CRYOSURVIVAL OF BUFFALO SEMEN EXTENDED IN TRIS-EGG YOLK SUPPLEMENTED WITH A COMBINATION OF DIFFERENT CONCENTRATIONS OF BUTYLATED HYDROXYTOLUENE AND TAURINE

Reda Ibrahim El-Sheshtawy*

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ABSTRACT

Cryopreservation is accompanied with much detrimental premature sperm capacitation, decreased livability and fertility consequences. Taurine is considered as an antioxidant ameliorating the semen value post freeze-thawing. The purpose of this investigation was to elucidate the impact of Tris-diluent enriched with a mixture of taurine and different concentrations of butylated hydroxytoluene on buffalo bull semen preservability. Semen samples were extended in a Tris extender. Variable concentrations of butylated hydroxytoluene (BHT) (0.5, 1.0 and 2.0 mM) were set in ethyl alcohol in prewarmed (37^{o}C) test tubes. Then taurine (60 mM) was added into each tube. Extended semen samples were added into the test tubes and put at 37^{o}C for 5 minutes to permit the permeation of BHT into the spermatozoa. The control test tubes were Tris extender with (zero BHT and zero taurine). The test tubes were exposed to the freezing protocol. Semen was assessed and conception rate was carried out. The sperm membrane integrity (HOST) was eminently higher in TTB_1 and TTB_2 if compared to the control post cooling (Table 1). The post thawing results (Table 2) revealed significant improvement in sperm motility in TTB_2 if compared to the control and other concentrations. Alive sperm % was kept in all concentrations as the control. Sperm abnormalities were kept in TTB_2 and TTB_3 as the control. Sperm membrane integrity (HOST) was significantly improved in all significantly as compared to the control. The acrosomal integrity was kept in TTB_1 and TTB_2 concentrations the same as the control The conception percent (Table 3) was higher in TTB_2. It could be concluded that, the post cooling results exhibited the best semen quality in $TTB_1$and TTB_2. The post -thawing results revealed that, most improved sperm parameters and conception rate were in TTB_2.

Keywords: *Bubalus bubalis*, buffaloes, semen, preservation, taurine, butylated hydroxytoluene

INTRODUCTION

Artificial insemination is a reproduction technology extensively functional in buffalo breeding with frozen semen (Vishwanath and Shannon 2000; Kubkomawa, 2018). Semen freezing ensures storage of sperm for long periods with consequent application in the breeding

Department of Animal Reproduction and Artificial Insemination, Veterinary Research Institute, National Research Center, Giza, Egypt, *E-mail: rielsheshtawy@gmail.com

of farm animals. Semen freezing also exerts functional stress on the spermatozoal membrane, linked with oxidative stress and ROS released by dead spermatozoa and atmospheric oxygen, causing decreases in the sperm motility, membrane flexibility and fertilizing capacity of spermatozoa for AI (de Lamirande and Gagnon, 1992; Chatterjee *et al*., 2001; Upreti *et al*., 1998).

Oxygen free radical accumulation is a principal determinant at the procedures of sperm freezing, which is elaborated from the dead sperms and also from the ingredients of the extenders, which involve the molecular oxygen (Thomson *et al*., 2009). The oxygen free radicals deteriorate the sperm plasma membrane especially the polyunsaturated fatty acids, membrane proteins, sperm DNA and acrosome causing oxidative damage of sperm membrane and DNA (Tremellen, 2008) with consequent reduction in the sperm quality. The release of free radicals through cryopreservation can be minimized by the addition of appropriate antioxidants in the semen extenders used for sperm freezing (Ball *et al*., 2001; Andreea and Stela, 2010).

