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RESEARCH ARTICLE

Effects of Storage Duration on the Quality and DNA Integrity of Nili-Ravi Bull Spermatozoa Frozen and Stored in Liquid Nitrogen

Muhammad Shoaib, Nazir Ahmad*, Maqbool Ahmad, Ijaz Ahmad, Sajid Iqbal[1] and Shamim Akhter[2]

Department of Theriogenology, University of Agriculture, Faisalabad, Pakistan; [1]Semen Production Unit, Qadirabad, Sahiwal, Pakistan; [2]Department of Zoology, Pir Mehr Ali Shah Arid Agricultural University, Rawalpindi, Pakistan
*Corresponding author: profnazir53@hotmail.com

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A B S T R A C T

In the present study, effects of storage duration on the quality and DNA integrity of Nili-Ravi buffalo bull spermatozoa frozen and stored in liquid nitrogen for up to three years were investigated. According to duration of storage, the semen samples were categorized into 10 groups, viz. i) 0 day, ii) one day, iii) two weeks, iv) one month, v) three months, vi) six months, vii) nine months, viii) one year, ix) two years and x) three years. A total of 50 straws, with 5 straws for each of the 10 groups were used. After thawing for 30 seconds at 37°C, these samples were evaluated for sperm motility, livability, livability index, plasma membrane integrity, acrosome damage and DNA damage. At 0 day, mean values for sperm motility (%), livability (hrs), livability index, plasma membrane integrity (%), acrosome damage (%) and DNA damage (%) were 53±1.22, 6.0±0.0, 130±5.2, 45.4±1.6, 3.8±0.6 and 2.2±0.4. After 3 years of storage, the corresponding values were 39±3.32, 4.4±0.2, 78.5±5.7, 28.2±2.2, 12.0±0.7 and 5.8±0.6. Sperm motility and livability remained unaffected up to 3 months of storage, followed by a decrease at 6th month, while absolute index of livability and membrane integrity started decreasing at 1 month ($P<0.05$). Sperm with damaged acrosome increased at 6th month ($P<0.05$). Similarly, sperm with damaged DNA increased after one year of storage ($P<0.05$). Thus, post-thaw motility, livability and acrosome integrity of spermatozoa of Nili-Ravi bulls started decreasing at 6th month of storage, while sperm DNA integrity remained unaffected for one year of storage in liquid nitrogen.

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INTRODUCTION

Buffalo is the main dairy animal in many countries of the world, including Pakistan (Hussain *et al*., 2012; Mahmood *et al*., 2012; Bhutto *et al*., 2012; Hussain *et al*., 2013). About 62% of milk produced in Pakistan is contributed by buffaloes, 35% by cows and the remaining by goats and camels (Anonymous, 2011-12; Khan *et al*., 2013; Chattha *et al*., 2013). However, the average daily milk production of our buffaloes is much less than majority of the exotic cattle breeds. In addition to other factors, low genetic potential of our dairy buffaloes seems to be responsible for their low productivity.

There are two ways for the improvement in the reproductive and productive potential of our dairy animals. Firstly, by improving the feeding and management conditions of animals; secondly, by improving their genetic make-up. Improvements achieved through feeding and management are quick but are not permanent and animals mostly return to their previous low producing state as soon as improvement of feeding and management is with-drawn. On the other hand, the improvement achieved through genetic modification is slow and time consuming but tends to be permanent when achieved (Rahman *et al*., 2012).

Rapid improvement in the genetic make-up of dairy and beef animals can be achieved through the use of artificial insemination. This is possible because semen collected from a few highly selected elite bulls is used to inseminate thousands of females per year. In artificial insemination (AI), semen from elite bulls is collected, diluted/processed and deposited in the uterus of the female. In order to achieve full benefits of AI, preservation of spermatozoa for long term without much

damage to their fertilizing abilities is imperative. Preservation of spermatozoa in frozen form in liquid nitrogen (-196°C) is generally practiced for their long-term storage (Rahman *et al*., 2012; Waheed *et al*., 2012). It is most important procedure for breeding programs in domestic cows and buffaloes, and this practice is also used for establishment of gene-banks and to preserve the genetic diversity.

