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Research Article

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Comparison on Pregnancy Rate of Frozen-thawed Bull Semen Processed by One and Two-Step Dilution Methods

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Abstract

The comparison of one and two-step dilution techniques of frozen semen was scrutinized based on post-thaw sperm quality and the fertility rate of cows after insemination in natural heat. The semen characteristics of two different pure breeds and two different crossbreed bulls, namely Harison (Friesian 100%), George (Friesian X), Bahadur (Sahiwal 100%) and Lalchand (Sahiwal X) were compared. Semen was diluted in a Tris-based extender with glycerol containing 20% egg yolk to obtain 20×10^6 spermatozoa per milliliter. In one step dilution, TRIS-egg yolk extender was added at 37°C, and in two steps dilution, TRIS-egg yolk extender with glycerol was added at 5°C. After two hours of equilibration with glycerol, all semen samples were frozen by box method placing the straws horizontally above the liquid N2 surface in a Styrofoam box. Sperm motility was assessed by using a CASA system before and after thawing. Results indicated that semen collected from individual bulls varies in quality and kinetic velocity but these variations were insignificant. In addition, the parameters of semen quality have been reduced after freezing and thawing but most of these variations were insignificant between dilution techniques. On the other hand, no significant variation was observed in pregnancy rates considering the bulls and dilution methods. So, the one-step dilution method could be a method of choice in the processing of semen during cryopreservation because of its easy operating technique than the two-step dilution method.

Keywords: Cryopreservation, dilution, kinetic velocity, semen.

INTRODUCTION

Artificial insemination (AI), a useful assisted reproductive biotechnology, has made possible effective use of best-breeding males (Januskauskas and Zilinskas, 2002). It is considered a significant vehicle to improve the existing reproductive performance of breeds by implementing cross-breeding. This impact could have not been possible without long-standing cryopreservation of bull semen. The success of AI depends on the maintenance of viability, motility, fertilizing capacity of spermatozoa and quality of frozen-thawed semen used for AI. Semen cryopreservation techniques have been in practice for the past 50 years and allow long-term storage of semen and hence its virtually unlimited availability (Sathe and Shipley, 2014). Cryopreservation of semen has become a valuable technique for facilitating extensive utilization of frozen semen from genetically superior male animals. The ability to produce good post-thawed semen quality, in turn, depends on many other factors which include the composition of the cryopreservation media used as semen extenders. Generally, semen extenders include a non-permeating cryoprotectant (milk or egg yolk), a penetrating cryoprotectant (glycerol, ethylene glycol, or dimethyl sulfoxide), a buffer (Tris), one or more sugars (glucose, lac-

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tose, raffinose, saccharose or trehalose), salts (sodium citrate, citric acid) and antibiotics (penicillin, streptomycin) (Evans and Maxwell, 1987). In bull and buck, as semen extenders dried skimmed milk or Tris-glucose-based hypertonic diluents are used frequently (Evans and Maxwell, 1987). The techniques for cryopreservation of spermatozoa have also slowly progressed over the past several decades (Hammerstedt *et al.*, 1990) and are now fairly standardized. During cryopreservation, cooling is a major stressor to the spermatozoa referred to as cold shock, is a phenomenon that causes decreased cell metabolism, altered membrane integrity, lower sperm viability and finally decreased sperm fertility (Watson, 2000). Extenders with cryoprotectant agent (CPA) viz. glycerol, egg yolk, milk, TRIS etc. have been used to provide some protection to spermatozoa and to minimize the adverse effects of cryopreservation (Katila, 1997). The success of cryopreservation depends not only on preserving the motility of the spermatozoa but also on maintaining their metabolic function (Watson, 2000). The success of cryopreservation depends upon many factors, these are types of extender, cooling rate, thawing rate, packaging, interactions between cryoprotectant and even the individual animal variation (Andrabi and Maxwell, 2007). In most cryopreservation protocols for bull spermatozoa the semen is diluted in one step, whereas in others, a two-step dilution is performed. In the single-step seminal cryopreservation, a sperm extender containing cryoprotectant is added to semen at 37°C, and the mixture is cooled to 4–5°C (Pena *et al.*, 1998). Conversely, the two-step cryopreservation is carried out with an initial extender without cryoprotectant until cooling to 4–5°C and then the cryoprotectant is added and the sample is further kept at 4–5°C to be glycerolized (Pena *et al.*, 1998). However, single-step freezing is advantageous due to less handling of the semen, reduced risks of accidents and contamination, easiness of work and a reduction of the time required for sperm cryopreservation (Godim *et al.*, 2009). The two-step method needs more handling, which increases the risk of contamination, accidental manipulation, and temperature oscillation. Many researchers are trying to develop a sustainable protocol for semen cryopreservation. However, the development of a reliable method to cryopreserve sperm is extremely important for the preservation of superior genes from valuable animals. However, the effects of doing the dilution in one or two steps have not been studied for bull spermatozoa in Bangladesh. Considering those facts this study was designed to investigate and compare the effects of one-step and two-step dilution with TRIS based extender on pre and post freezing progressive motility, plasma membrane integrity and kinetic velocity of bull spermatozoa after the cryopreservation of bull semen. We also investigated the pregnancy rates obtained in local crossbred cows inseminated with frozen-thawed semen after one step and two-step dilution with TRIS based extender.

