# Determination Of The Fertility Rate Of Frozen Thawed Kundhi Buffalo Bull Semen With Skimmed Milk And Tris Based Semen Extenders

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**Abstract**
The present study explored the fertility rate of frozen-thawed kundhi buffalo bull semen with skimmed milk and tris based extenders. Total four healthy khundi buffalo bulls of 3 to 4 years of age were selected. After collection the semen was examined for volume, colour, pH, wave motion, motility, Sperm concentration, morphology, viability and membrane integrity.After initial examination semen sample having motility, morphology and viability ≥ 75 % were diluted in Tris based extender consist of 6, 8, 10, 12, and 14gm concentration of skim milk. After dilatation Post chilled semen characteristics result showed Motility was (58.55±5.47), morphology (66.75±3.30), membrane integrity (66.75±1.25) and viability (58.25±6.4). Frozen thawed result showed motility (54.75±4.03), morphology (64.75±2.50), membrane integrity (62.25±3.86) and viability (64.00±4.47). Skim milk treatment group conception rate is significantly higher 40% than tris base extender 20%.Overall, skim milk added groups showed improved frozen thawed quality of semen than control. However highest values were obtained in 10gm concentration of skim milk added in extender.

**Key words:** Fertility rate, Frozen Thawed Semen, Skimmed Milk, Tris Based Extenders, Kundi Buffalo

## INTRODUCTION

Buffalo is the black gold and has been domesticated from pre-historic times(Khan *et al.,* 2007). Buffalo is well-known for their habit of wallowing in water, during the hot hours of the day; hence they are also denoted as water buffalo. The domestic buffalo has been mostly classified on the basis of habitat as river and swamp types. The buffalo can be described as a semi-aquatic mammalthis characteristic is so noticeable. The riverine buffalo favors to immerse itself in running water or ponds. The swamp buffalo likes immobile water or mud (Marai and Haeeb, 2010).The world population of buffalo is increasing in 2007. It was estimated to be over 180.70 million (FAO, 2008). Buffalo statistics have increased by 50 million,Since 1981/2 (Drost, 2007; Andrabi, 2009). Buffalo rearing and breeding is growing in numerous countries where these animal previously neglected – and even unknown. More than 97% of the population isfound in Asia.It's a main foundation of power for farm operation and transport but it alsoIt provides meat, milk and dung essential for soil fertility and fuel for farm house holds. Buffalo is well-thought-out to be four times more productive than the average cow (Khan *et al.,* 2007). In recent timesbuffalo farming is extended widely to theMediterranean areas and Latin America and Australia for the specific dairy products such as Mozzarella cheese.Buffalo are also raised for meat in non-traditional countries because of the good nutritional value of the meat, which contains less saturated fat than beef and pork.According to the latest Economic Survey of Pakistan the buffalo population is 36.8 million. The Average meat and milk production is 33,137 tons and 2,017 tons annually in the country (Misra *et al.* 2013).Pakistan is homeland of Nilli- Ravi and Kundhi breeds of buffalo and are popular for their high milk production. In Sindh province of Pakistan Amongst dairy breeds kundhi is the best dairy buffalo breed of the world and good source of milk production. It is originate inHyderabad, Karachi,Dadu Larkana, Nawabshah, Sanghar and Thatta

districts. The horns are spirally twisted and small. The udder is strong, large and the production is good. Milk yield per lactation is 1700-2200 litres with over 6.5% butter fat (Afridi *et al*., 2013).

For enhancement of high milk yield in buffaloes by the propagation of animals with better genetic potentialArtificial insemination (AI) is advance technology. Only one ejaculate can give us sufficient AI doses to increase the genetic potential of a herd (Atiq *et al*., 2011; Ciptadi *et al*., 2012). It is a vital tool for genetic improvement of dairy animal and have beenwell-thought-out the very first useful reproductive technology (Kaka *et al*., 2012). Artificial insemination (AI) and semen cryopreservation offer many benefits to the livestock industry, particularly in conjunction with genetic assessment and selection programs (Mahmoud et *al*., 2013). The stress produced by transportation for mating and the risk of disease transmission during copulation was protects by Artificial insemination in addition to supporting the protection of high-value genetic material (Silva *et al*., 2000). Extenders used for spermatozoa cryopreservation nowadays are of two types, one which are commercially

