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Effect of *in vitro* Selenium Addition to the Semen Extender on the Spermatozoa Characteristics before and after Freezing in Kundhi Buffalo Bull and *in vivo* Fertility Rate

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NUJ conducted experiments and wrote the article. AK designed the experiment. PK and MM proofread the article. MN analysed the data. AAM cheked refrences. RRK, DHK and HJ helped in writing the article.

Key words
Buffalo bull, Semen, Selenium supplementation, Frozen.

ABSTRACT

Present study was conducted to determine the effect of in vitro selenium addition to the semen extender on chilled and frozen thawed quality of Kundhi buffalo bull semen. After collection ejaculate was brought in Semen Production Unit. Semen was evaluated as fresh for volume, colour, motility, morphology, live dead ratio, membrane integrity and sperm concentration, post chilling for motility, morphology, live dead ratio, membrane integrity and frozen thawed for motility, morphology, live dead ratio and membrane integrity. Semen volume and colour were determined by visual examination, pH was determined by digital pH meter. Motility was determined under microscope at10x magnification, morphology and live dead ratio was assessed using eosin nigrosin stain under 40x magnification, concentration was calculated by hemocytometer, membrane integrity was examined by HOST solution. Conception rate was determined by inseminating 20 buffaloes (10 control and 10 best treatment group). After initial examination semen sample having motility ≥ 70% and morphology live dead ratio ≥ 80 % were diluted in Tris based extender consist of 0,2,4 and 6mM concentration of selenium. Four hours after dilution chilled semen was examined for post chilled semen quality, motility% (74.75±1.9), morphology% (86.62±1.2), membrane integrity% (87.87±0.6), live dead ratio% (87.62±0.7). After that samples were frozen for 24 hours, frozen thawed results showed motility (57.00±0.5), morphology (73.50±1.5), membrane integrity (70.00±0.01), live dead ratio (72.00±0.8 were high in 2mM/ml concentration of selenium. Overall results showed that 2mM/ml concentration of selenium improved chilled and frozen thawed quality of semen than control and other treatment groups. In conclusion, supplementation of selenium in Tris extender improved the chilled and frozen thawed quality characteristics and conception rate in Kundhi Buffalo bull semen.

INTRODUCTION

The buffalo has been domesticated in the Indus valley from pre-historic times (Khan *et al*., 2007). The domestic water buffalo (*Bubalus bubalis*) has an important contribution in the agricultural economy of Asia and other developing countries (Ferdousi *et al*., 2013). It provides milk and meat and is a major source of power for farm operation and transport. Semen cryopreservation and artificial insemination (AI) offer many advantages to the livestock industry, particularly in conjunction with genetic evaluation and selection programs. Use of these different extenders is recommended because of their role in protection of sperm cells (Sanchez-Gutierrez *et al*.,

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2008). Selenium (Se) is found to be a basic trace element for the production and health of the all types of livestock and its deficiency causes the low production and poor health (Datt, 2014). Selenium is found to be an essential trace element for regular spermatogenesis and it is part of seleno-proteins phospholipid. Most of the selenium present in the testis is associated with glutathione peroxidase (GPX). Glutathione peroxidase seen to be involved as an essential protein in the normal sperm motility and it is necessary that a variant to this protein is important for regular chromatin condensation and subsequent normal spermatozoa head conformation (Bindari *et al*., 2013). As selenium required for regular testicular growth and sperm formation and deficiency of selenium leads to retarded testicular growth (Sanchez-Gutierrez *et al*., 2008). Selenium supplementation in the male may alter testis structure, testosterone formation, or sperm characteristics (Behne *et al*., 2015). Selenium (Se) is found to be a basic trace element for the production and health of the all types of livestock and its deficiency causes the low production and poor health (Datt, 2014). The earlier studies has been reported on dietary selenium, however limited has been reported on *in vitro* addition of selenium (Se) in extender to determine the frozen thawed quality and *in vivo* fertility rate. Keeping in the view present study was designed with hypothesis that *in vitro* addition of selenium into extender would improve the frozen thawed quality of Kundhi buffalo bull semen.

MATERIALS AND METHODS

Four healthy and fertile Kundhi buffalo bulls of 3 to 4 years of age were used in the study. The bulls were housed at Department of Animal Reproduction, Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agriculture University, Tandojam.

Semen collection

Semen was collected early in the morning twice a week for 4 weeks. Immediately after collection, the semen sample were transferred into laboratory and kept in water bath at 37°C. Initially semen was evaluated for volume, colour, pH, Wave motion, motility, sperm concentration, morphology, viability, membrane integrity and Lipid peroxidation.