MATERIALS AND METHODS

Selection and management of breeding bulls

Two adult pure-bred (Holstein Friesian 100%, Sahiwal 100%) and two adults crossbred (Holstein Friesian X, Sahiwal X) bulls were selected for the study. These bulls had normal physical and reproductive health with the age ranging from 24 to 36 months and body weight from 280-320kg. The animals were provided with a balanced ration to provide their daily requirements for energy, protein and micro-nutrients twice daily. The concentrate was supplied at a rate of 1kg/100 kg body weight per day. The animals were kept in individual pens and provided plenty of green fodder (20 kg/bull/day), freshwater was supplied ad libitum.

Preparation of Tris-based Semen Extender

The basic extender is TRIS fructose citric glycerol. Extender T was made of TRIS (2.42% w/v), citric acid (1.36%w/v), fructose (1%w/v), 20% (v/v) egg yolk, glycerol 7% (v/v), penicillin (100,000 IU) and streptomycin (100mg). Then added water up to the mark. For one-step dilution whole extender was kept at 37°C in the water bath until use. For two-step dilution, the base solution was divided into Part-T1 (without glycerol) and Part-T2 (with 14% glycerol). Extenders Part-T1 was kept at 37°C in a water bath whereas Part-T2 was kept at 4°C until use.

Collection of Semen

Semen was collected by the Artificial Vagina (AV) method with the help of a homosexual mount. All the apparatus used for semen collection was sterilized before collection. The inner liner temperature of AV was maintained at 42 – 43°C temperature by loading the two-third area of the jacket with water of 52 – 54°C temperature. The rest of one-third area of the water jacket was filled with air. The sterile non-spermicidal water-soluble lubricant was smeared into the inner side of the artificial vagina by a glass rod.

Process of semen collection

Before collection, the prepuce of the bull was wiped clean to prevent semen contamination. Donor bulls were allowed at least 1-2 false mounts before collection of each ejaculation. Semen was collected two times a week during the study period. Five ejaculates were collected from each bull during the total session of collection. The collection was always performed in the morning between 7- 8 AM. The collected semen was shifted immediately to a water bath at 37°C and evaluated.

Evaluation of collected semen

Macroscopic evaluation

The volume of semen was recorded by reading the graduated mark of the collection vial in a milliliter. The colour was observed by the naked eye in the collecting tube immediately after collection and was scored 1-5 (from worse to best quality). Only those sample scored 3 or more was accepted for further processing.

Microscopic evaluation

Mass activity: To evaluate the mass activity, a drop (0.5µl) of semen was placed on a slide (37°C) without any coverslip and examined under a microscope equipped with phase-contrast optics (100x). The mass activity was scored into 5 scales: Scale 1: no motion; Scale 2: free spermatozoa moving without forming any waves; Scale 3: small, slow-moving waves; Scale 4: vigorous movement with moderately rapid waves and eddies; Scale 5: dense, very rapidly moving waves and distinct eddies.

Progressive Motility: A drop of 0.5µl semen diluted with TRIS at 1:8 ratios was placed on a clean pre-warmed slide (+37°C) and covered with a coverslip. The motility was determined by eye-estimation of the proportion of spermatozoa moving progressively straight forward at higher magnification (400x) and expressed as a percentage.

The concentration of spermatozoa: The concentration of spermatozoa (million/ml) was determined by using a spectrophotometer. Semen samples were diluted with distilled water (1:400) to kill the spermatozoa. The photometer was set at zero by using normal saline water and then 20 µL semen samples were mixed in 2 ml of normal saline water. Concentration was measured with a photometer following the manufacturer's instruction.