extenders and other are laboratory. Commercially extenders aretriladyl®, bovidyl®,bullxcell®,romed® and BioXcell®. The other are prepared in laboratory such as Tris, egg yolk,citrate extenders and skim milk. Extenders composition, cooling, freezing and thawing processes are important factor affecting buffalo semen freezability and fertility.It also function as sub-physiologic temperatures,membrane stabilizers, energy for sperm metabolism,balancer for ionand buffering of pH. The glycerol to extenders addition has been effective for cryopreservation of various mammalian species sperm, and sugars are added (such as, sucrose,trehalose andraffinose,) to take out intracellular water byproviding cryoprotection and increasing osmotic pressure.(Varisli *et al*, 2009).Extender Tris-based are commonly used for semen preservation in most farm animalsincluding buffalo research (El-Sheshtawy *et al.,* 2015). Extender Skim milk has been known as an appropriate storage medium in liquid state for buffalo bull spermatozoa because of its higher ability to preserve semen quality, availability and economic suitability (Akhter, 2006).

The earlier studies have been reported on Tris based semen extenders. However, the current studies have been reported on to determine the fertility rate of frozen-thawed Kundhi Buffalo bull semen with skimmed milk based and Tris extenders. Therefore, hypothesis for the present study is that addition of skim milk extender would improve the fertility rate of frozen-thawed Kundhi Buffalo bull semen with following specific object.

## MATERIAL AND METHOD

### *Study site*

The proposed study was conducted to investigate the fertility rate of frozen thawed semen of kundhi buffalo bull using skimmed milk and Tris based extenders at the Department of Animal Reproduction, Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agriculture University, Tando Jam.

### Experimental animal and their manage

In present study Four Kundhi Buffalo bulls of 3 to 4 years of age were used. The bulls were housed individualy. During study period Seasonal green fodder, Wheat straw, cotton seed cake, wheat barn and clean water was provide. Regular vaccinationtaken according to the standard protocols against seasonal Disease.

### Preparation of bull for semen collection

The bulls were cleaned properly. The preputial hairs were clipped and preputial area was wash properly before semen collection. The back and rear quarters of the teaser animals were washed properly. The cleaning of donor bulls and teaser animal were followed on every day before semen collection.

### Preparation of artificial vagina

The artificial vagina was washed and sterilized properly. The temperature of artificial vagina was maintained by filling with warm water at 42-45$^0$C, air at the pressure of 35 mmg and inner surface of inner liner will be lubricated with petroleum jelly (Sarsaifi *et al.,* 2013).

### Semen collection

Twice in a week semen was collected early in the morning for 8 weeks. Immediately after collection, the semen sample were moved into laboratory and kept in water bath at 37$^0$C. Initially semen was evaluated for volume, colour, pH, Wave motion, motility, sperm concentration, morphology, viability and membrane integrity with following methodology.

### Volume:

Volume was recorded directly from garduated collection tube per ejuculate.

### Colour

Semen colour was evaluated by visual examination direct from the collected tube and was categorized as white, milky, creamy, yellowish, watery and translucent as described by Hafez and Hafez (2000).

### pH

pH of the semen was determined by using digital pH meter.

**Wave motion**

The wave motion was evaluated in a drop of fresh semen without using cover slip under low magnification 10x with phase contrast microscope. Wave patterns of the semen sample were recorded and graded the observations as following (Sarsaifi *et al.,* 2013). Result was expressed in percentage (%).

- Mass activity nil = 0
- Slow wave motion = +
- Rapid wave motion = ++
- Eddies = +++(Sarsaifi *et al.,* 2013).

**Motility**

Motility was assessed by standard subjective ranking method. A drop of diluted semen was placed with the help of glass rod. Similarly, a drop the sodium citrate was placed near to drop fresh semen and gently was mixed. Cover slip was placed over it and observed under low magnification (20x) of phase contrast microscope. From the selected field microscopic area, At least 10 spermatozoa was selected randomly where the percent motile germ cells were observed in straight forward progressive movement. However, sperms moving in circles or in backward direction were excluded from count. The results were expressed in motility percentage. Motility percentage were recorded on warm stage maintaining the temperature about $37^0$C. The sample having 80% and above motility were used for freezing and further investigation (Hafez, 2002).