Prolonged storage of spermatozoa in liquid nitrogen can affect their viability and DNA integrity. Haugan *et al.* (2007) investigated the effect of storage duration between 1000 and 2400 days and observed that frozen semen stored in liquid nitrogen for shorter period resulted in higher conception rates in dairy cows than when the semen was stored for longer duration. According to Prinosilova *et al.* (2012), long term cryopreservation of canine semen resulted in adverse effects on sperm DNA integrity. However, there is relatively little information regarding the effects of storage duration on the quality and DNA integrity of spermatozoa of Nili-Ravi bulls stored in liquid nitrogen. Therefore, possible effects of storage duration on the quality and DNA integrity of spermatozoa from Nili-Ravi bulls frozen and stored in liquid nitrogen (-196°C) for up to three years have been described in this paper. Efforts were also made to see the correlations among various post-thaw parameters of semen quality.

MATERIALS AND METHODS

Experimental animals: Semen samples collected from five adult Nili-Ravi bulls housed at the Semen Production Unit (SPU) Qadirababad, Pakistan were used in this study. This area is located at an altitude of about 173 m above sea and lies between latitude 30 and 30.15ºN and longitudes 37 and 74ºE (Ahmad *et al.*, 1981). At this SPU, ejaculates with progressive sperm motility of at least 60% are diluted with tris-citric acid-fructose-egg yolk-glycerol extender (Younis *et al.*, 1999), filled in 0.5 ml French straws and cryopreserved in liquid nitrogen. Semen samples collected from different bulls are not pooled, rather, they are processed separately for each bull. Before freezing, an equilibration period of 04 hrs at 4°C is allowed to filled-in straws. This is followed by freezing of straws by holding them for 10 minutes in vapors 5 cm above the surface of the liquid nitrogen. These straws are then dipped in the liquid nitrogen for storage (Jalme *et al.*, 2003; Han *et al*., 2005).

Collection of samples: For this study, semen straws frozen and stored in liquid nitrogen for different time periods were selected. According to the duration of storage period, these selected samples were categorized into 10 groups, viz. i) 0 day, ii) 1 day, iii) 2 weeks, iv) 1 month, v) 3 months, vi) 6 months, vii) 9 months, viii) 1 year, ix) 2 years and x) 3 years. A total of 50 straws were selected in such a way that there were 5 straws in each of the 10 groups, with one straw from each bull.

Post- thawed evaluation of semen: Post-thaw evaluation of semen was carried out in terms of sperm motility, livability, livability index, plasma membrane integrity, acrosome integrity and DNA integrity. Before evaluation, semen samples were thawed at 37ºC for 30 seconds. Post-thaw motility of spermatozoa was measured by examining a small drop of sample under bright field microscope (X 400). Livability and livability index of spermatozoa at 37ºC for each sample were determined following Younis *et al.* (1999).

Hypo-osmotic swelling test (HOST) was applied for the determination of sperm plasma membrane integrity, using solution of 150 mOsm/L osmolarity (Jeyendran *et al.*, 1984). For semen evaluation, 0.1 ml of frozen-thawed sample was mixed with 1 ml of hypo-osmotic solution, incubated for 1 hr at 37°C and examined under microscope. Spermatozoa with swollen tail were assumed to have intact plasma membrane. Acrosome integrity was determined after fixing 100 µl of semen in 500 µl of 1% formal citrate solution and examining under phase contrast microscope (Akhter *et al.*, 2010). Sperm with normal apical ridge were assumed to having intact acrosome.

Non-fluorescent toluidine blue staining technique (Beletti *et al.*, 2005) was applied for assessing sperm with intact DNA. A thin smear of semen was fixed in 96% ethanol-acetone (1:1) at 4ºC for 30 minutes, stained with 0.05% toluidine blue for 10 minutes and examined under microscope (1000X). Spermatozoa exhibiting dark purple-colored head were considered as having fragmented DNA. On the other hand, spermatozoa with light blue colored-head were taken to be exhibiting intact DNA.

Statistical analysis: Data on various parameters were subjected to one-way ANOVA, using completely randomized design. Duncan's multiple range test was applied for multiple means comparisons, where necessary. Pearson's correlation coefficients among various physical parameters of semen quality were also computed.

