The Journal of Animal & Plant Sciences, 26(3): 2016, Page:605-611
ISSN: 1018-7081

EFFECT OF EXTENDER OSMOLALITY AND BUTYLATED HYDROXY TOLUENE SUPPLEMENTATION ON POST THAW QUALITY AND FERTILITY OF NILI RAVI BUFFALO BULL (*BUBALUS BUBALIS*) SEMEN

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ABSTRACT

Aim of the present study was to develop semen extender for cryopreservation of buffalo bull semen to improve the post thaw quality and conception rate. Study consisted of three experiments, single ejaculate of four Nili Ravi buffalo bulls were collected for a period of 5 weeks for each of experiment I and II. In experiment-I, osmotic pressure of tris citric acid extender (TCAE) was optimized. Semen of four bulls was pooled and calculated quantity was added to five solutions of variable osmotic pressures of TCAE (260, 270, 280, 290 and 300 mOsm/kg). In experiment-II, TCAE with best osmotic pressure as recorded in experiment-I was supplemented with butylated hydroxyl toluene (1.75, 2.0, 2.25, 0.0 mM and DMSO). As butylated hydroxyl toluene (BHT) was dissolved in dimethyl sulph oxide (DMSO), DMSO control was also run to rule out its reported effect as antioxidant. Post thaw spermatozoa motility, viability, plasma membrane integrity (PMI), normal apical ridge (NAR),DNA damage rate and lipid peroxidation were assessed in first two experiments. In experiment-III, pregnancy rate comparison between Trial and Control extenders was done. In experiment-I, at 300 and 280 mOsm/kg spermatozoa motility was significantly higher ($P<0.05$) compared to 260 mOsm/kg, and NAR at 300 mOsm/kg was significantly higher than other osmotic pressures. In experiment-II, a higher spermatozoa motility was recorded at 2.0mM BHT than other groups except 0.0mM BHT. At 2.25mM BHT, spermatozoa viability and PMI rates were lower compared to 1.75 and 2.0mM, respectively. Moreover, higher DNA damage was observed at 2.25mM as compared to 0.0, 2.0mM BHT and DMSO. A lower lipid peroxidation in BHT treated groups was also noted. Pregnancy rate was 24 and 42% in Comparative and Trial extenders, respectively but difference was not significant. In conclusion, TCAE (300 mOsm/kg) with BHT (2.0 mM) improved post-thaw semen quality of Nili-Ravi buffalo bulls.

Keywords: Buffalo, Spermatozoa, Cryopreservation, Extender, Osmolality, antioxidant.

INTRODUCTION

The basic purpose of semen extender is to extend the semen volume and to maintain semen fertility after cryopreservation. Buffalo spermatozoa are more susceptible to damage during freezing than cattle spermatozoa. Cryo-preserved semen commonly has impaired fertility compared to fresh semen as 40-50 % spermatozoa die during cryopreservation (Watson, 2000). Moreover, cryopreservation reduces number of motile spermatozoa and also damages spermatozoa plasma membrane (Yildiz*et al*., 2007). Osmotic stress (Watson, 2000) and oxidative stress (Agarwal *et al*., 2003) are among the factors that contributes towards spermatozoa damage during cryopreservation. Oxidative stress also adversely affects spermatozoa membrane integrity, motility, DNA and proteins that results into cell apoptosis.

Change in osmotic pressure during cryopreservation exerts osmotic stress (Watson, 2000) that results in intra and extra cellular ice crystals formation and leads to irreversible damage to spermatozoa integrity (Hammerstedt *et al*., 1990). The highest metabolic activity of spermatozoa was noted when osmotic pressure of semen extender was appropriate. There is contentious information regarding buffalo semen osmotic pressure; 293.33±3.39, 268.8 1.±1.17 and 289.4 mOsm/kg osmotic pressures have been reported by Ibrahim *et al*. (1985), Khan and Ijaz (2008) and Mughal *et al*. (2013), respectively. Ignoring osmolality of semen extender can expose spermatozoa to osmotic stress that affects structure and function of plasma membrane, nucleus, mitochondria, flagella and cell signaling which may lead to spermatozoa death (Khan and Ijaz, 2008). These damages can be minimized by developing an iso-osmotic semen extender.

