98[a]	133.31±13.55[ab]	117.22±1.18[bc]	102.10±1.54[bcd]	88.91±1.71[cd]	80.53±1.37[de]
BCF (Hz)	42.19±0.57[a]	41.32±1.01[ab]	39.75±0.62[ab]	38.26±1.65[abc]	37.48±0.21[c]	34.56±0.71[c]
STR (%)	93.50±1.02[a]	89.11±2.23[ab]	84.33±2.33[ab]	81.44±3.07[bc]	79.00±2.08[bc]	74.33±2.19[c]
LIN (%)	70.65±3.55[a]	68.11±2.06[a]	64.96±2.85[ab]	61.56±2.08[ab]	58.67±1.84[b]	54.11±2.41[b]
SM (%)	76.67±1.67[a]	68.33±3.33[ab]	58.33±1.67[bc]	48.33±1.67[cd]	40.00±2.89[de]	16.67±1.67[f]
SV-PMI (%)	85.33±1.46[a]	78.67±1.86[a]	68.67±2.03[b]	60.33±1.45[bc]	45.33±2.40[d]	15.67±1.45[e]
V/HP (%)	89.22±1.97[a]	81.78±1.93[a]	69.56±1.11[b]	45.89±0.89[c]	26.00±2.67[d]	11.00±0.67[e]
V/IACR (%)	88.33±1.86[a]	82.67±1.76[ab]	75.33±2.03[bc]	67.33±1.76[cd]	53.33±1.76[e]	24.33±2.19[f]

Values lacking common superscripts in a row differ significantly ($P < 0.05$). CASA = Computer Aided sperm motion analysis, PM = Progressive motility, RV = Rapid velocity, VAP = Average path velocity, VSL = Straight line velocity, VCL = Curvilinear velocity, BCF = Beat cross frequency, STR = Straightness, LIN = Linearity, SM = Subjective motility, SV–PMI = Supra–vital plasma membrane integrity, V/HP = Viable with high mitochondrial transmembrane potential, V/IACR = Viable with intact acrosome

Table 16. Effects of different concentrations of H_2O_2 exposure on DNA fragmentation indices of buffalo bull spermatozoa (n= 5 bulls and n= 3 replicates)

Semen quality parameters	**H_2O_2 Concentrations (mM)**				
	0	**25**	**50**	**75**	**100**
CL (µm)	187.91 ± 6.33^{a}	263.08 ± 13.18^{b}	334.34 ± 11.98^{c}	418.75 ± 10.66^{d}	537.20 ± 2.68^{e}
HDNA (%)	86.88 ± 0.96^{a}	82.82 ± 0.35^{b}	77.10 ± 0.42^{c}	67.99 ± 0.62^{d}	52.43 ± 1.10^{e}
TL (µm)	76.40 ± 2.22^{a}	85.55 ± 0.95^{b}	92.45 ± 0.73^{c}	101.00 ± 1.38^{d}	110.83 ± 0.68^{e}
TDNA (%)	15.12 ± 1.03^{a}	17.18 ± 0.35^{a}	22.90 ± 0.39^{b}	32.01 ± 0.76^{c}	47.53 ± 1.07^{d}
TMom	18.08 ± 0.87^{a}	23.19 ± 0.82^{b}	30.23 ± 1.10^{c}	37.51 ± 0.99^{d}	44.27 ± 0.64^{e}
OM	12.47 ± 0.88^{a}	18.60 ± 1.24^{b}	24.78 ± 1.25^{c}	32.05 ± 1.46^{d}	39.92 ± 0.74^{e}

Values lacking common superscripts in a row differ significantly ($P < 0.05$).

CL = Comet length, HDNA = DNA in head, TL = Tail length, TDNA = DNA in tail, TMom = Tail moment, OM = Olive moment