Experiment design

Semen samples with motility of at least ≥ 70% and normal morphology and viability ≥ 80% were used for freezing. Ejaculates of all four bulls were pooled and extended in Tris base extender which were divided into four groups A, B, C and D consist of 0, 2, 4, 6 mM/ concentraion of selenium, respectivily. The semen was diluted at 5°C in cold cabinet. Rate of dilution was based on initial sperm concentration and adjusted to 20 million sperm/straw (one dose). After dilution semen was chilled at 5°C for 4 h. The French straws of 0.25ml of four assorted colors white (control), red (6mM), blue (4mM) and yellow (6mM) corresponding to each Group. The filling of straws was carried out with the help of filling machine (Repulsion Motor, MULTIFLEX Betriebsart: DB Schutzart: IP 21). Sealing of open ends of straws was closed manually with polyvenyl chloride powder (PVC). The straws were frozen in Liquid nitrogen vapors for 10 min. After that, the semen straws were immersed in liquid nitrogen for 24 h. Post thawed evaluation was carried out after thawing of frozen semen samples were done by keeping semen straw in water bath at 37°C for 30 seconds. Semen was evaluated for after chilling and frozen thawed for sperm motility, morphology, membrane integrity live dead ration.

Volume

Ejaculate volume per ejaculate was recorded directly from graduated collection tube.

pH

pH of the semen was obtained by using digital pH meter made up of RoHS company having serial number pH-107.

Preparation of extender

The composition of extender for all groups is given in Table I.

Table I.- Composition of extender.

Compoanents	Treatments groups			
	A	B	C	D
Tris (g)	3.81	3.81	3.81	3.81
Citric acid (g)	1.97	1.97	1.97	1.97
D (-) fructose (g)	1.25	1.25	1.25	1.25
Egg yolk (ml)	20	20	20	20
Glycerol (ml)	7	7	7	7
Penicillin (I.U/ml)	1000	1000	1000	1000
Streptomycin (µg/ml)	1000	1000	1000	1000
Distilled water (ml)	100	100	100	100
Selenium (mM)	0 (control)	2	4	6

Wave motion

Wave motion was evaluated in a drop of undiluent semen without using cover slip under low magnification (20X) with phase contrast microscope. Wave patterns of the semen sample were recorded and graded following Kaka *et al*. (2016). 0 is for no mass activity, + is slow wave

motion, ++ is rapid wave motion with formation of eddies at the end of wave and +++ is Eddies.

Motility

Motility was assessed by standard subjective ranking method. A wet mount of diluted semen was prepared by placing a drop of fresh semen under cover slip at magnification of 20x under phase contrast microscope. At least 100 spermatozoa, selected randomly, were observed for percent motile spermatozoa in forward progression movement. The results were expressed in motility%. The wave motion and motility % were recorded on warm stage maintaining the temperature about 37°C. The sample having 60% and above motility were used for freezing and further investigation.

Sperm concentration

Sperm concentration was determined using a hemocytometer as described by Kaka *et al*. (2012). Fixing solution contained sodium chloride (30g), farm aldehyde (4 ml) and distilled water (1000 ml). Semen was diluted at 1:100 with 3% NaCL solution in test tube. Shake the test at least 2 minutes. A small drop of suspension was dropped on both chambers of hemocytometer, covered with a cover slide and observed under light microscope at 40 x magnifications. Sperms were counted in 5 squares one middle and four corner squares. Following formula was used to determine the cell count. Sperms/ ml = n x 5 x df (100) x10 x 1000 (n is number of sperm counted, 5 is number of chambers counted on hem cytometer, df is dilution factor that is equal to 100, 10 id depth of counting chambers and 1000 is unit conversion from µl to ml).

Assessment of membrane integrity (ORT)

Fresh semen sample was used for the hypo osmotic swelling (HOS) test by the method developed by Revell and Mrode (1994) in cattle. The test solution for fresh semen was prepared using Fructose13.51g Tri-sodium-citrate 7.35g Distilled water 1000ml. 100 µl semen was added to 1 ml of a hypo-osmotic solution. After incubation for 60 min at 37°C, sperm swelling was assessed by placing 15µl of well mixed sample on a warm slide (37°C), covered with a cover slide and observed under light microscope at 40x magnification. The spermatozoa swell in the response of the test solution were the cells having intact membrane and were considered as normal fertile cells. Three hundred spermatozoa per slide were counted and expressed in percentage.