Antioxidants neutralize oxidants present in semen and improve post thaw semen quality by reducing oxidative stress (Bucak*et al*., 2008). Cryopreservation reduces natural semen antioxidants and spermatozoa are exposed to oxidative stress. Moreover, due to high membrane contents of poly unsaturated fatty acids, buffalo have higher lipid oxidation rate compared to cattle (Chatterjee and Gagnon, 2001). Addition of

antioxidants in semen extender prevents spermatozoa integrity during cryopreservation. Butylated hydroxytoluene (BHT), a phenolic organic antioxidant that is a synthetic analogue of vitamin E, significantly decreases the membrane permeability changes during cryopreservation by acting as membrane protectant (Khalifa*et al*., 2008).

Presently, buffalo farmers are not satisfied with fertility rates of cryopreserved semen. Low conception rate in buffaloes may be due to acrosomal damage, reduced spermatozoa motility and alterations in spermatozoa membrane integrity during cryopreservation. Therefore, this study was designed to develop a suitable semen extender for cryopreservation of buffalo semen that can improve conception rate.

MATERIALS AND METHODS

Animals and semen collection: Four buffalo bulls housed at the Semen Production Unit, Qadirabad, Sahiwal, Pakistan were used for semen collection. Information regarding feeding and management conditions of these bulls has been given elsewhere (Mughal *et al*., 2013). Single ejaculate at weekly intervals was collected for a period of 5 weeks for experiment I and II, respectively. Artificial vagina maintained at 42˚C was used for semen collection and semen collection tubes were shifted to the water bath (37˚C) and holding time (15 minutes) was provided. Preliminary semen assessment e.g. color, motility, volume and sperm concentration were carried out.

Experimental Design: In experiment-I, optimal osmotic pressure for buffalo bull semen out of TCAE different osmotic pressures (260, 270, 280, 290 and 300 mOsm/kg) was selected by evaluating post thawed spermatozoa quality parameters. In experiment-II, extender with optimal osmotic pressure chosen from experiment-I was tried to improve by the addition of BHT at different concentrations (1.75, 2.0, 2.25, 0.0 mM and DMSO). In experiment-III, fertility trial comparison was carried out under field conditions by using TCAE having optimal osmotic pressure and supplemented with best BHT concentration (50 inseminations) of experiment-II (Trial group) and 50 inseminations (Control group) using semen diluted with extender presently used for buffalo semen cryopreservation by Semen Production Unit, Qadirabad, Pakistan.

Experimental Stations: Buffalo bull semen was collected, processed and cryopreserved at the Semen Production Unit (SPU), Qadirabad, Pakistan. Post-thaw semen quality analysis of experiment I and II was carried out in post graduate laboratory, department of Physiology, University of Veterinary and Animal Sciences, Lahore. Fertility trial was conducted at artificial insemination (A.I.) Centers; district Bahawal Nagar, Punjab, Pakistan.

Experiment-I: Extender preparation and semen processing: After modifications to the composition (Table 1) used by Liete *et al*. (2010), stock solution (500 mL: pH 7.0 and osmotic pressure > 300 mOsm/kg) for TCAE was prepared. This stock solution of TCAE then further subdivided into six parts. Five subdivided parts of stock solutions were used to prepare desired osmotic pressure levels of TCAEi. e. 260, 270, 280, 290 and 300 mOsm/kg (Gonotec 030, Cryoscopic Osmometer, Berlin, Germany). Double distilled water was used for lowering osmotic pressure of stock solution. To uplift osmotic pressure spared sixth part of stock solution (> 300 mOsm/kg) was used. Ejaculates collected from four bulls on each collection day (≥70 % motility) were pooled and calculated semen quantity was diluted at 37˚C with each osmotic pressure based extender to achieve $20x10^6$ spermatozoa/0.5 ml straw. Diluted semen cooled from 37˚C to 4 ˚C in cold cabinet in two hours. Then equilibration time of 4 hours was given to the diluted semen at 4˚C. After equilibration at 4˚C, semen was filled in polyvinyl straws. These straws were cooled @ 3˚C/minutes from 4˚C to -15˚C and then @ 10˚C/minutes from -15˚C to -80˚C. Finally, these straws were placed at 4 cm above liquid nitrogen for 10 minutes before plunging into liquid nitrogen. Post thaw evaluation of these straws was started after 24 hours of cryopreservation.