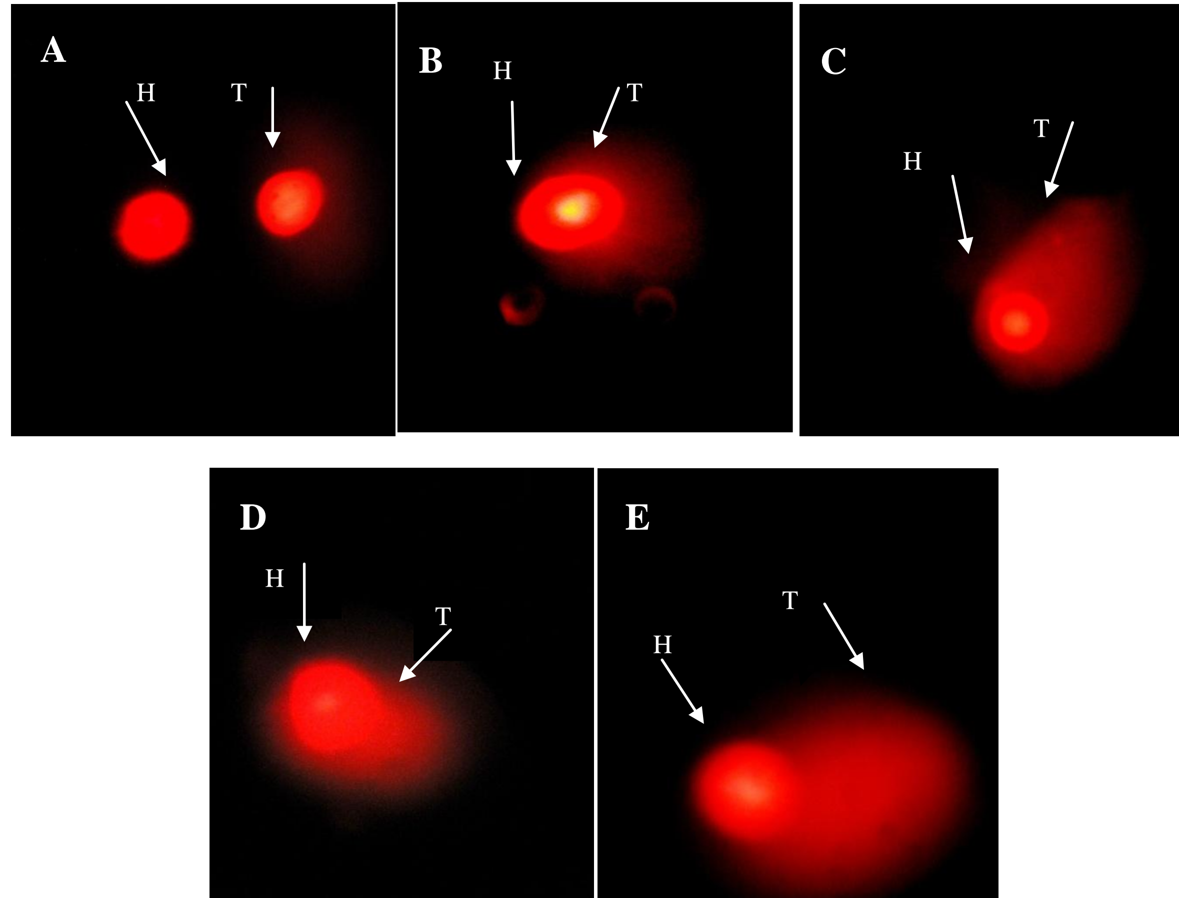

Figure 20. Fluorescent photomicrograph (x 400) of buffalo spermatozoa subjected to neutral comet assay protocol. The brightly fluorescent circular ‘‘comet head’’ (H) corresponding to the nucleus of the sperm, and to the left the diffusely stained ‘‘comet tail’’ (T) of damaged DNA. (A) Spermatozoa under control conditions (lacking H_2O_2 exposure). B, C, D and E spermatozoa of buffalo bull exposed to different concentrations of H_2O_2 (25, 50, 75 and 100 mM, respectively. Exposure of H_2O_2 caused a dose dependent increase ($P < 0.05$) in DNA fragmentation as compared to control.

DISCUSSION

The present study was carried out to investigate the effects of H_2O_2 on semen quality parameters having the capability of prediction of fertility with ultimate aim to validate them for buffalo bull spermatozoa.