Morphological characteristics

These were estimated as per standard staining procedure described by Kaka *et al*. (2015a,b, 2017) and Memon *et al*. (2012). The staining solution was prepared as follow. Eosin 0.67g, Nigrosin 5.0g Distilled water to 100 ml. A drop of fresh semen was mixed with eight drops of stain solution. After standard incubation time (3 min) at 37°C, a thin smear was made on pre warmed slides and allowed to dry at 30°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 morphological percentages.

Live and dead sperm count

It was estimated as per standard staining procedure using nitrogen eosin solution as described by Khumran *et al*. (2015, 2017). A drop of diluted semen mixed with 8 drops of stain was incubated at 30°C for 5 min. Thin mounted smear was observed under 40x objective of light microscope. Approximately 50 sperms were counted. The dead sperms were staining's red with blue background, whereas live sperm appears transparent. Every ejuculate4 of semen contain some morphologically abnormal spermatozoa. The expected range 8-10% has no adverse effect on the fertility. If abnormal spermatozoa exceed 25% of the total ejaculate, recorded fertility can anticipated.

Conception rate

The conception rate was determined after inseminating 20 females' buffaloes. Ten females with best treatment group and 10 with control. Pregnancy was confirmed by cessation of estrus at 21 days and through pregnancy diagnosis by rectal palpation after 60 days of inseminations.

Statistical analysis

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

RESULTS

Assessment of fresh semen

The mean (±SE) volume of each bull is presented in Table II. The significant difference ($P<0.05$) was observed among the bulls 1, 2 3 and 4. Significantly lower volume were recorded in bull 3. Furthermore, significantly higher volumes were recorded in the bull 1. The colour of semen varies from milky to translucent. In current study all bulls produced creamy white semen. It was thick in consistency and the appearance of sample was very clear to record colour of each sample accurately (Table II).

Table II.- Assessment of fresh semen.

Bulls	Volume	Colour	pH	Motility	Wave motion	Concentration million (ml)	Morphology	Live dead ratio	Membrane integrity
1	6.2±0.05*	Creamy white	6.1±0.01	92.9±1.86*	+++	2769.3±0.08*	91.43±0.51*	87.84±0.21*	87.75±1.90*
2	6.0 ±0.04	Creamy white	6.8±0.09*	86.8±1.32	+++	2423.7±0.06	89.90±0.48	85.23±1.59	85.39±1.39
3	4.9±0.03	Creamy white	6.7±0.04	87.3±1.07	++±	2161.2±0.81	85.93±0.34	86.65±1.35	84.01±1.13
4	6.00±0.03	Creamy white	6.7±0.01	86.3±1.05	++±	2261.0±0.82	85.90±0.31	85.60±1.30	81.01±1.13

*Significant difference.

The mean (±SE) pH of each bull is presented in Table II. The significant difference (P< 0.05) was observed among bull 1, 2, 3 and 4. Significantly high pH was recorded in group 2. Furthermore, the low pH was recorded in group 1. The mean (± SE) motility percentage of semen of each bull is present in Table II. The significant difference (P< 0.05) was observed among bulls 1, 2, 3 and 4. The significantly (92.96±1.86) better motility was observed in group 1. The normal fresh sperm exhibit swirling movement under microscope field called wave motion. It was found to be +++ in all the bulls. The sperms were making rapid swirling motion forming eddies at the end of each motion (Table II). The mean (±SE) sperm concentration of each bull is presented in Table II. The significant difference (P< 0.05) was observed among all bulls 1, 2, 3 and 4. Significantly low concentration (2161.2±0.81) was recorded in bull 3. Furthermore, the significantly higher (2769.3±0.08) sperm concentration was recorded in bull 1. The mean (±SE) sperm morphology of each bull is presented in Table II. The significant difference (P< 0.05) was observed among all bulls 1, 2, 3 and 4. Significantly low morphology (85.93±0.34) percentage was recorded in group 4. The better morphology (91.43±0.51) was recorded in bull 1. The mean (±SE) live dead ratio sperm each bull is present in Table II. All 4 bulls were significantly different (P< 0.05) from one another 1, 2, 3 and 4. The significantly better (87.75±0.21) live sperm rate was found in bull 1. The mean (±SE) membrane integrity each bull is present in Table II. All 4 bulls were significantly different (P< 0.05) from one another 1, 2, 3 and 4. The better membrane integrity (87.75±1.90) was found in bull 1.