**Sperm concentration:**

Concentration of sperm was assessed by preparing dilution of sample in normal saline and counting total sperms by hemocytometer (Hafez, 2002).

**Fixing solution**

Sodium chloride 3% 30, Formaldehyde 37% 4ml, Distilled wat 1000ml

A 9.99 ml of fixing solution was added in glass test tube. Added 0.01ml of diluted semen into it and allowed for fixation for 5 minutes. A small drop of suspension was dropped on both chamber of hemocytometer. A cover slide was placed in the center of chambers that covers both chambers and observed under light microscope at 4x gradually increased up to 40x magnification. Sperms were counted in central chamber of nine large squares. Total 5 squares of central chamber were counted out of 25 small chambers that were one middle and four corner squares. Following formula was used to determine the cell count.

- Sperms (ml) = n x 5 x df x 10000
- No of sperm counted =N
- No of chambers counted on hemocytometer = 5
- Dilution factor that equal to 100 = df

**Morphological characteristics:**

Sperm morphology will be determined in a smear of Eosin nigrosin (India ink) staining technique.
The staining solution was prepared as follow

- Eosin 0.67g
- Nigrosine 5.0g
- Distilled/water 100ml

A drop of fresh semen was mixed with eight drops of stain solution. After standard incubation time (3 minutes) at 37 $^0$C, a thin smear was made on pre-warmed slide and allowed to dry at $30^0$C. The excess stain was washed off in running tap water. The slide was then immersed in Ethanol to remove water. The dried film was examined using the oil immersion lens of light microscope. 100 sperms were counted and scored as normal and abnormal (Memon *et al.,* 2012, kaka *et al.,* 2015). Result was expressed in percentage.

**Membrane integrity:**

The membrane integrity of sperm was evaluated by using ORT method of assessment (Kaka *et al*., 2015:2012). The test solution for fresh semen was prepared as follows.

- Fructose 13.51g
- Tri-sodium-citrate 7.35g
- Distilled water 1000ml

Total 100 ul semen was added into 1ml of a hypo-osmotic solution. After incubation for 60 min at $37^0$C, the sperm swelling was assessed by placing 15ul of well mixed sample on a warm slide ($37^0$C). And covered with a coverslip and observed under light microscope at 40x magnification. The spermatozoa were swell in the response of test solution and cells having intact membrane were considered as normal fertile cells. One hundred spermatozoa per slide were counted and expressed in percentage.

**Extension of semen**

**Preparation of diluents**.

Tris based buffer system was prepared and used as under (Rasul *et al*., 2000; Kaka *et al*., 2012).

**Tris extender**

- Tris (hydroxymethyl-amino-methane) 3.81gm
- Citric acid 1.97gm
- D (-) fructose 1.25g
- Egg yolk 20ml
- Glycerol 7ml
- Penicillin 1000 i.u
- Streptomycin 1.00gm
- Water 100ml

Skimmed milk buffer system was prepared and used as under. (Akhter *et al*., 2011)

**Skimmed milk extender**

- Skimmed milk 6, 8, 10, 12,14gm
- D (-) fructose 1.25g
- Glycerol 7ml
- Penicillin 1000 i.u
- Streptomycin 1.00gm
- Water 100ml

**Equilibration**

After cooling, the duration of equilibrium period was 4 hour for 5$^{o}$C.

**Filling and sealing of straws**

The French straws of 0.25ml of three different colors corresponding to each Group. The filling of straws was carried out with the help of filling machine. Sealing of open ends of straws was closed manually with polyvinyl chloride powder (PVC).

**Freezing of semen**

The straws were freezed in Liquid Nitrogen vapors for 10 minutes (-40$^{o}$c). After that, the semen straws were immersed in liquid nitrogen at temperature -196$^{o}$C till usage.

**Thawing of semen**

Thawing of frozen samples was carried out after 24 hours post freezing by emerging the straws in a water bath at 37 ℃ for fifteen seconds. The thawed sample were subjected to the post thawing motility, morphology, and membrane integrity using ORT method of assessment (Kaka *et al.*, 2015: 2012).