RESULTS

At 0 day, mean values (±SE) for sperm motility (%), livability (hrs), livability index, plasma membrane integrity (%), acrosome damage (%) and DNA damage (%) were 53±1.22, 6.0±0.0, 130.0±5.2, 45.4±1.6, 3.8±0.6 and 2.2±0.4. After 3 years of storage, the corresponding values were 39±3.32, 4.4±0.2, 78.5±5.7, 28.2±2.2, 12.0±0.7 and 5.8±0.6 (Table 1). Post-thaw sperm motility was highest at 0 day of storage; it started decreasing at the 6th month, reaching the lowest value after 3 years of storage ($P<0.05$). A similar trend was seen for the livability of spermatozoa at 37ºC. After higher value at 0 day to 2 weeks of storage ($P<0.05$), livability index of spermatozoa showed a decreasing trend with the increase in storage duration. An almost similar trend was noted for the plasma membrane integrity (Table 1).

The percentage of sperm with damaged acrosome was the lowest at 0 day of storage, and remained unchanged up to 3 months; from the 6th month it exhibited an increasing trend, reaching the highest value 3 years after storage ($P<0.05$). Percentage of sperm with damaged DNA was also lowest at 0 day of storage; however, it remained unaffected up to one year of storage, and increased thereafter.

The correlation analysis (Table 2) revealed that post thaw sperm motility, livability, livability index and plasma membrane integrity had significant positive

Table 1: Mean (±SE) values for various parameters of post-thaw semen quality of Nili-Ravi bulls at different storage intervals in liquid nitrogen

Storage duration	Sperm motility (%)	Sperm livability (hrs)	Sperm livability index	Sperm plasma membrane integrity (%)	Sperm with acrosome damage (%)	Sperm with DNA damage (%)
0 D	53±1.22a	6.0±0.0a	130.0±5.2a	45.4±1.6a	3.8±0.6e	2.2±0.4c
1 D	52±1.22a	6.0±0.0a	121.8±10.7ab	43.6±0.6ab	3.8±0.4e	2.2±0.6c
2 W	50±2.24ab	6.0±0.0a	103.8±9.3abc	41.4±1.5abc	4.6±0.6e	2.4±0.4c
1 M	47±1.22abc	6.0±0.0a	91.0±11.1bcd	38.6±1.9bcd	5.0±0.8de	3.6±0.6bc
3 M	47±2.55abc	5.4±0.2ab	82.4±17.4cd	37.4±2.3cde	5.2±0.6de	3.0±0.7c
6 M	44±3.67bcd	5.0±0.0bc	97.6±16.0abcd	34.2±2.7def	6.4±0.5cd	3.0±0.7c
9 M	43±2.55bcd	5.0±0.0bc	71.1±15.7d	32.6±1.5efg	7.0±0.6c	3.8±0.7bc
1 Yr	43±3.39bcd	5.0±0.0bc	92.3±7.3bcd	31.6±2.2fg	8.8±0.4b	3.8±0.4bc
2 Yr	40±3.54cd	4.6±0.2cd	81.6±6.9cd	29.0±2.2fg	10.4±0.5ab	5.0±0.5ab
3 Yr	39±3.32d	4.4±0.2cd	78.5±5.7d	28.2±2.2g	12.00±0.7a	5.8±0.6a

D = Day(s); W = Week(s); M = Month(s); Yr = Year(s); Mean values with different letters within a column differ significantly from one another (P<0.05).

Table 2: Values of correlation coefficients among different parameters of post-thawed Nili-Ravi bull spermatozoa

Parameters	Correlation coefficients	Parameters	Correlation coefficients
Sperm motility & livability	0.683**	Sperm livability & sperm with DNA damage	-0.444**
Sperm motility & livability index	0.695**	Sperm livability index & sperm membrane integrity	0.672**
Sperm motility & plasma membrane integrity	0.907**	Sperm livability index & sperm with acrosome damage	-0.332*
Sperm motility & sperm with acrosome damage	-0.561**	Sperm livability index & sperm with DNA damage	-0.330*
Sperm motility & sperm with DNA damage	-0.398**	Sperm plasma membrane integrity & sperm with acrosome damage	-0.707**
Sperm livability & livability index	0.474**	Sperm plasma membrane integrity & sperm with DNA damage	-0.472**
Sperm livability & plasma membrane integrity	0.772**	Sperm with acrosome damage & sperm with DNA damage	0.566**
Sperm livability & sperm with acrosome damage	-0.794**	-----	--

* = Significant at P<0.05; ** = Significant at P<0.01.

correlations with one another. Similarly, sperm with damaged acrosome and those with damaged DNA also showed significant positive correlation with each other. However, the correlations between latter two parameters and former four parameters were significant but negative (P<0.01).