However, the low success rates of the cryopreserved semen, and poor success rate of IVF are referred to temperature decrease, sperm dehydration, freeze - thawing procedures (Medeiros *et al*., 2002). Freezing of bovine semen often exerts a hazardous source for the oxidative stress on spermatozoa owing to lowered antioxidant enzymes activities and the sperm membrane become more liable to oxidative damage (El-Sisy *et al*., 2007) which deteriorates the sperm membrane integrity (Awda *et al*., 2009). Cryopreservation exerts over accumulation of oxygen free radicals causing oxidative injury to spermatozoa with subsequent decrease in sperm viability (Aumuller and Seitz, 1990; Mammoto *et al*., 1996; Anderson *et al*., 1994). Therefore, addition of antioxidant to the extender is of a great value by reducing the cryodamage induced by these oxygen free radicals (Khalifa and El Saidy, 2006; Khan and Ijaz, 2007). Cryopreservation is associated with increased spermatozoal unwanted premature capacitation. These changes may not reduce sperm motility but decrease lifespan, capability to adapt with the female reproductive tract and sperm fertilizing potential (Medeiros *et al*., 2002). In buffalo semen, ROS is released mainly by dead spermatozoa through an aromatic amino acid oxidase catalyzed reaction (Upreti *et al*., 1998). Low concentrations of ROS are physiologically involved in the maintenance of the fertilizing capacity and sperm capacitation (acrosome reaction) of spermatozoa. But excessive ROS damage sperm function and enzymatic activity (Baumber *et al*., 2000). Cryopreservation process results in the liberation of reactive oxygen species (ROS) and over release of ROS among cryopreservation impairs post-freeze-thaw sperm motility, viability, membrane fluidity, antioxidant condition, fertility potential, sperm vitality and DNA status (Aitken and Krausz, 2001; Mughal *et al*., 2013; Bilodeau *et al*., 2001). Spermatozoa contain high levels of polyunsaturated fatty acids (PUFA), which are highly vulnerable to lipid peroxidation (LPO) with subsequent impairment of motility, membrane integrity, fertilizing capacity and metabolic alterations of sperm (Cassani *et al*., 2005). There is a great request for semen cryopreservation to conserve the genetic material of the selected sires to be used in artificial insemination (AI) program to increase the animal productivity (Medeiros *et al*., 2002). The use of suitable extenders, additives, cryoprotectants, freezing and thawing regimes relative to cryopreservation of semen is of a great demand to improve the fertility rate of buffalo

(Mittal *et al*., 2019). Tris-based diluents are commonly applied for semen freezing protocol in farm animals (Purdy, 2006) as well as in buffaloes. Cryoprotectants as taurine were added to the bull freezing diluent (Chen *et al*., 1993; Sarıozkan *et al*., 2009; Uysal *et al*., 2007), boar (Funahashi and Sano, 2005; Gutierrez-Perez *et al*., 2009; Hu *et al*., 2009), ram (Bucak *et al*., 2007; 2008), goat (Atessahin *et al*., 2008), dog spermatozoa (Martins-Bessa *et al*., 2009; Michael *et al*., 2007) to enhance the semen quality post cryopreservation. Taurine as a sulfonic amino acid, exerts an antioxidant effect and can permeate the sperm membrane and reduces oxidative damage and fatty acids peroxidation and keeps the cells from the over accumulation of ROS (Chen *et al*., 1993; Foote *et al*., 2002), reactions with sperm membrane lipids, creating hypertonic extracellular medium, with subsequent sperm cells osmotic dehydration and so reducing the degree of sperm deterioration by ice crystals formation (Liu *et al*., 1998; Storey *et al*., 1998). The influence of semen extender cryoprotectants as taurine on the frozen buffalo sperm features has not been sufficiently interpreted. BHT is a synthetic analogue of vitamin E that improved post-thawing semen quality in bulls (Shoae and Zamiri, 2008), goat (Khalifa *et al*., 2007), boar (Roca *et al*., 2004) and rams (Bucak and Tekin, 2007). So, the objective of our study was to evaluate buffalo semen quality post-cooling and post-freezing upon using a combination of taurine and BHT in the extender.

MATERIALS AND METHODS

Semen collection and initial evaluation

Five mature buffalo-bulls with superior genetics and semen quality characteristics kept at The Semen Freezing Center, Egypt, were involved in this study as semen supply. Semen samples were harvested from bulls using an artificial vagina every week for five weeks. The semen ejaculates were primarily assessed for volume, concentration using Thoma rulling of the Neubaur haemocytometer and sperm forward motility. The freezing process was implemented on semen specimens with more than 70% motility and 80% normal morphology. The semen samples were pooled so as to have adequate semen for a replicate and to get rid of the individual bull effect. The semen was put for 10 minutes at 37ºC in a water bath before dilution to be evaluated for sperm forward motility, alive, total morphological abnormalities, and acrosome and membrane integrities before freezing.