Sperm progressive motility is indispensable to penetrate the zona pellucida *in vivo* or in vitro, and thus has been considered as one of the most important factors influencing fertilization rates (Liu *et al.*, 1991; Donnelly *et al.*, 1998; Turner, 2006). Hydrogen peroxide is a known genotoxic agent that is believed to generate ROS and ultimately deteriorate semen quality in terms of motility pattern and motion kinematics in a dose dependent manner (Dietrich *et al.*, 2005). The results of the present study revealed that H_2O_2 at a dose of (10 µM) diminished PM, RV and motion kinematics (VAP, VSL and VCL) of buffalo bull spermatozoa in a time dependent manner as compared to o min exposure of H_2O_2. Moreover, a very strong relationship was developed between exposure of H_2O_2 and decline in PM, RV and motion kinematics (VAP, VSL and VCL). Results of the present study are in agreement with those of O'Flahery *et al.* (1999) who incubated bull spermatozoa with different doses of H_2O_2 over a period of time and found a significant decline in motility pattern. Furthermore, Sharma (2015) found a huge decrease in motility of human spermatozoa by incubating semen samples with varying concentrations of H_2O_2. An inverse relationship has been observed between sperm motility and concentration of H_2O_2 in a time and dose dependent manner in buffalo (Garg *et al.*, 2009).

Structural and functional intactness of sperm plasma membrane is of fundamental importance in assessing the fertilizing capacity of semen samples (Cabrita *et al.*, 1999, Pintado *et al.*, 2000; Andrade *et al.*, 2007). Similarly, the presence of an intact acrosome and the induction of the acrosome reaction are also indispensable for fertilization process (Oura & Toshimori, 1990). The result of the present study showed that sperm structural cum functional integrity and acrosome integrity were significantly decreases by H_2O_2 exposure in a time dependent manner. Moreover, a highly significant relationship was noticed between exposure of H_2O_2 and percent declines in SV–PMI and V/IACR. Similar to the results of our study, Garg *et al.* (2009) conducted a comprehensive study on buffalo spermatozoa and noticed that H_2O_2 exposure is responsible to decrease sperm viability, plasma membrane integrity and acrosome integrity in a time and dose dependent manner.

It has been found that H_2O_2 reacts with other hydroxyl radicals that are assumed to cause loss in fatty acid content, viability and plasma membrane functionality of a cell (Jones *et al*., 1979; Halliwell & Gutteridge 1984).

It has been reported that mitochondrial transmembrane potential is directly associated with *in vivo* or *in vitro* fertilization rate in buffalo (Selvaraju *et al*., 2008) and human (Kasai *et al*., 2002). Moreover higher progressive motility is linked with higher mitochondrial transmembrane potential (Folger *et al*., 1993; Kao *et al*., 1998; Gąxzarzewicz *et al.*, 2003). Reduced motility is often associated with increased mitochondrial abnormalities and structural deformities in the flagella (Baccetti *et al*., 1993; Chemes *et al*., 1998). In the current study, it was found that H_2O_2 caused a highly significant decline in viability/ mitochondrial membrane potential of water buffalo bull spermatozoa as compared to control. These results suggest that with the passage of time H_2O_2 generate ROS which are responsible for decline in viability, motility, plasma membrane functionality in buffalo bull spermatozoa (Garg *et al*., 2009). Furthermore, a strong relationship was noticed between exposure of H_2O_2 and percent decline in viability/ mitochondrial transmembrane potential of buffalo bull spermatozoa. It has already been reported that H_2O_2 impaired mitochondrial membranes by lowering the redox potential (Bilodeau *et al*., 2001), with a subsequent decrease in the metabolic activity required for the maintenance of sperm motility and progression.

Normal sperm genetic material is required for successful fertilization, as well as for further embryo and fetal development that will result in a healthy offspring (Sailer *et al*., 1995; Evenson & Jost, 2000; Agarwal & Said, 2003). The neutral comet assay is reported to be more sensitive to detect dsDNA breaks, and more efficient in identifying DNA damages related to infertility (Morris *et al*., 2002; Fraser, 2004; Sharma *et al*., 2004). In this study, we measure CL, HDNA, TL, TDNA, TMom and OM parameters of neutral comet assay in water buffalo bull spermatozoa exposed to different concentrations of H_2O_2. The results demonstrated that exposure of H_2O_2 induced DNA damage in a dose dependent manner as compared to control. The DNA damage detected in this study is comparable to those reported with terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) and sperm chromatin structure assay in human (Aravindan *et al*., 1997; Donnelly *et al*., 2000). Spermatozoa with high levels of DNA damage have increased TL (Hughes *et al*., 1996), CL (Singh & Stephens, 1998), OM (Olive *et al*., 1993) and more

DNA in the tail region (Boe-hansen *et al*., 2005). On the basis of this study, it is suggested that determination of DNA damage at the single cell level can provide improvement in semen evaluation procedure.