Analysis of motility and kinetic velocity with CASA

The motility characteristics of fresh spermatozoa were analyzed by CASA after dilution with PBS (Phosphate buffer saline). Motility analysis was carried out in 5 µl of semen samples placed onto a pre-warmed (37°C) microscopic slide covered with an 18 mm × 18 mm coverslip. A minimum of 200 spermatozoa from one drop was analyzed for each sample. Various motion parameters of spermatozoa like motility, progressive motility, straight-line speed, curvilinear velocity, linearity, lateral head displacement and average path velocity, straightness, beat cross frequency were evaluated and recorded.

Plasma membrane integrity test by Hypo-osmotic swelling test (HOST)

HOST was used to evaluate the functional integrity of the sperm plasma membrane. This was performed by incubating 20µl semen with 200µl of a 75 mOsm/L hypo-osmotic solution (made of 13.5g fructose and 7.35g sodium citrate in 1000 ml distilled water) at 37°C for 60 min. After incubation, 5µl of the mixture was spread with a coverslip on a warm slide. A total of 200 sperms were evaluated in different microscopic fields at 40X objective. The percentage of spermatozoa with swollen and curled tails was recorded as HOST +ve cells.

Preservation of semen

One-step method: After collection and evaluation of bull semen, the ejaculates of each bull were diluted with a TRIS-based egg yolk extender and kept at 37°C in a water bath. Diluted semen samples were drawn into 0.25 ml French straws (Minitub, Germany), sealed with an automatic filling and sealing machine (SFN-1, Fujihira Industry Co. Ltd., Japan). White colour straws were used for TRIS based one-step method. The sealed straws were placed in the refrigerator at +4°C for further equilibration for 2 hours. After equilibration, Liquid nitrogen was poured in a special box where a rack was placed in the box keeping a gap of 5-6 cm above the surface of the liquid nitrogen and kept for 30 minutes to create vapour and to the stable bubbling of liquid nitrogen. Then the straws were frozen in liquid nitrogen vapours (temperature –80°C) in a special box for 5-6 minutes. After that straws were transferred into cryocan at –196°C.

Two steps method: A two-step dilution method was used to freeze the semen. After collection and proper

evaluation, the pooled sample was diluted with extenders Part T1. For cryopreservation, diluted semen and extender Part T2 were transferred to the refrigerator for two hours. After the designated time interval, the calculated amount of extenders Part T2 poured into the previously diluted semen in three divided parts. Then the semen was filled into the straws using micropipette. Green color straws were used for TRIS based two-step method. After filling, the straws were sealed with an automatic filling and sealing machine (SFN-1, Fujihira Industry Co. Ltd., Japan). The sealed straws were placed in the refrigerator at +4°C for further equilibration for 2 hours. Liquid nitrogen was poured in a special box where a rack was placed in the box keeping a gap of 5-6 cm above the surface of the liquid nitrogen and kept for 30 minutes to create vapour and to the stable bubbling of liquid nitrogen. The freezing was done in liquid nitrogen vapour (temperature –80°C) in a special box for 5-6 minutes. After that straws were transferred into cryocan at –196°C.

Evaluation of frozen-thawed semen

Semen was thawed by plunging the frozen straws in a water bath at 38° – 40°C for 10-12 seconds Salamon and Maxwell (2000). To study the preservation effects randomly selected straws were taken from different groups. Semen was thawed at 37°C for 15 seconds. Thawed semen straws were evaluated to observe motility and CASA parameters following the methods described earlier.

Artificial Insemination

AI was done to 206 local and crossbred cows with the frozen-thawed semen by the recto-vaginal method.

Pregnancy diagnosis

All inseminated cows were monitored for non-return to estrus 17-21 days following insemination. The cows which were in non-return to estrous were allowed for Transrectal ultrasonography (MUIV ultrasonography, Bionet®, Korea) using linear transducer frequency 7 MHz within 45-50 days of post insemination.

Statistical analysis

Independent samples T-test was used to compare pre and post freezing semen quality. A Chi-square test was also done to find out significant differences in the pregnancy rate of cows. All the statistical analyses were done using SPSS 20.0. The differences between the groups were regarded as significant or highly significant when the P-value was less than 0.05 ($P < 0.05$) or less than 0.01 ($P < 0.01$), respectively.

Table 1: General characteristics (Mean ± SME) of fresh bull's semen used in this study.