DISCUSSION

Long term storage of cryopreserved semen can adversely affect it post-thaw quality. In the present study, post-taw sperm motility and livability decreased, while percentage of sperm with damaged acrosome increased at 6th month of storage in liquid nitrogen. When human sperm were frozen and stored in liquid nitrogen or liquid nitrogen vapors for 1 week, 1 month or 3 months, no difference could be observed in sperm motility, viability, morphology, DNA integrity or active mitochondria among cryostorage periods (Lim *et al.*, 2010). However, Sugulle *et al.* (2006) stored frozen cross-bred bull semen in liquid nitrogen for 1 day, 7 days and 3 months and observed a significant decrease in sperm motility for 3 months storage duration than 1 day or 7 days. According to Buarpung *et al.* (2012), motility of cat spermatozoa was lower after long term storage (9-13 years) as compared to short term storage (1-5 years).

Sperm motility and acrosome integrity are generally considered to be fairly reliable indications of viability and fertility of semen (Hafez and Hafez, 2000; Janett *et al*., 2008). In the present study, mean post-thaw sperm motility of 39±3.32% was recorded in samples stored for 3 years. Considering 30 million spermatozoa in an insemination dose, a buffalo would receive 11.7 million motile spermatozoa per insemination dose, which is slightly higher than 10 million recommended per insemination dose using fresh/fluid semen.

Boe-Hansen *et al.* (2008) noted more than 20% DNA fragmentation in semen samples from 11 out of 145 boars (7.6%) cryo-preserved and stored for three days. In the present study, the percentage of sperm with damaged DNA remained unaffected up to 1 year of storage in liquid nitrogen. However, it increased significantly at 2 years of storage. A total of 5.8% sperm showed damaged DNA when stored for three years. Sperm DNA integrity can also vary due to type of extender used or the breed of the bull (Waterhouse *et al.*, 2010). DNA integrity in Norwegian Red bulls was higher in Tris egg yolk glycerol extender as compared to skimmed milk egg yolk glycerol extender. This may be due to interactions between Tris and glycerol present in the extender (Pickett, 1993; Kommisrud *et al.*, 1996). Moreover, changes in Holstein sperm DNA integrity were higher as compared to Norwegian Red bulls. The affinity of DNA fragmentation was different in different animals and DNA fragmentation even varied from sample to sample of the same individual (Cortes-Gutierrez *et al.*, 2008).

The present study revealed a decreasing trend in sperm motility, livability, absolute index of livability and sperm membrane integrity as the percentage of sperm with damaged DNA increased. Thus, significant negative correlations of sperm having damaged DNA were noted with sperm motility, livability and sperm membrane integrity. This is supported by the findings of Prinosilova *et al.* (2012), who observed a negative correlation between DNA fragmentation index and sperm motility, viability and morphology in the dog.

Long term storage of spermatozoa can also affect their fertilizing ability (Haugan *et al.*, 2007). Boe-Hansen *et al.* (2008) recorded decrease in litter size when DNA fragmentation values were more than 2.1%. In the present study, percentage of sperm with damaged DNA increased from 2.2% at 0 day to 3.8% at one year of storage, but the difference was non-significant. However, it increased (P<0.05) to 5.0 and 5.8% at two and three years of storage, respectively. DNA fragmentation has been shown to be negatively correlated with sperm viability (Cortes-Gutierrez *et al.*, 2008). However, the fertility of Nili-Ravi bull spermatozoa stored for variable durations could not be studied. Whether this high percentage of sperm with

damaged DNA would affect their fertilizing ability remains to be investigated.

In the present study, the method used to measure DNA integrity was quite subjective. It would have been better if the image analysis of stained sperm had been performed using suitable software.

Conclusion: The results of the present study show that post-thaw motility, livability and acrosome integrity of Nili-Ravi bull spermatozoa started decreasing at 6th month of storage, while sperm DNA integrity remained unaffected for one year of storage in liquid nitrogen. However, number of samples used for each storage duration was only five, which is too small to draw any conclusion. Moreover, fertility rates for semen samples stored for different durations in liquid nitrogen need to be investigated.

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