Conclusion

In conclusion, spermatozoa with higher PM are linked with viability/ mitochondrial transmembrane potential, viabity/ acrosome integrity and plasma membrane integrity and are essential to achieve fertilization, whereas sperm DNA is importantly involved in embryonic development. In a nutshell, these results suggest that CASA parameters and SM, SV–PMI, V/HP, V/IACR, and DNA compactness are negatively modulated by H_2O_2 and may be used for monitoring the effectiveness of buffalo bull semen quality and fertility and must be included in optimization procedures.

GENERAL DISCUSSION

In the present study, we have investigated various semen quality parameters of buffalo bull spermatozoa during low and peak breeding seasons. Pearson's correlation coefficients were used to explore the linear relationship between semen quality parameters and *in vivo* fertility during peak and low breeding seasons. Moreover, multiple regression analyses were performed to identify single semen quality parameter and a combination of semen quality parameters in the prediction of *in vivo* fertility during low and peak breeding seasons at post-thawing. In this study, we have also investigated the effect of season on various semen quality parameters in fresh and frozen-thawed buffalo bull spermatozoa during peak and low breeding seasons. Furthermore, the effect of season on *in vivo* fertility of buffalo bull spermatozoa have also been investigated in a fertility trial conducted during low breeding season. In the last study, we have investigated the effect of H_2O_2 on semen quality parameters having the capability of prediction of fertility with ultimate aim to validate them for buffalo bull spermatozoa.

Sperm CASA motility is generally considered to be one of the most significant features linked with the fertilizing potential (Kathiravan *et al*., 2008; Kathiravan *et al*., 2011). Results of the present study showed that CASA PM and RV of buffalo bull spermatozoa were positively correlated with *in vivo* fertility during low and peak breeding seasons. Previous studies conducted on bovine semen have shown a strong relationship between PM and fertilization ability (Farrell *et al*., 1998). Moreover, previous studies suggested that sperm PM can be used to estimate the fertility of frozen-thawed swamp buffalo spermatozoa (Koonjaenak *et al*., 2007a). Highly significant positive relationship between PM, RV and *in vivo* fertility during peak and low breeding seasons signify the ability of the spermatozoa with progressive and forward movement to have greater capability to fuse with oocytes.

The measurement of motion kinematics has been considered as an indicator of functionality of spermatozoa (Budworth *et al*., 1988). In the present study, sperm motion kinematics, that is, VAP and VSL have established positive association with *in vivo* fertility during low and peak breeding seasons. These results are in consonance with the findings of Budworth *et al*. (1988), Farrell *et al*. (1988) in bovine and Liu *et al*. (1991) in human. Sperm motion kinematics i.e., VAP and VSL are also relevant to several other

functions like diffusion through cervical mucus, zona and IVF outcomes (Mortimer *et al*., 1998; Verstegen *et al*., 2003). In the present study, four subpopulations of motile spermatozoa were defined in frozen-thawed semen samples of buffalo bull during low and peak breeding seasons. Our results reported that percentages of rapid subpopulation 1 (most rapid and progressive) of motile spermatozoa were significantly associated with *in vivo* fertility bull during low and peak breeding seasons. These results are comparable with the findings in bovine (Muino *et al*., 2008a; 2009) and equine (Quintero–Moreno *et al*., 2003) during their respective breeding seasons.

Plasma membrane and acrosome integrities are considered to be useful for predicting the fertilizing ability of semen sample of buffalo bull (Kumar *et al.,* 2012; Andrabi *et al*., 2016). Results of the present study revealed that viable spermatozoa with intact plasmalemma and intact acrosome were positively associated with *in vivo* fertility during low and peak breeding seasons. Previous studies have shown relationship between sperm membrane integrity and fertility in bovine (Januskauskas *et al*., 2001) and Swamp buffalo (Koonjaenak *et al*., 2007a) during their respective breeding seasons.

Mitochondrial transmembrane potential and viability is considered to be the most sensitive test for semen evaluation. Mitochondrion is located in mid-piece of the spermatozoa and delivers adenosine triphosphate (ATP) to the axenome, where it is used for flagellar movement (Finkel, 2001). In this study, viable spermatozoa with higher mitochondrial transmembrane potential have established positive relationship with *in vivo* fertility during peak breeding season. These findings are comparable with that of Selvaraju *et al.* (2008) in buffalo bull and human (Kasai *et al*., 2002; Marchetti *et al*., 2002; 2004).