Parameters	**Friesian 100%**	**Friesian X**	**Sahiwal 100%**	**Sahiwal X**
Colour	Milky white	Creamy white	Milky to creamy white	Creamy white
Volume (ml)	7.40 ± 0.29	6.70 ± 0.03	6.80 ± 0.51	6.90 ± 0.69
Mass activity (0-5 scale)	4.00 ± 0.44	3.80 ± 0.37	3.40 ± 0.50	3.60 ± 0.24
Concentration (X per ml)	1321.75 ± 91.34	1832.84 ± 351.68	1316.7 ± 444.2	1656.0 ± 357.9
Total motility (%)	83.64 ± 2.61	68.84 ± 6.62	72.24 ± 7.84	73.62 ± 4.32
Plasma membrane functional integrity (%)	69.40 ± 3.03	69.40 ± 3.03	58.20 ± 5.10	57.70 ± 4.60
Bent tail (%)	1.66 ± 0.59^{b}	2.34 ± 0.69^{b}	4.90 ± 0.95^{a}	1.96 ± 0.60^{b}
Coiled tail (%)	0.34 ± 0.13	0.48 ± 0.30	1.24 ± 0.37	0.36 ± 0.16
Proximal droplet (%)	2.66 ± 0.80^{b}	5.90 ± 0.94^{a}	2.16 ± 0.30^{b}	2.72 ± 0.50^{b}
Distal droplet (%)	1.70 ± 0.42	3.18 ± 0.45	3.06 ± 0.38	2.30 ± 0.66

Within a single row, values with different superscripts (a, b) differ significantly among the bulls.

RESULTS

The present study focused on the effect of the one and two-step dilution techniques on post-thaw sperm quality and the fertility rate of cows after insemination in natural heat. All the characteristics of freshly collected semen volume, mass activity, total motility and

Table 2: Kinetic parameters (Mean ± SME) of fresh bull's semen used in this study.

Parameters	**Friesian 100%**	**Friesian X**	**Sahiwal 100%**	**Sahiwal X**
Progressive motility (%)	66.46 ± 3.49	56.82 ± 5.34	57.70 ± 6.86	53.30 ± 5.52
ALH (μm)	9.83 ± 1.26	8.72 ± 0.70	10.48 ± 0.68	11.27 ± 0.52
BCF (Hz)	33.93 ± 2.22	33.37 ± 1.53	32.10 ± 2.27	25.79 ± 3.06
LIN (%)	50.46 ± 4.27	46.17 ± 2.89	44.14 ± 5.52	36.51 ± 1.86
STR (%)	85.52 ± 3.27	82.95 ± 2.21	76.30 ± 6.48	72.72 ± 2.66
VAP (μm/s)	141.76 ± 14.04	110.65 ± 4.49	129.70 ± 11.89	113.55 ± 11.45
VCL (μm/s)	251.71 ± 31.63	210.09 ± 12.00	235.03 ± 14.91	233.02 ± 23.44
VSL (μm/s)	120.73 ± 9.93	92.12 ± 2.13	103.56 ± 15.00	82.15 ± 8.55

Table 3: Comparison of Pregnancy rates of frozen-thawed bull semen diluted by one and two steps methods.

Freezing Techniques	**Pregnancy of different bulls**				
	Friesian 100% (n=62)	**Friesian x (n=52)**	**Sahiwal 100% (n=36)**	**Sahiwal X (n=56)**	**P-Value**
One-step (n=103)	71.8% (28/39)	76.9% (10/13)	68.4% (13/19)	68.8% (22/32)	0.94
Two-step (n=103)	73.9% (17/23)	66.7% (26/39)	82.4% (14/17)	75% (18/24)	0.66
P-Value	0.85	0.48	0.35	0.60	

plasma membrane functional integrity were found the highest in Friesian 100% than other bulls, except sperm concentration, which was highest in Friesian X. There was no significant difference among semen samples collected from the four bulls except morphological changes of semen (bent tail% and proximal droplet %). Significantly higher bent tail % and proximal droplet % (5.90 ± 0.94) were recorded (4.90 ± 0.95) in Sahiwal X and Friesian X respectively, in this study (Table 1).