**Conception Rate**

The conception rate was determined after inseminating 20 females' buffaloes of group having best frozen-thawed semen quality and pregnancy was confirmed by cessation of estrus and through pregnancy diagnosis by rectal palpation.

**STATISTICAL ANALYSIS**

Collected data on semen characteristics was subsequently subjected to a one way analysis of variance (ANOVA) and LSD using Statistics (2006).

**RESULTS**

**Fresh semen evaluation**

The mean (±SE) volume, color, pH, motility of each bull is presented in Table I. The significant differences (P< 0.05) were observed among all bulls 1, 2, 3and 4. Significantly lower volume was recorded in bull 3 and higher volume was recorded in the bull 1. The colour of semen varies within ejaculated from milky to translucent. In current study, 1, 2 and 4 bulls produce creamy white semen but no. 3 bulls produce milky and translucent semen colour. It was thick in consistency and the appearance of sample was very clear .The pH of each bull has significant differences (P< 0.05) were observed among all bulls 1, 2, 3 and 4 respectively. The highest pH value was observed in bull 2. Similarly, low value of pH was recorded in bull 3. The motility percentage of semen of each bull has significant differences (P< 0.05) were observed among all bulls 1, 2, 3 and 4 respectively. Individual percentage of bull 1 (86.00 ±0.91%) was significantly different among all bulls.

The normal fresh sperm exhibit swirling movement under microscope field called wave motion. It was found to be +++ in all the bulls. The sperms was making rapid swirling motion forming eddies at the end of each motion in Table-1.

**Table I. Mean (±SE) values of semen colour of Kundhi Buffalo Bull**

| Bull | Volume | Colour | pH | Motility | Wave motion |
|---|---|---|---|---|---|
| 1 | 6.00 ±0.29[a] | Creamy white | 5.90 ±0.94[a] | 86.00±0.91[a] | ++++ |
| 2 | 5.58 ±0.28[a] | Creamy white | 5.95 ±0.26[a] | 79.25 ±1.75[b] | +++ |
| 3 | 3.43 ±0.22[c] | Milky/translucent | 5.70 ±0.13[a] | 64.50±1.04[c] | ++ |
| 4 | 4.40 ±0.24[b] | Creamy white | 5.75 ±0.28[a] | 76.75±1.89[b] | ++± |

[a,b,c,d] superscripts within rows are significant difference $p < 0.05$.

The mean (±SE) sperm concentration, morphology, viability, membrane integrity of each bull is presented in Table. II. The significant differences (P< 0.05) were observed among all bulls 1, 2, 3 and 4, respectively. Statistically analyses showed that significantly low sperm concentration (2047.3 ±45.42) was recorded in bull 3 and higher (2679.75 ±58.77) sperm concentration was recorded in bull 1.The sperm morphology of each bull has significant differences (P< 0.05) were observed among all bulls 1, 2, 3 and 4, respectively. Statistically, the values of sperm morphology significantly low (63.75 ±1.31%) was recorded in bull 3 and better sperm morphology (83.25 ±1.11%) was recorded in bull 1.The viability sperm of each bull has significant differences (P< 0.05) were observed among all bulls 1, 2, 3 and 4, respectively. The significantly better (86.00 ±0.91) viable sperm rate was found in bull 1.
The membrane integrity of each bull has significant differences (P< 0.05) were observed among all bulls 1, 2, 3 and 4, respectively. The better membrane integrity (77.00 ±1.29) was found in bull 1.

**Table II. Mean (±SE) values of fresh semen of Kundhi Buffalo Bull**

| Bull | Concentration | Morphology (%) | Viability (%) | Membrane integrity |
|---|---|---|---|---|
| 1 | 2,679.75 ±58.77[a] | 83.25 ±1.11[a] | 86.00 ±0.91[a] | 77.00 ±1.29[a] |
| 2 | 2,380.75 ±18.17[b] | 79.25 ±1.11[b] | 79.25 ±1.75[b] | 74.00 ±1.68[a] |
| 3 | 2,047.50 ±45.42[c] | 63.75 ±1.31[d] | 64.50 ±1.04[c] | 63.25 ±0.85[b] |
| 4 | 2,457.50± 55.00[b] | 73.25 ±1.11[c] | 76.75 ±1.24[b] | 74.25 ±1.44[a] |

[a,b,c,d] superscripts within rows differ significantly $p < 0.05$.