Sperm DNA integrity is essential at the time of successful fertilization, embryo and fetal development that will result in a healthy offspring (Andrabi, 2007). In the present study, DNA fragmentation parameters i.e., CL and TL were negatively correlated with *in vivo* fertility during low and peak breeding seasons respectively. Similarly, Koonjaenak *et al.* (2007a) reported that higher DNA fragmentation of buffalo spermatozoa is linked with low-fertilization potential. Chan *et al.* (2001) have suggested that failed fertilization is due to DNA fragmentation of the spermatozoa.

It was observed that the assessment of a single parameter does not provide high predictive value of fertility. Therefore, several regression equations were obtained by combining

semen quality parameters for the prediction of fertility. In our study, prognostic values to predict the *in vivo* fertility of buffalo bull by the equation of VAP, VSL, VCL, rapid subpopulation 1, SV–PMI, V/IACR, and V/HP accounted for 81.30 % ($P < 0.001$) during low breeding season. Moreover, the best predictive equation (R^2 adjusted=83.50 %, $P < 0.000$) of fertility for frozen–thawed buffalo semen included PM, RV, VAP, VSL and SV–PMI during peak breeding season. Farrell *et al*. (1998) reported that combinations of PM, VSL, ALH, and BCF provided higher predictive value for fertility ($R^2 = 0.89$). It was reported earlier, that among the sperm motion characteristics, PM and velocity parameters (VCL, VSL and VAP) could be used to predict the fertility of the bull spermatozoa. However, the ALH and BCF had no correlation with fertility of the bull (Kathiravan *et al.,* 2011).

In the present study, we have investigated the effect of season on various semen quality parameters in fresh and frozen–thawed water buffalo during peak and low breeding seasons. It was observed that ejaculate volume and concentration of spermatozoa per mL in fresh semen were significantly higher during peak than low breeding season. Our results are in agreement with the previous studies conducted on buffalo (Tuli & Singh 1983; Bhosrekar *et al*., 1992). Percent TM, PM, VAP, VSL, STR, LIN and rapid subpopulation 1 in fresh semen of buffalo bull were significantly higher during peak than low breeding season. In similar studies, Bhosrekar *et al.* (1992b) in Surti buffalo and Ravimurugan *et al.* (2003) in Murrah buffalo bulls have found improved initial sperm motility. Moreover, sperm SV–PMI, V/IACR, V/HP and DNA integrity were significantly higher during peak than low breeding season. Correspondingly, Nandre *et al*. (2011) have found lower DNA damage in buffalo spermatozoa during winter than summer.

At post–thawing, sperm TM and rapid subpopulation 1 in frozen–thawed semen of buffalo bull were significantly higher during peak than low breeding season. High ambient temperature during summer seems to affect sperm viability and motility in buffalo bulls (Sukhato *et al*., 1988; Bahga & Khokar, 1991). Moreover, we found significantly higher sperm SV–PMI, V/IACR and V/HP during peak than low breeding season. These results are comparable with the previous studies in Murrah (Mandal *et al*., 2003) and Swamp buffaloes (Koonjaenak *et al*., 2007b), who have reported better sperm plasma membrane functionality during winter than summer season. The results of the present study showed that sperm DNA fragmentation was significantly lower during peak than low breeding

season, whereas DNA fragmentation indices i.e., CL, TL, TDNA, TMom and OM were higher during low than peak breeding season. Earlier studies conducted on buffalo have shown that season had greater impact on DNA integrity, being higher in winter than summer (Nandre *et al*., 2011; Alam *et al*., 2015).

Quality of frozen semen is one of the most influential factors to establish a reasonable conception rate in the farm animals (Saacke, 1984; Andrabi, 2009). In the present study, we found significantly higher *in vivo* fertility of frozen–thawed buffalo spermatozoa processed in peak compared to low breeding season in a fertility trial carried out during low breeding season. A similar trend in fertility was reported by Heuer *et al*. (1987) in water buffalo during low breeding season. It is therefore, suggested that the seasonality in buffalo bulls can be tackled by using the semen processed during peak breeding season for AI throughout the year.