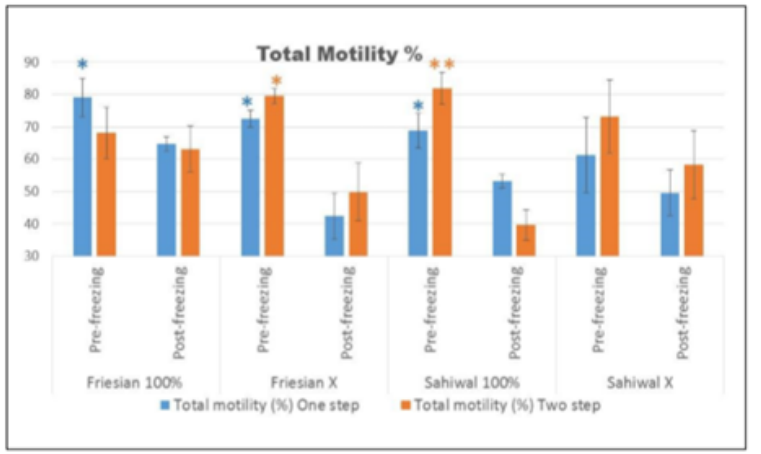
The highest value of progressive motility was observed in Friesian 100% (66.46 ± 3.49) compared to the value of Friesian X, Sahiwal 100% and Sahiwal X semen. But there were no significant differences in progressive motility among the four bulls. The highest values of BCF (33.93 ± 2.22), LIN (50.46 ± 4.27), STR (85.52 ± 3.27), VAP (141.76 ± 14.04), VCL (251.71 ± 31.63) and VSL (120.73 ± 9.93) were observed in Friesian 100%, whereas, the highest value of ALH (11.27 ± 0.52) was observed in Sahiwal X. But there were no significant differences in kinetic parameters among the four bulls (Table 2).

When we considered the one-step dilution effect on semen quality during pre and post freezing evaluation of semen, we found significant variations in total motility % and progressive motility % of Frisian 100%, Frisian x, Sahiwal 100%; ALH of Frisian 100% and Frisian cross; STR of Frisian 100%; VAP ($P < 0.01$) and VSL of Frisian 100% and Frisian x and BCF in Sahiwal X. In case of two-step dilution, significant variations were observed in total motility % of Frisian X and Sahiwal 100%; progressive motility% of Frisian X; VAP, VCL and VSL of Sahiwal X. (Figure 1).

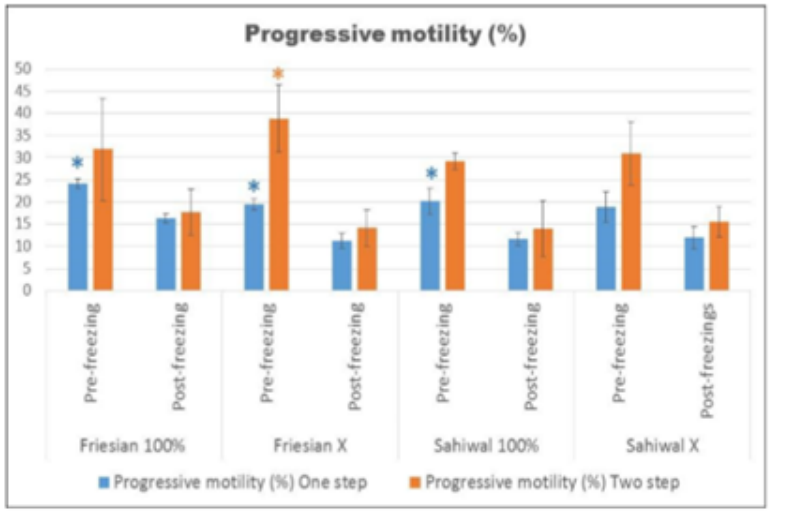
Though no significant variation was observed in other quality parameters during pre and post freezing evaluation of semen. In one step dilution method, pregnancy rates were the highest (76.9%) when Friesian X Bull semen was used. In Two step dilution methods, the pregnancy rate was the highest (82.4%) in Sahiwal 100%. We did not find any significant differences in pregnancy rate in regard to the bull and dilution steps (Table 3).

