

**EFFECT OF L-CYSTEINE ON POST THAW QUALITY OF NILI RAVI BUFFALO
(BUBALUS BUBALIS) BULL SPERMATOZOA CRYOPRESERVED IN TRIS CITRIC
ACID EXTENDER**

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ABSTRACT

This study was designed to determine effect of various concentrations of L- cysteine on frozen-thaw semen quality of Nili Ravi buffalo. Single ejaculate of four buffalo bulls (Nili-Ravi) was collected at weekly intervals for 5 weeks. Ejaculates of acceptable motility (70%) were pooled and semen was diluted by tris citric acid extender (TCAE; 300 mosm/kg osmotic pressure and ~7.0 pH) having 4 levels of L-cysteine (0.0, 2.0, 5.0 and 8.0 mM). Semen was equilibrated at 4°C and packaged in 0.5 mL French straws with 20×10⁶ spermatozoa/ straw and then frozen in liquid nitrogen. L-cysteine inclusion levels had non-significant (P> 0.05) effect on spermatozoa motility, viability, NAR, PMI and on lipid peroxidation. However, DNA damage in spermatozoa was significantly higher (P<0.05) in 8.0 mM L-cysteine. It was concluded that L-cysteine (2.0 and 5.0 mM) addition to cryopreserved semen had no significant effect on post thaw semen characteristics, except at 8 mM which adversely affected DNA integrity. This may be due to its toxicity at this concentration.

Key words: cryopreservation, semen, spermatozoa, antioxidants, buffalo, L-cysteine

INTRODUCTION

L-Cysteine is an amino acid (propionic acid), of thiols group of non-enzymatic antioxidants. It is a precursor of glutathione which plays an important role in hydrogen peroxide oxidation and can prevents DNA damage during cryopreservation. Addition of L-cysteine to semen extender prevents apoptosis, spermatozoa motility loss and lipid peroxidation during cryopreservation (Bilodeau *et al.*, 2001). Reactive oxygen species (ROS) affects semen quality (Thuwanut, 2007), however, certain concentration of ROS is useful for spermatozoa acrosome reaction and capacitation (de Lamirande and Gagnon, 1993). Imbalance of oxidants and antioxidants (Oxidative stress) adversely affects spermatozoa motility, proteins, DNA and membrane integrity which leads to cell apoptosis (Filho *et al.*, 2009).

Various reports indicate that addition of antioxidants to semen reduces oxidative stress and improves post thaw semen quality as antioxidants neutralize the oxidants present in the semen (Bucak *et al.*, 2008). Semen cryopreservation decrease natural antioxidants to an extent that spermatozoa are exposed to oxidative stress. Moreover, buffalo have higher lipid oxidation rate due to higher membrane contents of poly unsaturated fatty acids (Chatterjee and Gagnon, 2001) which further reduces activity of natural antioxidants. Therefore, antioxidants addition to semen extender helps preserving spermatozoa integrity during the process of cryopreservation (Andrabi *et al.*, 2008). Therefore, the

present study was designed to determine effect of various concentrations of L- cysteine on frozen-thaw semen quality of Nili Ravi buffalo.

MATERIALS AND METHODS

Experimental Animals and Semen Collection: During this study, four Nili-Ravi buffalo bulls of Semen Production Unit, Qadirabad, Sahiwal, Pakistan were used. Conditions of feeding and management were same as of Mughal *et al.* (2013). Single ejaculate was collected at weekly intervals for 5 weeks during spring months (March - April). Collected semen was provided 15 minutes holding time at 37 °C in water bath. Preliminary semen assessment e.g. color, motility and volume etc. were carried out.

Preparation of Extender and Semen Processing: Composition of TCA extender is given in Table 1. Suitable ejaculates (70% motility) of four bulls were pooled and then calculated semen quantity was added to the graduated tubes having specific amount of TCAE and L-cysteine (C7352, Sigma Aldrich, USA) concentrations of 0.0, 2.0, 5.0 and 8.0 mM to obtain 40×10⁶ spermatozoa/mL. Semen was equilibrated at 4°C and filled in 0.5 mL polyvinyl straws (IMV, France) in cold cabinet. These straws were cooled from 4°C to -15°C @ 3°C/min then from -15°C to -80°C @ 10°C/min. Subsequently, these straws were placed 4 cm above the liquid nitrogen for 10 minutes in straw grill and finally dipped into liquid nitrogen.

Semen Assessment: Procedure adopted by Ijaz *et al.* (2009) was used to assess spermatozoa motility after thawing semen at 37 °C for 30 seconds. Spermatozoa viability and PMI was determined as per Khan and Ijaz, 2008 while NAR by Rasul *et al.* 2000. Acridine orange staining technique following the procedure of Tejada *et al.* (1984) was used to determine spermatozoa DNA integrity. Two hundred spermatozoa were examined to assess their viability, PMI, NAR and DNA integrity. The mean of three observations was considered a single data point. For lipid peroxidation, thiobarbituric acid assay was used to determine Malondialdehyde (MDA: stable lipid peroxidation product) through a spectrophotometer (UV 2800, BMS, Canada) at 532 nm as per procedure of Ohkawa *et al.* (1979). The results were expressed in nm of MDA.