**Post Chilling Assessment**

The mean (± SE) motility, morphology, membrane integrity, viability of post chilling semen in each group is presented in Table III. The significant differences (P< 0.05) were observed among treatment experimental animals A, B, E but C and D are non-significant. Motility percentage characteristic increased from control to treatment groups A, B, C and decreased in D and E. However, highest values were observed in group C (10gm).
The morphological characteristic of post chilling semen in each group have significant differences (P< 0.05) were observed among treatment groups A, B, C, D and E, respectively. Morphological characteristic increased from control to treatment groups A, B, C and decreased in D and E, respectively .However, highest values were observed in group C (10gm) as compared with other groups. The membrane integrity of each bull has significant difference (P< 0.05) was observed among treatments groups A, B, C, D and E. Membrane integrity characteristic increased from control to treatment groups A, B, C and decreased in D and E. however, highest values were observed in group C (10gm).
The sperm viability percentage of each bull has significant difference (P< 0.05) was observed among treatments groups A, B, C, D and E. Viable percentage increased from control to treatment groups A, B, C and decreased in D and E. however, highest values were observed in group C (10gm).

**Table III. Comparison effect of Tris and skim milk based extenders on post chilled semen characteristics (Mean % ± SEM)**

| Sperm Parameters | Tris (control group) | Skim milk | | | | |
|---|---|---|---|---|---|---|
| | | 6gm | 8gm | 10gm | 12gm | 14gm |
| Motility | 40.55±1.32[c] | 43.75±5.67[b] | 45.00±6.4[b] | 58.55±5.47[a] | 58.00±2.25[a] | 56.55±6.54[a] |
| Morphology | 49.00±4.12[b] | 51.75±1.41[a] | 57.25±4.20[a] | 66.75±3.30[a] | 61.75±4.10[a] | 60.00±4.93[a] |
| Membrane integrity | 54.75±2.75[a] | 54.25±0.55[a] | 56.25±4.50[a] | 66.75±1.25[a] | 65.50±4.72[a] | 61.50±3.00[a] |
| Viability | 56.00±2.44[a] | 53.75±6.07[a] | 56.25±6.4[a] | 58.25±6.4[a] | 57.00±3.75[a] | 56.05±3.25[a] |

[a,b,c,d] superscripts within rows demonstrate significant difference $p < 0.05$.

**Post Thawing Assessment**

The mean (± SE) motility, morphology, membrane integrity, viability of post thaw semen in each group is present in Table IV. The significant difference (P< 0.05) were observed among treatments groups A, B, C, D and E. Motility percentage increased from control to treatment groups A, B, C and decreased in D and E. however, highest values were observed in group C (10gm).Themorphological characteristic of post chilling semen in each group have significant difference (P< 0.05) were observed among treatments groups A, B, C, D and E. Morphological characteristic increased from control to treatment groups A, B, C and decreased in D and E. however, highest values were observed in group C (10gm).

The membrane integrity of each bull has significant difference (P< 0.05) was observed among treatments groups A, B, C but D and E are non-significant. Membrane integrity increased from control to treatment groups A, B, C and decreased in D and E. however, highest values were observed in group C (10gm).
The sperm viability percentage of each bull has significant difference (P< 0.05) was observed among treatments groups A, B, C, D and E. Viable percentage increased from control to treatment groups A, B, C and decreased in D and E. however, highest values were observed in group C (10gm).