DISCUSSION

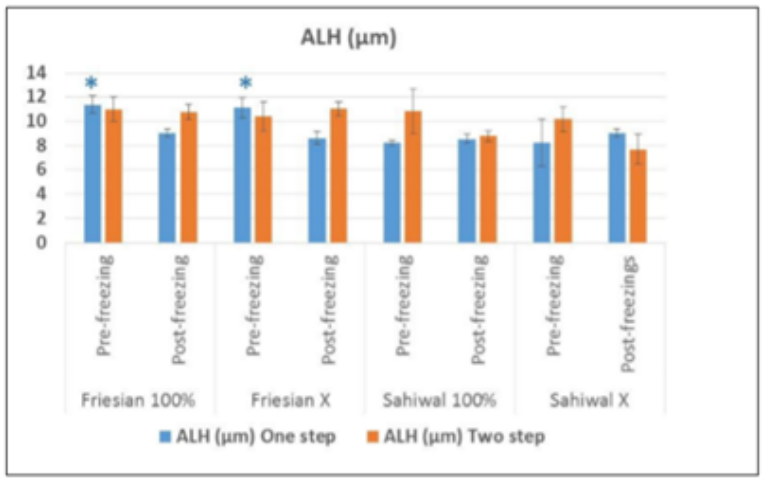
A complete understanding of sperm physiology during cryopreservation is mandatory to ensure maximum success in AI. During sperm cryopreservation, the addition of a cryoprotectant is an essential step for the feasibility of the process. Therefore, the moment in which the cryoprotectant is added during the cryopreservation process is the main difference between the one-step cryopreservation protocol and the two-steps


Total Motility %
90
80
70
60
50
40
30
Pre-freezing
Post-freezing
Friesian 100%
Friesian X
Sahiwal 100%
Sahiwal X
Total motility (%) One step
Total motility (%) Two step

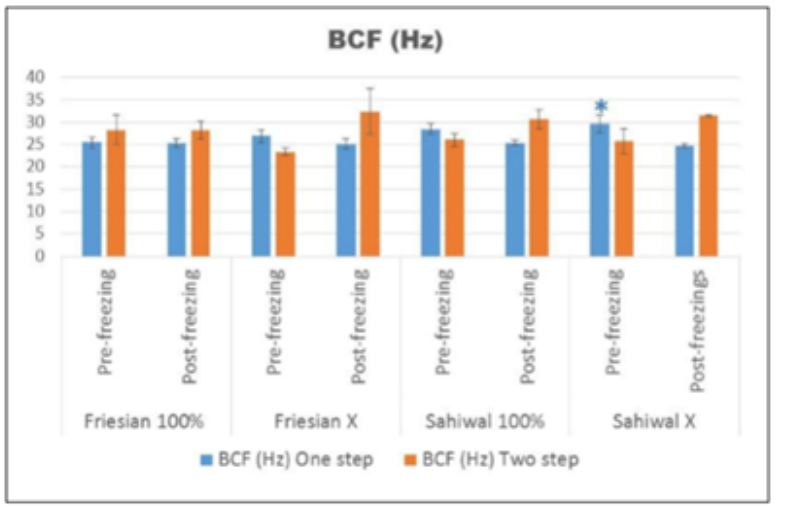
(a)


Progressive motility (%)
50
45
40
35
30
25
20
15
10
5
0
Pre-freezing
Post-freezing
Post-freezings
Friesian 100%
Friesian X
Sahiwal 100%
Sahiwal X
Progressive motility (%) One step
Progressive motility (%) Two step

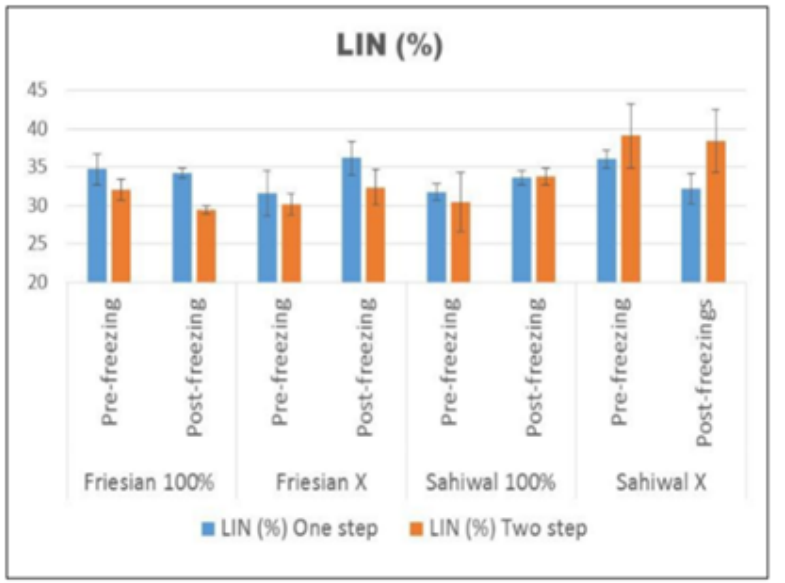
(b)