Semen processing

Semen ejaculates were diluted (1:7 dilution rate) in a Tris-citrate egg yolk diluent with 20% (v/v) egg yolk and 7% (v/v) glycerol at 37ºC (de Paz *et al*., 2010; Roof *et al*., 2012) to ensure 60 million motile spermatozoa mL-1. Variable concentrations of BHT (0.5, 1.0 and 2.0 mM) were prepared in ethanol in pre warmed (37ºC) test tubes. The ethanol was allowed to evaporate so that. A thin crystallized layer of BHT was deposited on the inner surface of the tubes. Then taurine (60 mM) was added into each tube. Diluted semen was added into the test tubes and kept at 37ºC for 5 minutes to permit permeation of BHT by spermatozoa (Ijaz *et al*., 2009). The control tubes were Tris containing (zero BHT and zero taurine). The tubes were slowly cooled (about for 2 h) till 5ºC and exposed to equilibration for four hours. Semen was packed into 0.25 mL polyvinyl French straws (IMV, France). After equilibration periods, the straws were kept horizontally on a rack and frozen 4 cm above liquid nitrogen vapors (LN2) for 10 minutes

and were then plunged in liquid nitrogen at -196ºC.

Estimation of semen quality characteristics

Frozen semen straws were thawed individually at 37ºC for 30 seconds in a water bath for microscopic examination. The criteria implemented were sperm motility, sperm viability, sperm morpholpgical abnormalities, sperm membrane fluidity (HOST), normal intact acrosome in cooled and frozen-thawed semen.

Sperm motility

Subjective motility was determined using phase contrast microscope (Olympus Optical Co. Ltd., Japan). Visual motility was microscopically evaluated with closed circuit television (Graham *et al*., 1970).

Live and abnormal spermatozoa (%)

The viability and abnormalities% of sperm were assessed using eosin-Nigrosin stained smear (Sidhu and Guraya, 1985).

Sperm membrane integrity

Sperm membrane integrity was assessed using the hyposmotic swelling test (Jeyendran *et al*., 1984). Two hundred spermatozoa were calculated and the percentage of spermatozoa with curled tails (swollen/intact plasma membrane) was estimated.

Intact normal acrosome percent

Acrosome integrity was estimated using giemsa stain (Watson, 1975). The % intact acrosome was recorded for two hundred spermatozoa that were examined under an immersion objective (×1000) by phase contract microscope.

***In vivo* fertility rate (CR)**

No. of buffalo females (n=290) were inseminated with the TTB post-thawed semen and with the post-thawed semen extended in TCFY (Control group). The insemination of females was carried out using the insemination gun and semen was deposited intra uterine. Pregnancy was recorded via animal rectal palpation post two months from insemination. The inseminated females were used by the collaboration in Beni-Suef Governorate. CR was calculated by the equation:

$$CR = \frac{\text{no.of conceived buffaloes}}{\text{Total no.of inseminated buffaloes}} \times 100$$

Statistical analysis

Output data were analyzed by one-way analysis of variance (ANOVA), followed by Duncan test to determine significant differences in all the parameters among all groups, with SPSS Version 14.0 for Windows (SPSS, 2005). Differences with values of $P<0.05$ were considered to be statistically significant.

RESULTS

The post- cooling results exhibited maintenance of sperm motility and alive sperm % in $TTB_1$and TTB_2 as compared to the control. Sperm abnormalities were kept in TTB_1 and TTB_3 as the control. The sperm membrane integrity (HOST) was significantly ($P<0.013$) higher in TTB_1 and TTB_2 if compared to the control (90.87±0.71, 85.85±0.72 and 56.61±8.93 respectively). Acrosome % was kept in all concentrations as the control.

The post- thawing results revealed significant ($P<0.000$) improvement in sperm

motility in TTB_2 (63.33±1.66) if compared to the control and other concentrations. Alive sperm % was kept in all concentrations as the control. Sperm abnormalities were kept in TTB_2 and TTB_3 as the control. Spermatozoal membrane status (HOST) was extremely ($P<0.013$) ameliorated in all significantly ($P<0.013$) as compared to the control (76.23±0.72, 73.17±0.61, 69.89±6.0 and 57.90±0.15 respectively). The acrosome % was kept in concentrations TTB_1 and TTB_2 as the control. The conception rate was the best in TTB_2 (65.7%).