Post thaw semen evaluation: Spermatozoa motility (%) after thawing of semen straws for 30 seconds at 37 ˚C was noted as mentioned by Ijaz *et al*. (2009). Spermatozoa viability (supravital staining technique) and PMI rates (HOS solution: 75 mOsm/kg) were evaluated as per method of Khan and Ijaz, (2008) while DNA damage (acridine orange staining technique) and NAR were evaluated as per Tejada *et al*. (1984) and Rasul *et al*. (2000), respectively. At least 200 spermatozoa were examined for assessment of their PMI, viability, NAR and DNA integrity. Mean of three observations was considered as single data point. Thiobarbituric acid assay mentioned by Ohkawa*et al*. (1979) was used to determine Malondialdehyde (MDA: stable lipid peroxidation product) using a spectrophotometer (UV 2800, BMS, Canada) at 532 nm. Results were expressed as nM of MDA.

Experiment-II: Semen processing and evaluation: Calculated pooled semen quantity was added in the graduated tubes having specific volume of TCAE and BHT (B1378, Sigma Aldrich, USA) to obtain $20x10^6$ spermatozoa/0.5 ml straws. As BHT was dissolved in DMSO and the latter has antioxidant properties (Donoghue and Donoghue, 1997), the DMSO volume equal to its volume present in 2.25 mM BHT

concentration was also run as DMSO control group. Semen cryopreservation and evaluation was same as mentioned for experiment-I.

Experiment-III: Semen doses and inseminations: Fifty inseminations were carried out for each Trial and Control group. Trial doses had TCAE (300 mOsm/Kg and pH ~7.0) and 2mM BHT. Semen doses for Control group were prepared by using the conventionally used semen extender of SPU, Qadirabad, Punjab, Pakistan (Table 1). Trial and control semen doses were prepared under uniform condition. Buffaloes were inseminated after 12-24 hours of observed estrus; all the inseminations were made in low breeding season (April - June) after thawing semen doses at 37˚C for 30 seconds. Buffaloes were fed same fodder (alfalfa with wheat straw + concentrate), concentrate was provided according to the milk production. Pregnancy rate was decided on the basis of rectal palpation of all the inseminated animals at day 60±10 post insemination.

Statistical Analysis: Statistical analysis was done using the Statistical Package for Social Science (SPSS for Windows version 12, SPSS Inc., Chicago, IL, USA). Data were presented as mean ± S.E. In experiment I and II, the data were analyzed using analysis of variance (ANOVA), the group differences were compared by the Duncan's Multiple Range Test. In experiment III, pregnancy rates were analyzed by using chi square. Difference in results was considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

In experiment-I, at 300 and 280 mOsm/kg spermatozoa motility was significantly higher ($P<0.05$) compared to 260 mOsm/kg. Spermatozoa NAR at 300 mOsm/kg of TCAE was significantly ($P<0.05$)higher than <300 mOsm/kg. In TCAE, spermatozoa DNA damage was higher ($P<0.05$) at both 260 and 290 mOsm/kg compared to other osmotic pressures. Mean post thaw spermatozoa characteristics at different osmotic pressures are presented in Table 2.