ALH (µm)
14
12
10
8
6
4
2
0
Pre-freezing
Post-freezing
Post-freezings
Friesian 100%
Friesian X
Sahiwal 100%
Sahiwal X
ALH (µm) One step
ALH (µm) Two step

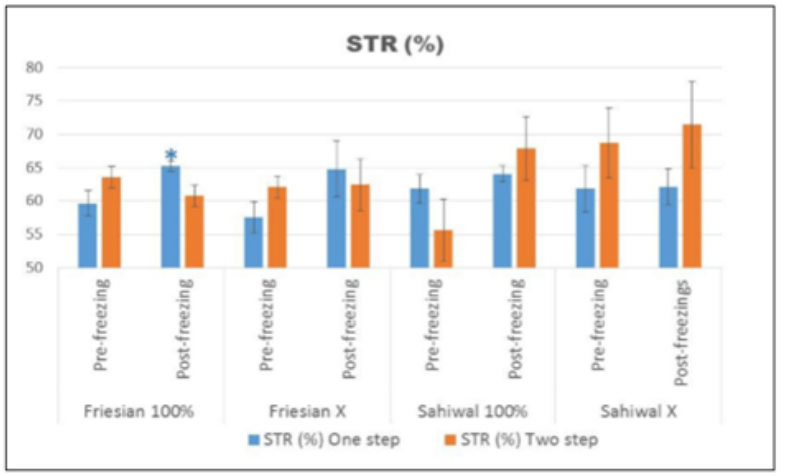
(c)


BCF (Hz)
40
35
30
25
20
15
10
5
0
Pre-freezing
Post-freezing
Post-freezings
Friesian 100%
Friesian X
Sahiwal 100%
Sahiwal X
BCF (Hz) One step
BCF (Hz) Two step

(d)



(e)



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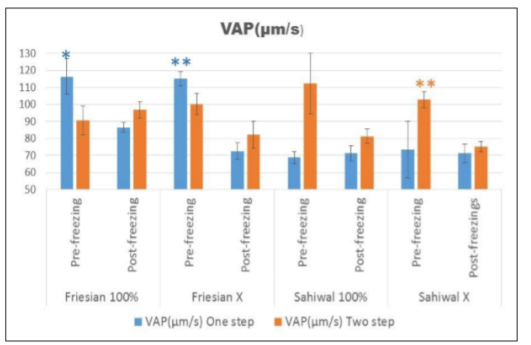

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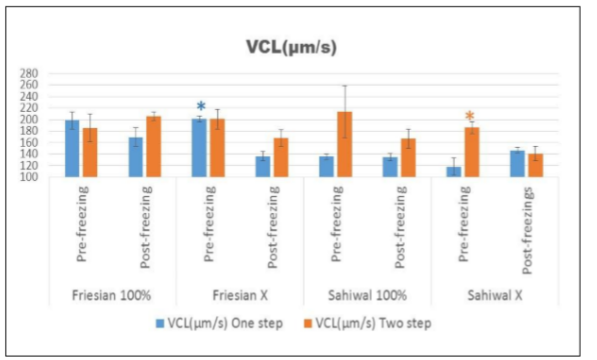
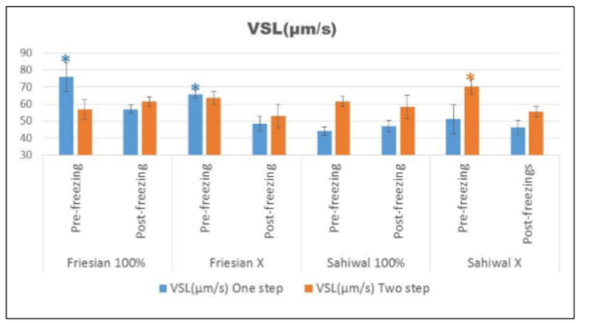


(h)



(i)

Figure 1: Comparison of Kinetic parameters of pre-freezing and post-freezing bull semen Processed by one and two steps dilution method. (a) Total motility (%) (b) Progressive motility (%) (c) ALH (µm) (d) BCF (Hz) (e) LIN (%) (f) STR (%) (g) VAP (µm/s (h) VCL (µm/s) and (i) VSL (µm/s. ** indicates highly significant ($P < 0.01$) differences and * indicates significant ($P < 0.05$) differences between pre-freezing and post freezing kinetic parameters of bull semen within the dilution group.