In the last experiment, we have investigated the effect of H_2O_2 on semen quality parameters having the capability of prediction of fertility with ultimate aim to validate them for buffalo bull spermatozoa. It was found that H_2O_2 at a dose of (10 µM) diminished sperm PM, RV and motion kinematics (VAP, VSL and VCL) in a time dependent manner as compared to control. Results of the present study are in agreement with those of O'Flahery *et al*. (1999) who incubated bull spermatozoa with different doses of H_2O_2 over a period of time and found a significant decline in motility pattern.

This study found that sperm structural cum functional integrity and acrosome integrity were significantly decreased by H_2O_2 exposure in a time dependent manner. Similar to the results of our study, Garg *et al*. (2009) conducted a comprehensive study on buffalo spermatozoa and noticed that H_2O_2 exposure caused decrease in sperm viability, plasma membrane/ acrosome integrities in a time and dose dependent manner. In the current study, it was found that H_2O_2 caused a highly significant decline in V/HP of buffalo bull spermatozoa as compared to control. It has already been reported that H_2O_2 impaired mitochondrial potential by lowering the redox potential (Bilodeau *et al*., 2001), followed by decrease in sperm metabolic activity and motility. Furthermore, this study found that exposure of H_2O_2 induced DNA fragmentation in a dose dependent manner compared to control. Earlier studies have reported that spermatozoa with more DNA damage have higher CL (Hughes *et al*., 1996), TL (Singh & Stephens, 1998), OM (Olive *et al*., 1993) and more DNA in the tail region (Boe-hansen *et al*., 2005).

General conclusion and future perspectives

It is concluded that assessment of CASA parameters and sperm structural and functional parameters i.e., integrity of plasma membrane and acrosome, and viability/ transmembrane potential of mitochondria at post-thawing were able to predict the *in vivo* fertility of water buffalo bull during peak and low breeding seasons. Moreover, ejaculates collected/ processed during peak breeding season (September, October and November) demonstrated better semen quality i.e., viability/ plasma membrane integrity, acrosome, DNA integrities and viabity/ mitochondrial transmembrane potential after freezing thawing process. Furthermore, the *in vivo* fertility of frozen-thawed buffalo spermatozoa processed during peak breeding season was significantly higher than semen cryopreserved during low breeding season in a fertility trial carried out during low breeding season and hence was more suitable for AI than ejaculates collected during low breeding season (May, June and July). It is therefore, suggested that in order to increase fertility rate in buffalo, semen should be collected and preserved during cooler months and used for AI throughout the year.

Hydrogen peroxide (H_2O_2) is a potent membrane–permeable oxidizing agent and is considered as one of the major ROS having dual effects on structural and functional integrity of mammalian spermatozoa. Lower concentrations of H_2O_2 added exogenously are believed to play a stimulatory role in sperm physiology. However, at higher concentration, H_2O_2 behave as a genotoxic agent that is believed to deteriorate semen quality in terms of SM, SV-PMI, V/IACR, V/HP and DNA fragmentation indices in a time and dose dependent manner respectively. In a nutshell, these results recommended that semen quality of buffalo bull is negatively modulated by H_2O_2 and may be used for monitoring the effectiveness of buffalo bull semen quality and fertility and must be included in optimization procedures.

CASA motility assessment is a very good first step on the exact relation between semen motility and fertility. Little is known about subpopulations of motile spermatozoa within a given ejaculate and if unique motility patterns in those subpopulations have specific abnormalities influencing fertility of buffalo bull. Identifying different semen populations by means of CASA (Holt & Van Look, 2004; Satake *et al.*, 2006) is still at a research level and waits for further development. More detailed studies could be added to current results,

being additive for a final use in semen quality assessment. Furthermore, i*n vitro* fertilization rates may also be studied to predict the fertility of buffalo bull spermatozoa.

Fertility of cryopreserved water buffalo spermatozoa through AI is reported to be affected by seasonality (Andrabi, 2014). Several studies (Tuli & Mehar, 1983; Heuer *et al.*, 1987; Bahga & Khokar, 1991; Koonjaenak *et al.,* 2007a, b, c) have reported lower cryo-damage and subsequently higher fertility of buffalo spermatozoa during the winter (peak breeding season) compared with that processed during the summer (low-breeding season). In our study, we found a highly significant increase in semen quality and *in vivo* fertility of buffalo bull spermatozoa during peak than low breeding season. The ultimate reason for this increase in semen quality and fertility may be the difference in ambient temperature, photoperiodism and even improved quality of green fodder in prevailing environmental conditions in Pakistan during winter than summer.

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