**Table IV. Comparison effect of Tris and skim milk based extenders on frozen-thawed semen characteristics (Mean % ± SEM)**

| Sperm | Skim milk | | | | | |
|---|---|---|---|---|---|---|
| Group | Tris | A | B | C | D | E |
| Parameters | Control | 6gm | 8gm | 10gm | 12gm | 14gm |
| Motility | 40.55±1.21[b] | 49.00±3.21[a] | 50.50±4.25[a] | 54.75±4.03[b] | 52.25±4.35[b] | 50.50±3.75[a] |
| Morphology | 49.07±3.72[a] | 49.75±3.77[a] | 52.50±3.69[a] | 64.75±2.50[a] | 62.75±4.11[a] | 60.25±3.35[a] |
| Membrane integrity | 47.75±4.57[ab] | 49.00±4.21[a] | 50.50±3.21[a] | 62.25±3.86[a] | 60.05±3.77[a] | 60.80±4.65[a] |
| Viability | 49.15±4.35[a] | 49.89±3.87[a] | 56.75±4.22[a] | 64.00±4.47[a] | 62.00±4.50[ab] | 60.75±4.82[a] |

[a,b,c,d] superscripts with in rows demonstrate significant difference p < 0.05.

**Fertility/ conception Rate**
After AI results indicate that conception rate was high in group C (10 gm) followed by group A, B, D and E.

**Table V. Determination of the fertility rate of frozen thawed kundhi buffalo bull semen With skimmed milk and tris based semen extenders.**

| Groups | Animals | No of Pregnant animals | Percentage |
|---|---|---|---|
| **A (tris based extender)** | 10 | 02 | 20% |
| **B (skim mik extender)** | 10 | 04 | 40% |

Skim milk treatment group conception rate is significantly higher than tris base extender

**DISCUSSION**
The current study was carried on four Kundhi buffalo bulls. For this study Semen were collected twice in a week ,semen samples with motility, morphology and viability ≥ 70% were pooled extended in Tris base extender and skim milk concentration of 6,8,10,12 and 14 gm respectivily. The volume of semen obtained in this study wasranged from 3.43±0.22 – 6.00±0.29. The vales of volume in experimental buffalos were higher than previously reported studies in the Kundhi buffalo bulls by Samo *et al*., 2004; Rahoo *etal*., 2007; and Kaka *et al*., 2016. The result of heigest semen volume of current investigation in both treated and controlexperimental animals may be due to environmental and low frequency of ejaculations and as well as good nutrition.Kaka *et al.,* 2016, who reported that the colour of buffalo semen usually varies from white to translucent. The results of current investigation showed that the bulls produced creamy white semen except of bull 3 which produced milky and translucent colored semen. The similar results were observed in Kundhi buffalo by kaka *et al.*, 2016; and Rehman *et al.*, 2012. This variation in thecolor of semen may be due to effect ofseasonal, libido, age, sexual desire as well as sexual excitement of the animals.
Inpresent study, the pH of buffalo semen was observed ranges from 5.70 to 5.90. However, Mahfouz *et al*., 2009 and Kumar *et al*., 2004 has reported average pH of Kundhi bull ranges from 6.4 to 7.1. The variation in pH might have been due to the activity of the seminal vesicles and estrogens of semen, these results are in agreement with (Fatih *et al*., 2010). Similarly, Kaka et al., (2016) who reported same valuves of pH in buffalo ranges from 6.4 to 7.4.
The spermmotility of semen used for freezing in most of the A.I organization is more than 60 percent (Kaka *et al*., 2016). The results of sperm motility in present study were 64.50±1.04 to 86.00±0.91. The observed values of sperm motility in present study were 64.50±1.04 to 86.00±0.91. Better values were obtained in current study and these results are better for semen cryopreservation as well as good for getting higher fertility rate in animals. Change in motility supposed due to age and season of bulls, as the sperm in warm climatic condition are more active utilizing more energy more sources.
The analysis of wave motion was then performed following two complementary strategies. First, sperm movement was measured directly by observation using phase contrast microscopy at low magnification. The wave motion observed in the glass chamber wasaltered from that observed from drops by exhibiting a laminated flow structure (Creppy *et al.* 2015). Wave pattern of buffalo bull has been investigated and indicates (+++) was analogous in present investigation. The results of current study were in agreement with (Kaka *et al*., 2016; Rehman *et al.,* 2012) in Kundhi buffalo bulls. Similarly, sperms were making rapid swirling motion forming eddies at the end of each motion during semen evaluation of experimental buffaloes. It has been reported that vital criteria for the artificial insemination can be applied to assure the assessment of quality of semen. The mean values were so different but not significant it may be because of animal age. In buffaloes, some animals get maturity at age between 2.5-3.5 years, however those were found deficient in sperm concentration after semen evaluation. In this study, the overall sperm concentration of semen was in good ranged from (2047.50±0.03 to 2679.5±58.77 million/mL), which is good for freezable sperm concentration as well as preservation the semen of Kundhi buffalo bulls. These sperm concentration values are higher than the values reported in earlier