In experiment-II, a higher ($P<0.05$) spermatozoa motility was recorded at 2.0 mM BHT than other groups including DMSO, except 0.0 mM BHT. At 2.25 mM BHT, spermatozoa viability and PMI rates were lower ($P<0.05$) compared to 1.75 mM and 2.0 mM BHT, respectively. Moreover, higher ($P<0.05$) DNA damage at 2.25 mM compared to 0.0, 2.0 mM BHT and DMSO group was also noted. A lower ($P<0.05$) lipid peroxidation in BHT treated groups compared to DMSO group and 0.0 mM BHT was also noted. Mean post thaw spermatozoa characteristics at different concentrations of butylated hydroxy toluene are presented in Table 3.

In experiment III, pregnancy rate of 24 and 42% was recorded in Control and Trial groups, respectively and this difference was statistically non significant (Fig. 1).

Decline in spermatozoa motility at osmotic pressures <270 mOsm/kg may be correlated to the fact that motility depends on ATP contents of spermatozoal mitochondria (Perchek *et al*., 1995) which were damaged by hypo osmotic stress and consequently reduces the ATP availability to spermatozoa. Mitochondrial damage in hypo osmotic extender is primarily due to increased intracellular ice formation during cryopreservation. Similarly, Liu and Foote (1998) noted higher spermatozoa motility in cattle bull semen at 300 mOsm/kg compared to hypo-osmotic tris extender i.e., 200 mOsm/kg. These workers also noted that spermatozoa motility had decreasing trend as osmotic pressure increased or decreased from 300 mOsm/kg in tris extender.

Higher percentage of NAR at 300 mOsm/kg may be due to relatively hypertonic extenders comparing to the ~293 mOsm/kg; reported by Ibrahim *et al*. (1985). Hypertonic extender enhances spermatozoa dehydration and reduces intracellular ice that improves spermatozoa survival (Pommer *et al*., 2002). Results reported by Andrabi *et al*. (2008) by using buffer of 320 mOsm/kg are also similar to present study. The difference of spermatozoa acrosomal integrity might be due to the change in osmotic pressure of the media.

El-Sisy*et al*. (2010) in buffalo noted 10.4% spermatozoa DNA damage by acridine orange staining technique that was 8-9% higher compared to this study. In that study osmotic pressure was not monitored and an-isotonic extender could be a reason for higher DNA damage of buffalo semen. Higher DNA damage at 260 and 290 mOsm/kg may be due to hypotonic extender that results in higher intracellular ice formation and oxidative stress (Lewis and Aitken, 2005). Higher unsaturated fatty acids contents of buffalo plasma membrane may be responsible for higher oxidative stress.

Changes in osmotic pressure did not affect ($P>0.05$) viability, PMI and lipid peroxidation which may be due to the smaller increments in osmotic pressures in this study (10 mOsm/kg). Secondly, may be because sperm has capacity to tolerate an-isotonic conditions to a certain extent as osmotic tolerance of spermatozoa is variable for different species. Stallion spermatozoa can tolerate osmolality variations up to 100 mOsm/kg from isotonic condition (Ball and Vo, 2001). Smaller range of osmotic pressure (260-300 mOsm/kg) in this study was to determine the suitable osmolality rather than the osmotic tolerance of buffalo spermatozoa.

Higher spermatozoa motility at 2.0 mM BHT may be due to the fact that osmotic pressure of semen extender used in this study was optimized in last experiment. Lower spermatozoa motility noted at 2.25 mM compared to 2 mM BHT is speculated due to damage of internal organells at higher BHT level. Ijaz*et*

al. (2009) reported similar buffalo spermatozoa motility at 1.0 and 2.0 mM BHT but in that study 2 mM BHT improved the spermatozoa motility than both less and above levels of BHT. Shoae and Zamiri (2008) results were in our opposition in which 0.5-1.0 mM BHT addition in cattle semen showed higher spermatozoa motility than 2.0mM. This difference in spermatozoa motility may be due to higher buffalo plasma membrane contents of PUFA which were responsible for higher lipid per-oxidation in buffalo semen than cattle during cryopreservation. It is suggested that antioxidants maintained the spermatozoa motility by scavenging the ROS molecules (Bucak *et al*., 2008) which causes lipid peroxidation of the spermatozoa plasma membrane. Membrane damage due to high ROS further contributes in spermatozoa axosome and mitochondria damage and ultimately affect spermatozoa motility as motility depends on ATP contents of spermatozoa mitochondria (Perchek *et al*., 1995).