Statistical analysis: Statistical Package for Social Science (SPSS for Windows version 12, SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Data were presented as mean \pm S.E. One way analysis of variance was used. Duncan's Multiple Range Test was employed to compare group differences. Difference in results was considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Present study was designed to determine effect of various concentrations of L- cysteine on frozen-thaw semen quality of Nili Ravi buffalo. The results regarding the effect of varying concentrations of L-cysteine on seminal parameters are presented in Table 2. It was revealed that L-cysteine had significant ($P < 0.05$) effect on spermatozoa DNA damage at 8.0 mM L-cysteine compared to 2.0, 5.0 and 0.0 mM, however, effect on all other parameters was non-significant ($P > 0.05$). Non-significant results of percentage spermatozoa motility, viability, acrosomal integrity, PMI and lipid peroxidation may be correlated to the fact that L- cysteine is a water soluble antioxidant and due to this characteristic do not bind properly to plasma membrane (Khalifa *et al.*, 2008). Moreover, in this study a suitable semen extender and optimized osmotic pressure based on previous

experiments (data not shown) was selected and it could be another reason for the differences of our findings from other investigators (extender osmotic pressure not mentioned in their studies) as semen extender with optimized osmotic pressure decreases the oxidation rate (Aitken and Krausz, 2001).

Over compaction of spermatozoa chromatin occurs due to oxidative stress that break DNA strands (Cordova *et al.*, 2002). These spermatozoa do not attach to ova as they do not decondense during fertilization and transmit defective genome (Sakkas and Alvarez, 2010). Higher ($P < 0.05$) DNA damage at 8.0 mM of L-cysteine might be due to toxic effects of antioxidant as excessive antioxidant decreases physiological level of oxidants which is considered essential for normal spermatozoa function (Roca *et al.*, 2004), moreover, higher concentrations of antioxidant make spermatozoa vulnerable to cryo-injury by increasing the plasma membrane fluidity (Shoe and Zamiri, 2008). As higher concentrations of antioxidant decrease the oxidation rate of PUFA (in spermatozoa membrane) and consequently enhance the membrane fluidity (Sikka, 2004). Enhanced fluidity of spermatozoa membrane weakens the function of ionic channels/ATPases in membrane that ultimately affects nutrients availability to the spermatozoa. Higher

Table 1. Composition of TCA Extender

Solution A	
Tris (Hydroxymethyl) aminomethane	16.78 g
citric acid monohydrate	9.32 g
D-Fructose	7.53 g
Bi-distilled Water	500 mL
Solution B	
Solution A	73 mL
Egg Yolk	20 mL
Glycerol	7 mL
Pencillin	1000 I.U./mL
Streptomycin	1.0 mg/mL
Solution A	73 mL

Table 2. Effect of Different Concentrations of L-Cysteine on Post Thaw Spermatozoa Characteristics of Buffalo Bull (n=5).

L - Cysteine (mM)	Motility (%)	Viability (%)	NAR (%)	PMI (%)	Damaged DNA (%)	Lipid Peroxidation (nm)
2.0	47.00 \pm 2.33 ^a	65.27 \pm 2.10 ^a	57.55 \pm 2.05 ^a	56.24 \pm 3.49 ^a	0.50 \pm 0.15 ^a	35.17 \pm 5.23 ^a
5.0	43.67 \pm 1.79 ^a	65.70 \pm 2.16 ^a	53.20 \pm 3.78 ^a	56.27 \pm 3.07 ^a	0.60 \pm 0.12 ^a	29.61 \pm 3.99 ^a
8.0	40.33 \pm 2.69 ^a	62.97 \pm 3.02 ^a	56.40 \pm 3.70 ^a	53.88 \pm 2.37 ^a	1.20 \pm 0.17 ^b	27.05 \pm 2.22 ^a
0.0	43.00 \pm 3.22 ^a	61.69 \pm 3.32 ^a	54.56 \pm 3.48 ^a	52.55 \pm 3.38 ^a	0.33 \pm 0.12 ^a	31.59 \pm 3.95 ^a

n= Number of collections

Values are represented as mean \pm standard error (S.E.)

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

oxidative stress during buffalo semen cryopreservation due to its higher PUFA contents results into DNA damage (Aitken and Krausz, 2001). Our results were contrary to Thuwanut (2007) work which may be primarily due to difference in extender composition, osmotic pressure and species. Tuncer *et al.* (2010) noted higher cattle spermatozoa DNA damage at 10.0 mM cysteine concentration compared to control as reported in this study.

In conclusion, L-cysteine 2.0 and 5.0 mM inclusion to semen cryopreserved in TCAE had non-significant influence on post thaw semen characteristics, except at 8 mM which adversely affected DNA integrity which may be due to its toxic effect.