Post chilling assessment

The mean (±SE) motility of post chilling semen in each group is present in Table III. The significant difference (P< 0.05) was observed among treatments groups 1, 2, 3 and 4. Motility percentage increased linearly from control to treatment groups however, highest values were observed in group B (2mM/ml). The mean (±SE) morphological characteristic of post chilling semen in each group is present in Table III. The significant difference (P< 0.05) was observed among treatments groups 1, 2, 3 and 4. Morphological characteristic increased linearly from control to treatment groups however, highest values were observed in group B (2mM/ml). The mean (±SE) membrane integrity of each bull presented in Table III. The significant difference (P< 0.05) was observed among treatments groups 1, 2, 3 and 4. Membrane integrity increased linearly from control to treatment groups however, highest values were observed in group 2 (2mM/ml). The mean (±SE) sperm live percentage of each bull is presented in Table III. The significant difference (P< 0.05) was observed among treatments groups 1, 2, 3 and 4. Live percentage increased linearly from control to treatment groups however, highest values were observed in group B (2mM/ml).

Table III.- Effect of combination of selenium Tris with selenium on post chilled semen characteristics (Mean % ± SEM).

Sperm parameters %	Selenium (mM/ml)			
	0	2	4	6
Motility	63.87± 2.10	74.75± 1.9*	72.37± 2.20	65.18± 1.40
Morphology	82.19± 0.90	86.62± 1.2*	82.93± 2.20	78.06± 2.30
Membrane integrity	82.87± 1.00	87.87± 0.6*	86.37± 1.20	82.50± 1.70
Live dead ratio	83.25± 1.10	87.62± 0.7*	86.37± 1.30	83.37± 1.50

*Significant difference.

Post thawing assessment

The mean (±SE) motility of post thaw semen in each group is present in Table IV. The significant difference (P< 0.05) was observed among treatments groups 1, 2, 3 and 4. The motility percentage increased linearly from control to treatment groups however, highest values were observed in group 2 (2mM/ml). The mean (±SE) morphological characteristic of post thaw semen in each group is present in Table IV. The significant difference (P< 0.05) was observed among treatments groups 1, 2, 3 and 4. Morphological characteristic increased linearly from control to treatment

groups however, highest values were observed in group 2 (2mM/ml). The mean (±SE) membrane integrity of each bull presented in Table IV. The significant difference (P< 0.05) was observed among treatments groups 1, 2, 3 and 4. Membrane integrity increased linearly from control to treatment groups however, highest values were observed in group 2 (2mM/ml). The mean (±SE) sperm live dead ratio of each bull is presented in Table IV. The significant difference (P< 0.05) was observed among treatments groups 1, 2, 3 and 4. Live percentage increased linearly from control to treatment groups however, highest values were observed in group 2 (2mM/ml).

Table IV.- Effect of combination of selenium Tris with selenium on frozen-thawed semen characteristics (Mean % ± SEM).

Sperm parameters %	Selenium (mM/ml)			
	0	2	4	6
Motility	41.50±1.2	57.00±0.5*	47.50±0.8	49.75±1.1
Morphology	59.00±1.5	73.50±1.5*	63.75±2.5	65.00±3.4
Membrane integrity	57.25±3.4	70.00±0.1*	60.25±3.4	65.25±0.3
Live dead ratio	57.75±2.9	72.00±0.8*	64.25±0.4	67.25±1.3

*Significant difference.

Table V.- Conception rate.

Groups	Animals	No. of animals pregnant	Percentage
A	10	03	30 %
B	10	06	60%*

*Significant difference .

Fertility/conception rate

In our results pregnancy was confirmed by cessation of estrus at 21 days and through pregnancy diagnosis by rectal palpation after 60 days of inseminations the results for conception after performing the AI results indicate that conception rate was observed higher in group B (0.2mM/ml) as compared to other groups A, C and in this study (Table V).