DISCUSSIONS

Many factors have been recorded to control the cryovitality of spermatozoa involving osmotic damage, ice crystal aggregation, toxicity of the added cryoprotectants and the individual variability (Neild *et al*., 2003; Ferrusola *et al*., 2009). Among various causes, oxidative damage has been postulated to influence the fertilitypotential and function of frozen/thawed spermatozoa (Agarwal *et al*., 2008; O'Flaherty, 2014; Smith *et al*., 2006). Oxidative injury takes place as a result of inequality between the concentration of reactive oxygen species (ROS) release and the antioxidant activity of the sperm cell (Halliwell, 2006). Extreme levels of ROS are hazardous to the spermatozoa (Halliwell and Gutteridge, 2007), low concenrations of these molecules are necessary to stimulate sperm capacitation in human, a process that is mandatory for the spermatozoa to gain their fertilizing capacity (O'Flaherty *et al*., 2003). Upon oxidative damage, spermatozoa experience severe deterioration as peroxidation of membrane fatty acids, DNA damage, (Barroso *et al*., 2000), inferior mitochondrial activity (Gallon *et al*., 2006; Koppers *et al*., 2008) and reduced activation of enzymes related to sperm motility (de Lamirande and Gagnon, 1992a, b).

A diversity of antioxidants is found in the spermatozoa and seminal plasma especially antioxidant enzymes (SOD, CAT and GSH). Their antioxidant activity is inadequate and slowly declines on progress of the freezing process, so antioxidants enrichment should be available in the semen diluent (Bilodeau *et al*., 2001). Taurine has a beneficial effect in improving CAT level and consequently enhancing the antioxidant effect (Bucak *et al*., 2007).

In the current study, taurine enrichment to the freezing extender improved semen characteristics as represented by post- thaw sperm motility, sperm membrane integrity, acrosome status and viability. Our results agreed with Reddy *et al*. (2010) who recorded improved semen parameters by the effect of taurine.

In the present study, tris extender enriched with (60 mM) taurine and concentrations of BHT (0.5, 1.0 mM) improved sperm membrane integrity (HOST) post cooling. Sperm motility improved with BHT (1.0 mM) and sperm membrane integrity (HOST) improved with all concentrations of BHT post freezing. The conception rate was the best in TTB_2, and this result come in accordance with the best sperm motility at this concentration. Our results were similar to that obtained in buffalo (Ijaz *et al*., 2009; Reddy *et al*., 2010), ram (Alverez and story, 1983), rabbits (Bucak *et al*., 2007) and boar spermatozoa (Hu *et al*., 2009). These results are in accordance with those obtained in bulls (Shoae and Zamiri, 2008) who recorded enhanced postfreezing-thawing semen characteristics with concentration 0.5 to 1 mM BHT in bulls and that higher concentrations induced hazardous effect.

Cryopreservation process lead to over accumulation of oxygen free radicals that lead

to decreased motility, membrane integrity and fertilizing potential (Cassani *et al*., 2005).

Many studies clarified the improving effect of taurine on post- thaw semen quality (Bucak *et al*., 2007; Sariozkan *et al*., 2009). Taurine is a sulfuric amino acid that plays an important role as non enzymatic scavenger of the oxygen free radicals, so protect sperm from oxidative damage during cryopreservation (Sariozkan *et al*., 2009; saleh and Agarwal, 2002).

Taurine addition may exert protective post-freezing outcome on the functional status of acrosome and mitochondria with subsequent energy release from intracellular ATP that lead to superior sperm motility. The evaluation of viability, acrosome and membrane integrities are also indispensable as motility only is insufficient for sperm assessment post freezing.

The enrichment of BHT as an antioxidant in buffalo semen diluents improved post-thawing semen quality (Ijaz *et al*., 2009). Also, this improvement was recorded in ram (Maia *et al*., 2010), goat (Memon *et al*., 2012) and in canines (Neagu *et al*., 2010). Patel *et al*. (2015) stated that, the addition of BHT has improved the quality of Hariana bull sperm after dilution, equilibration, and thawing with a significant improvement in the quality of sperm in all the three stages with the addition of 0.5 mM and 1 mM BHT.