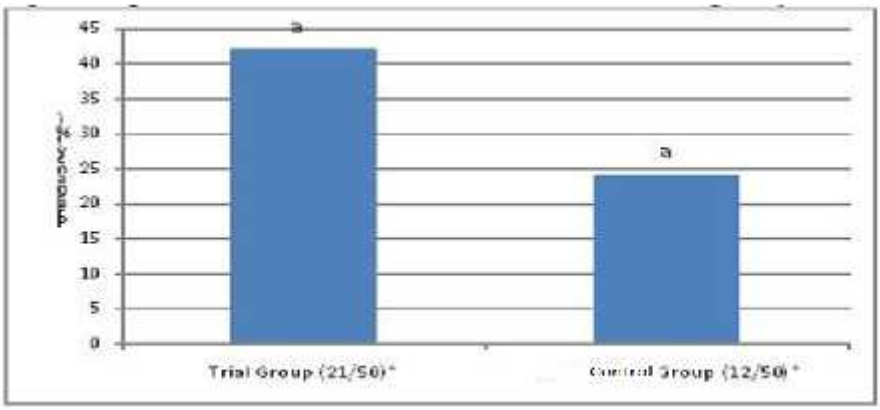
At 2.25 mM BHT significantly lower spermatozoa viability and higher DNA damage (%) might be due to toxic effects of BHT as excessive antioxidant decreases physiological level of oxidants which is considered essential for normal spermatozoa function (Roca *et al*., 2004), moreover, higher concentrations of BHT make spermatozoa vulnerable to cryo-injury by decreasing the oxidation rate of PUFA in spermatozoa membrane that ultimately increases plasma membrane fluidity (Shoae and Zamiri, 2008). Enhanced fluidity of spermatozoa membrane weakens the function of ionic channels/ATPases in membrane that ultimately damages the spermatozoa as entry of nutrients into the spermatozoa is impaired. Moreover, higher oxidative stress during buffalo semen cryopreservation due to its higher PUFA contents results into DNA damage (Aitken and Krausz, 2001). Over compaction of spermatozoa chromatin occurs due to oxidative stress (Lewis and Aitken, 2005) that breakDNA strands (Cordova *et al*., 2002). Spermatozoa with broken DNA strands not attach to oviduct as it not decondence during fertilization and if attach then transmit defective genome (Sakkas and Alvarez, 2010) that causes fertilization failure, poor embryo growth or even embryo death. Similarly toxic effect of higher BHT concentrations were reported by Ijaz *et al*. (2009) and Shoae and Zamiri (2008) in buffalo and cattle, respectively.

Similar to our study, Roca *et al*. (2004) claimed that addition of 1.6 mM BHT to boar semen significantly decreased the lipid peroxidation. Buffalo sperm membranes have relatively greater concentrations of membrane PUFA and are more susceptible to peroxidative damage (Chatterjee and Gagnon, 2001), that is why; antioxidants decreased the lipid peroxidation which is associated with good semen quality and fertility (Chaudhari *et al*., 2008).

Positive influence of BHT on post thaw semen quality may be due to the fact that BHT protected plasma membrane fluidity due to its lipid solubility (Khalifa *et al*., 2008). Secondly, BHT is a synthetic analogue of vitamin E and is a reactive oxygen species production chain breaker and not a scavenging antioxidant, thus it protects plasma membrane integrity without affecting ROS production system (Sharma and Agarwal, 1996). Another possible mechanism for BHT positive influence is due to its antiviral properties that inactivate viruses in semen (Shoae and Zamiri, 2008).

Difference in pregnancy rates between Trial and Control extender was non significant. Less number of inseminations in trial and control groups may be a reason for these non significant results.