DISCUSSION

The study was conducted on four fertile Kundhi buffalo bulls. Semen collected twice a week, Semen samples with motility of at least ≥ 70% and normal morphology and viability ≥ 80% were pooled and extended in Tris base extender consist of 0, 2, 4, 6 mM/ concentraion of selenium, respectivily. The results are described below. In this study volume of semen ranged from 4.9±0.03 to 6.2±0.05. However, the all bulls were kept in the similar management and feeding systems, the findings of our research indicates the results similar to the results of Baiee *et al*. (2015) and (2017) in the Holstein and Crossbred bulls. However, Kaka *et al*. (2016) reported less volume (2.25 ± 0.015ml) than value in current study. The difference may be due to age of bull and season. Bull used in the current study (3-4years) are older than earlier study (2years). The present is conducted in winter months of the year compared to the earlier studies (Baiee *et al*., 2017; Ahmed *et al*., 1984; Bajwa *et al*., 1992; Kaka *et al*., 2016) conducted in summer season. It is also reported by the above researchers that the volume always varies with management, feeding, age of bull and breed of animals. Because young and fully trained bull produce large amount of semen as compared to old and un-trained bull 1-3ml (Vale, 1997). Busffalo semen usually varies from white to translucent (Kaka *et al*., 2016). In current study all bulls produced creamy white semen. The same was also observed by Brohi (1993), Kumar *et al*. (2004), Kaka *et al*. (2016), Rehman *et al*. (2012) and Rahoo *et al*. (2011) in Kundhi buffalo. The pH of buffalo semen ranges 6.4 to 7.4 (Kaka *et al*., 2016). The results for pH in current study were observed 6.1 to 6.7 which is related to findings of Brohi (1993), Kumar *et al*. (2004) and Kaka *et al*. (2016). However, Kumar *et al*. (2004), reported average pH of Kundhi bull 6.4 to 7.1. The slight variation in the pH may because of old age of bull or season during the collection of semen. Motility of the spermatozoa used for freezing in most of the A.I organization is above 60 percent (Kaka *et al*., 2016). Motility percentage (85.27±1.05-92.96±1.86%) observed in the current study is higher than that reported earlier by Brohi (1993), Kumar *et al*. (2004) and Kaka *et al*. (2016) in Kundhi buffalo breed. Difference in motility supposed to due to age and season of bulls, as the sperm in warm climatic condition are more active utilizing more energy more sources. Wave motion found (+++) was similar as reported by Brohi (1993), Kumar *et al*. (2004), Kaka *et al*. (2016), Rehman *et al*. (2012) and Rahoo *et al*. (2011) in Kundhi buffalo bulls. The sperms were making rapid swirling motion forming eddies at the end of each motion. It has been reported that important criteria for the artificial insemination can be applied to assure the quality of semen. Whereas it is subjective type of assessment has poor relationship with fertility (Kaka *et al*., 2016). In this research the overall sperm concentration of semen was observed ranged from 2161.2±0.81 to 2769.3±0.08 million per ml in freezable Kundhi buffalo bulls. These values are higher than the values reported in earlier studies in same breed by Brohi (1993) (1161.2±0.71), Kumar *et al*. (2004) (1261.1±0.72), Kaka *et al*. (2016) (1548 ±19.85x106), Rehman *et al*. (2012) (1542±9.201x106) and Rahoo *et al*.

(2011) (1542± 9.20x10^6). This slight variation in values may be due to difference in the age of bull and season in current and earlier studies. Buffalo sperm has distinct morphological features having plasma membrane of prime significance. A typical buffalo bull spermatozoon is shorter than *Boss taurus* bulls and measures 62µ (Kaka *et al*., 2016). The morphology membrane integrity and live dead sperm found the current study ranged from 85.90±0.31 to 91.43±0.51%. These results are higher than reported by Brohi (1993), Kumar *et al*. (2004), Kaka *et al*. (2016) (79.35±1.372%), Rehman *et al*. (2012) (81.2 ±0.8) and Rahoo *et al*. (2011) (82.2±1.02). 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 aspects to determine the fertility of sperm after freezing and thawing. In the current study frozen thawed motility and morphology live dead ratio and integrity of plasma membrane were assessed. Result of the present study are higher than results of previous studies of same breed by Brohi (1993), Kumar *et al*. (2004), Kaka *et al*. (2012), Rehman *et al*. (2012) and Rahoo *et al*. (2011). This improvement in result may due the addition of selenium which worked as antioxidant which resulted in decrease lipid per oxidation which improved frozen thawed quality of bull semen. The results of present study are confirmed by Drostkar *et al*. (2012), who reported that supplementation of selenium improved frozen thawed quality of bull semen in Tris extender. Conception rate was ranged highest to lowest from 16.6% to 66.6%. The findings of Griveau *et al*. (1995) are partially supporting the results of current research. However, present results are higher than results reported by Nair *et al*. (2017), who reported results with and without bio stimulation in the same breed.

CONCLUSION

Supplementation of Selenium improved chilled and frozen thawed quality of Kundhi Buffalo bull semen. In addition to quality improvement of semen and fertility rate was high in treated group as compared to control.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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