The improved post-cooled and post-thawed semen value in this study may be attributed to the antioxidant capacity of the BHT (Hammerstedt *et al*., 1976; Williams *et al*., 1999; Pursel, 1979; Watson, 2000), the permeation into the cell membrane improving its integrity (Shoae and Zamiri, 2008). Butylated hydroxytoluene (BHT) is a phenolic antioxidant being supplemented in the semen extender to reduce the sperm membrane permeability impairment during cryopreservation (Neagu *et al*., 2010). BHT reduces in the lipid peroxidation with consequenct antioxidant effect (Shoae and Zamiri, 2008; Stradaioli *et al*., 2007). BHT is chemically a synthetic analog of Vitamin E, and is essential in the auto-oxidation reaction, through converting the peroxy radicals to hydroxyperoxides (Fujisawa *et al*., 2004). BHT also exerts antiviral effect and has been implicated with the inactivation of lipid-containing viruses (Graham and Hammerstedt, 1992). Our findings are contradictory to those observed by Ball *et al*. (2001) who stated detrimental effect of BHT in stallion semen preservation. This antagonistic observation may be due to species variation. It could be concluded that, the post cooling results exhibited the best semen quality in TTB_1 and TTB_2. The post -thawing results revealed that, most improved sperm parameters and conception rate were in TTB_2.

Abbreviations

ROS: reactive oxygen species

HOST: Hypo-osmotic swelling test/ sperm membrane integrity test

AI: Artificial insemination

PUFA: polyunsaturated fatty acids

LPO: Lipid peroxidation

BHT: Butylated Hydroxy Toluene

SOD: Superoxide Dismutase enzyme

CAT: Catalase enzyme

GSH: Glutathione Hydrogenase enzyme

TTB: Tris Taurine Butylated Hydroxy Toluene

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Table 1. Effect of Tris extender enriched with taurine and BHT on the post-cooled extended buffalo bull semen (Mean±SE).

Diluent	Motility	Alive	Abnormality	HOST	Acrosome
TTB_1	91.66±1.66^{b}	90.66±0.66^{b}	7.66±1.33^{ab}	90.87±0.71^{b}	88.66±1.85^{a}
TTB_2	91.66±1.66^{b}	86.00±1.00^{ab}	9.33±0.33^{b}	85.85±0.72^{b}	82.66±2.66^{a}
TTB_3	83.33±3.33^{a}	85.33±2.02^{a}	8.66±1.33^{ab}	71.62±7.46^{ab}	81.66±1.66^{a}
Control (TCFYG)	90.00±0.00^{b}	88.33±1.66^{ab}	6.66±0.33^{a}	56.61±8.93^{a}	85.00±5.00^{a}
Total	89.16±1.35	87.58±0.88	8.08±0.43	76.24±4.73	84.45±1.40
P-value	0.059	0.107	0.126	0.013	0.263

Means bearing different superscripts between different extenders and differ at 5% and 1% levels of probability.

TCFYG = Control Tris-citrate-fructose-egg yolk-glycerol;

TTB_1 = $TrisTB_1$; TTB_2 = $TrisTB_2$; TTB_3 = $TrisTB_3$.

Table 2. Effect of Tris extender enriched with taurine and BHT on the post- thawed extended buffalo bull semen (Mean±SE).

Diluent	Motility	Alive	Abnormality	HOST	Acrosome
TB_1	48.33±1.66^{a}	81.66±1.66^{a}	13.33±0.88^{b}	76.23±0.72^{b}	79.33±2.33^{b}
TB_2	63.33±1.66^{b}	83.66±1.85^{a}	10.66±0.66^{ab}	73.17±0.61^{b}	85.33±0.33^{b}
TB_3	48.33±1.66^{a}	82.33±2.33^{a}	12.00±1.15^{ab}	69.89±6.00^{b}	69.33±2.90^{a}
Control	43.33±1.66^{a}	80.66±0.66^{a}	10.33±0.33^{a}	57.90±0.15^{a}	87.50±2.50.0^{b}
Total	50.83±2.37	82.08±0.81	11.58±0.49	69.30±2.46	79.72±2.40
P-value	0.000	0.678	0.107	0.013	0.004

Means bearing different superscripts between different extenders and differ at 5% and 1% levels of probability.

TCFYG = Control Tris-citrate-fructose-egg yolk-glycerol;

TTB_1 = $TrisTB_1$; TTB_2 = $TrisTB_2$; TTB_3 = $TrisTB_3$.

Table 3. Effect of taurine and different concentrations of butylated hydroxytoluene enriched extender on a field conception rate test in buffalo.

Treatment	No of inseminated females	No of conceived females	*In vivo* fertility rate (CR, %)
TTB_1	77	42	54.5 %
TTB_2	70	46	65.7%
TTB_3	75	43	57.3 %
Control (TCFYG)	68	38	55.9%

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