studies in same breed by (Kaka *et al*., 2016; Rehman *et al.,* 2012).This slight variation in sperm concentration values may due to differences present in the age of bull as well as in environmental season.

Buffalo's sperm has different morphological features having plasma membrane is of prime significance. A distinctive buffalo bull spermatozoon is shorter than Boss taurus bulls and measures 62 micron (Kaka *et al*., 2016). The morphology membrane integrity and vaibility found the current study ranged from (63.75±1.31-83.25±1.11%), (63.25±0.85-77.00±1.29%) and (64.50±1.04-86.00±0.91%).These results are higher than stated by (Kaka *et al*., 2016; Rehman *et al.,* 2012; Rahoo *et al.,* 2011).This slight variation in values may due to differences in age of buffalo bull and season in current and earlier studies.

Freezing and thawing of semen leads to the decrease in the percentage of intact sperms, and reduces 50% viable sperm (Kaka *et al*., 2012). Assessment of post thawed quality parameter is important regarding aspects to determine the sperm fertility after adopting freezing and thawing procedures.in present investigations, statistical analyses showed that the values of motility, morphology, viability and integrity of plasma membrane were higher in thawed semen during evaluation in experimental buffaloes. In contrast, these values were lower in previous studies of same breed (Kaka *et al*., 2016; Rehman *et al.,* 2012; Rahoo *et al.,* 2011). These findings showed that the improvement was seen may be due to the addition of skim milk which enhances the quality of frozen thawed bull semen. The results of present study are confirmed by the (Akhter *et al*., 2011) who reported that skim milk based extender improved frozen thawed quality of buffalo bull semen. Conception rate was ranged highest to lowest from 25.00%$^{b}$ to 50.00%$^{a}$.The findings of faith *et al*.,(2000) are partially supporting the results of current research.

The conception rate obtain in this study is 50%$^{a}$, which is higher than previously reported study in buffalo by (Paul and Parkash, 2005) who reported that the ovulation happened earlier and lasted for longer time in cyclic animals as compared to acyclic buffalo heifers. Findings of current research are in agreement with (Karen and Darwish, 2010), who reported initial ovulation in acyclic buffalo's heifers as compared to cyclic buffaloes. Our research are in line to (Perry *et al*., 2007) who reported that maximum estrus response has been observed when follicle size reached between 11 to 12 mm. The maximum size of follicles at the time of ovulation has reported by (Sharma *et al*., 2012) was 16.07±0.99 mm which is more close to inthis study. The result of this study are analogous to (Mirmahmoudi *et al*., 2014) who gave the range of 11 to 15 mm size of follicles at the time of ovulation. Observations of current study is contrast to to Sartori *et al*., (2001), who reported that when the diameter of follicle is less than 10 mm then it is impotent to ovulate in cows. This difference might be due to variation from species to species.

The poor semen quality, freezability and higher sperm abnormality are the serious problem in production of value frozen semen from buffalo bulls. Due to these serious issues, numerous ejaculates are rejected for additional processing (de Castro *et al*., 2017). It has been important for definite utilization of germ plasm to investigate the quality of fresh ejaculates collected with different rates (Kanchan *et al*., 2010).

## CONCLUSION

Comparison of Skim milk and Tris improved frozen thawed quality of Kundhi Buffalo bull semen.Fertility rate was high in treated group as compared to control.

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*Statement of conflict of interest*

The mentioned authors have declared no conflict of interest.

## CONTRIBUTION

AS, AK, SF and HB conceived and designed the experiments.AS performed the experiments. SBK, and JK analyzed the data. LK, IAP, SA, and AH revised the manuscript .MS, SK, and MF wrote the manuscript.

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