freezing protocol (Pena *et al.*, 1998). Therefore, we investigated the efficacy of one and two-step dilution effects on the pre and post freezing semen quality and fertility. We compared the fresh semen characteristics in four bulls. It is reported that volume per ejaculates, motility, sperm concentration and the percentage of normal sperm are common criteria for evaluating semen quality. All the characteristics of freshly collected semen volume, mass activity and total motility and plasma membrane functional integrity were found the highest in Friesian 100% in the study. The values o of volume, concentrations, motility % and progressive motility were in range as described by Pradeep *et al.* (2013). Variation due to individual bull, size of testes, age of bull, frequency of collection, feeding, condition and health of bull. The concentration of spermatozoa reflects the status of testicular function and hormonal inter-relationship. The average concentration of spermatozoa (×106/ml) in bull to be 1455 ± 56.21 (Pathak *et al.*, 1990) and 1047.45 ± 83.88 (Ulfina and Raina, 2002). The mean concentration (X106 per ml) ranged from 1832.84 ± 351.68 to 1316.74 ± 444.24 in the present study, which is relevant to the findings of Hafez (1993).

In the case of Friesian X semen volume was higher but the sperm concentration was lower than those reported by Munsi *et al.* (2007) who have stated that Volume (ml), concentration (x106/ml), mass activity, motility (%) were 4.5 ± 0.4, 1410.0 ± 70.0, 3.8 ± 0.3, 77.5 ± 1.5, 93.5 ± 0.5. respectively in fresh semen of HF Crossbred bull Whereas, ejaculate volume (ml), sperm motility (%), concentration (X106/ml), total abnormality (%), total viability, HOST and pH were 5.03 ± 0.2, 83.5 ± 2.4, 1480.0 ± 70.0, 5.40 ± 0.6, 82.30 ± 2.8, 83.80 ± 3.3 and 6.59 ± 0.0, respectively in fresh semen of bull (Uysal *et al.*, 2007). Generally, *Bos indicus* bulls have a higher sperm concentration and higher sperm morphologic defects than Bos taurus (Brito *et al.*, 2002). In this study, the mean mass activity was 3.37 ± 0.48, which goes with the finding of Pal and Chatterjee (2006). ALH (μm), BCF (Hz), LIN (%) and STR (%) were more or less similar to the findings of Matas *et al.* (2011). VAP (μm/sec) VCL (μm/s) and VSL (μm/s) in fresh semen were higher than those described by Matas *et al.* (2011).

In this study, we observed significant variation in total sperm motility % and progressive motility % before and after freezing in all bulls. Zou and Yang (2000) reported that pre-freezing semen quality parameters, such as sperm motility and the abstinence period of sperm donors, can also affect the cryosurvival rate of post-thaw sperm. Spermatozoa with abnormal motility traits (e.g., asthenozoospermic, oligoasthenozoospermic) are particularly susceptible to cryo-damage, possibly reducing their fertilizing ability. No significant variation was observed in post-thaw semen quality and pregnancy rate in the study. These findings are in agreement with the statement of Salamon and Maxwell (2000), who reported that a single step addition of glycerol to the semen has similar effects as effective as a two steps dilution. The use of glycerol to preserve spermatozoa during freezing is most widespread. Glycerol acts as a synergistic protective effect with egg yolk in extenders during cooling and freezing. Most workers preferred a $4-5^{\circ}$C temperature for adding glycerol containing diluents to the semen (Colas and Reprod, 1975). The two-step freezing presents, confirmedly, minimal toxicity by adding glycerol to sperm with reduced metabolism (Pena *et al.*, 1998). In this study, 20×10^6 spermatozoa were used for AI and 66.7% to 82.4% pregnancy rates were observed. Watson (2000) stated that good fertility rates are obtained with sperm doses containing approximately 20 million spermatozoa, making AI commercially viable in cattle. In this study, individual male variation was observed in semen quality and kinetic velocity, though this variation is insignificant. Values of pre-freezing semen quality parameters were reduced after freezing and thawing, though variations were insignificant in most of the semen parameters of one and two steps dilution groups. No significant variation was observed in pregnancy rates considering the bulls and dilution method. So, one step dilution method could be used in the processing of semen with Tris - based extender during cryopreservation. Although cryopreserved spermatozoa have been effectively used to assist reproduction for decades, it is recommended that long-term follow-up studies be performed on offspring obtained from cryopreserved spermatozoa in future generations to fully assess their biological safety.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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