Results of the study, clearly indicate that osmotic stress primarily damaged the spermatozoa internal structures more severely (mitochondria, acrosome and DNA) rather than plasma membrane. TCA extender having 300 mOsm/kg osmotic pressure and optimal inclusion of BHT (2.0 mM) improved post thaw semen quality. However, pregnancy rates between Trial and Control extender did not differ.



* indicates number of pregnant animals/ total number of animals inseminated

Figure 1. Comparative effect of trial and control doses on the pregnancy rate

Table 1. Composition of Trial and Control Extender.

Ingredients	Trial Extender	Control Extender
	Quantity	Quantity
	Solution A	
tris (Hydroxymethyl) aminomethane (g)	16.78	24.2
citric acid monohydrate (g)	9.32	13.4
D-Fructose (g)	7.53	10
Bi-distilled Water (ml)	500	730
-	**Solution B**	-
-	73 ml-Solution A	-
Egg Yolk (ml)	20	200
Glycerol (ml)	7	70
Penicillin (I.U./ml)	1000	-
Streptomycin (mg/ml)	1.0	-
Streptopenicillin (g)	-	1

Table 2. Mean Post Thaw Spermatozoa Characteristics of Buffalo at Different Osmotic Pressures of Tris Citric Acid Extender (TCAE, n=5).

Osmotic Pressure (mOsm /kg)	Motility (%)	Viability (%)	NAR (%)	PMI (%)	Damaged DNA (%)	Lipid Peroxidation (nMm)
260	41.33±2.86^{a}	69.90±2.57^{a}	60.61±2.32^{a}	61.81±2.25^{a}	1.78±0.15^{b}	37.20±7.44^{a}
270	47.67±3.48^{ab}	72.22±2.36^{a}	61.74±0.98^{a}	61.14±2.47^{a}	1.17±0.13^{a}	37.00±9.98^{a}
280	53.67±3.57^{b}	73.18±2.05^{a}	62.23±2.33^{a}	61.52±3.06^{a}	1.10±0.14^{a}	33.70±6.34^{a}
290	50.33±3.53^{ab}	72.63±1.91^{a}	62.26±2.65^{a}	64.49±3.77^{a}	1.60±0.11^{b}	35.90±10.70^{a}
300	54.00±3.66^{b}	73.37±2.47^{a}	69.78±2.31^{b}	59.91±2.96^{a}	1.14±0.15^{a}	29.30±6.19^{a}

n=No. of collections

$^{a-b,}$ Means in columns with different letters are significant (P< 0.05).

Table 3. Effect of Different Concentrations of Butylated Hydroxy Toluene (BHT) on Post Thaw Spermatozoa Characteristics of Buffalo Bull (n=5).

BHT (mM)	Motility (%)	Viability (%)	NAR (%)	PMI (%)	Damaged DNA (%)	Lipid Peroxidation (nMm)
1.75	36.67±2.79 ^{a}	63.06±2.73^{b}	60.11±3.32^{a}	50.62±2.16^{ab}	0.60±0.20^{ab}	10.61±2.34^{a}
2.0	46.33±2.36^{b}	58.89±2.39^{ab}	55.40±3.89^{a}	57.76±3.06^{b}	0.30±0.12^{a}	8.22±1.06^{a}
2.25	38.00±2.33^{a}	54.26±2.60^{a}	54.19±3.39^{a}	47.94±3.16^{a}	1.00±0.21^{b}	12.61±3.60^{a}
DMSO	37.33±2.67^{a}	57.16±2.80^{ab}	55.84±2.71^{a}	47.49±2.79^{a}	0.50±0.16^{a}	24.56±2.41^{b}
0.0	43.33±3.22^{ab}	61.69±3.32^{ab}	54.56±3.48^{a}	52.55±3.38^{ab}	0.33±0.12^{a}	31.59±3.95^{b}

n= No. of collections

$^{a-b,}$ Means in Columns with different letters are significant (P< 0.05).

Acknowledgements: Authors thanks Semen Production Unit, Qadirabad, Pakistan authorities to grant permission to conduct this study. Authors also thanks to Higher Education Commission, Islamabad, Pakistan for the provision of monetary support to conduct this study.

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