REFERENCES

- Aitken, R. J. and C. Krausz (2001). Oxidative stress, DNA damage and the Y chromosome. *Reproduction* 122: 497-506.
- Andrabi, S. M. H., M. S, Ansari, N. Ullah and M. Afzal (2008). Effect of non-enzymatic antioxidants in extender on post-thaw quality of buffalo (*Bubalus bubalis*) bull spermatozoa. *Pak. Vet. J.* 28: 159-162.
- Bilodeau, J. F., S. Blanchette, C. Gagnon and M. A. Sirard (2001). Thiols prevent H₂O₂-mediated loss of sperm motility in cryopreserved bull semen. *Theriogenology* 56 (2): 275-286.
- Bucak M. N., A. Atessahin and A. Yuce (2008). Effect of antioxidants and oxidative stress parameters on ram semen after the freeze-thawing process. *Small Rum. Res.* 75: 128-134.
- Chatterjee S. and C. Gagnon (2001). Production of reactive oxygen species by spermatozoa undergoing cooling, freezing and thawing. *Mol. Reprod. Dev.* 59: 451-458.
- Cordova, A., J. F. Perez-Gutierrez, B. Lleo, C. Garcia-Artiga, A. Alvarez, V. Drobchak and R. S. Martin (2002). In vitro fertilizing capacity and chromatin condensation of deep frozen semen packaged in 0.5 and 5 mL straws. *Theriogenology* 57: 2119-2128.
- De Lamirande, E. and C. Gagnon (1993). Human sperm hyperactivation and capacitation as parts of an oxidative process. *Free Radic. Biol. Med.* 14: 157-66.
- Filho, I. C. B., C. D. Pederzoli, A. M. Sgaravatti, R. M. Gregory, C. S. D. Filho, M. I. M. Jobim and R. C. Mattos (2009). Skim milk-egg yolk based semen extender compensates for non enzymatic antioxidant activity loss during equine semen cryopreservation. *Anim. Reprod.* 6 (2): 392-399.
- Ijaz A., A. Hussain, M. Aleem, M. S. Yousaf and H. Rehman (2009). Butylated hydroxytoluene inclusion in semen extender improves the post-thawed semen quality of Nili-Ravi buffalo (*Bubalus bubalis*). *Theriogenology* 71: 1326-1329.
- Khalifa, T. A. A., A. G. Lymberopoulos and B. E. El-Saidy (2008). Testing usability of butylated hydroxytoluene in conservation of goat semen. *Reprod. Dom. Anim.* 43: 525-30.
- Khan, M. I. R. and A. Ijaz (2008). Effects of osmotic pressure on motility, plasma membrane integrity and viability in fresh and frozen-thawed buffalo spermatozoa. *Animal* 2 (4): 548-553.
- Mughal, D. H., A. Ijaz, M. S. Yousaf, H. Rehman, M. Aleem. H. Zaneb and F. Wadood (2013). Assessment of optimal osmotic pressure of citrate egg yolk extender for cryopreservation of buffalo bull (*Bubalus bubalis*) semen. *J. Anim. Plant Sci.* 23 (4): 964-968.
- Ohkawa, H., H. Kawa, N. Ohishi and K. Yagi (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* 95: 351-358.
- Rasul, Z., M. Anzar, S. Jalali and N. Ahmad (2000). Effect of buffering systems on post-thaw motion characteristics, plasma membrane integrity, and acrosome morphology of buffalo spermatozoa. *Anim. Reprod. Sci.* 59: 31-41.
- Roca, J., M. A. Gil, M. Hernandez, I. Parrilla, J. M. Vazquez and E. A. Martinez (2004). Survival and fertility of boar spermatozoa after freezethawing in extender supplemented with butylated hydroxytoluene. *J Androl.* 25: 397-405.
- Sakkas, D. and J. G. Alvarez (2010). Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. *Fertil. Steril.* 93: 1027-1036.
- Shoe, A. and M. J. Zamiri (2008). Effect of butylated hydroxytoluene on bull spermatozoa frozen in egg yolk-citrate extender. *Anim. Reprod. Sci.* 104: 414-418.extr
- Sikka, S. C. (2004). Role of oxidative stress and antioxidants in andrology and assisted reproductive technology. *J. Androl.* 25 (1): 5-18.
- Tejada, R. I., J. C. Mitchell, A. Norman, J. J. Marik and S. Friedman (1984). A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence. *Fertil Steril.* 42:87-91.
- Thuwanut, P. (2007). The effect of antioxidants on motility, viability, acrosome integrity and DNA integrity of post-thaw epididymal cat spermatozoa. Masters thesis, Swedish University of Agricultural Sciences, Upsala, Sweden.
- Tuncer, P. B., M. N. Bucak, S. Buyukleblebici, S. Sariozkan, D. Yeni, A. Eken, P. P. Akalin, H. Kinet, F. Avdatek, A. F. Fidan and M. Gundogan (2010). The effect of cysteine and glutathione on sperm and oxidative stress parameters of post-thawed bull semen. *Cryobiology* 61: 303-307.