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EFFECT OF LACTOSE EGG YOLK GLYCEROL EXTENDER SUPPLEMENTED WITH TREHALOSE ON POST-THAW CHARACTERISTICS AND FERTILITY OF BUFFALO BULL SPERMATOZOA

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Abstract

The present study was designed to: 1) study the influence of various concentrations of trehalose on buffalo bull spermatozoa extended in lactose- egg yolk-glycerol extender (LEYGE) and 2) compare the fertility rate between LEYGE supplemented with an optimal level of trehalose and traditionally used tris-citric-acid-fructose extender. Semen of Nili-Ravi buffalo bulls (n = 04) routinely used for artificial insemination, was collected using an AV at SPU once a week, for eight weeks. Semen samples with ≥70 % spermatozoa motility were pooled and diluted at 37°C in LEYGE extender containing trehalose at 0.0, 30.0, 50.0 and 70.0 mM. The sperm motility, viability, acrosomal integrity, plasma membrane integrity, and DNA integrity were significantly (P≤0.05) higher in LEYGE supplemented with 70.0 mM trehalose as compared to other groups. The fertility rate was significantly (P≤0.05) higher when semen doses extended in LEYGE and supplemented with 70.0 mM trehalose were used (n = 19; 38%) as compared to semen doses extended in the conventional tris-citric-acid-fructose extender (n = 27; 54%). In conclusion, 70 mM is an optimal inclusion level in buffalo bull semen for trehalose in LEYGE in respect to seminal attributes. Furthermore, LEYGE supplemented with 70 mM trehalose presented higher fertility rate in buffaloes as compared to traditionally used tris-citric-acid-fructose extender under field conditions.

Key words*: buffalo bull, spermatozoa, trehalose, lactose, cryopreservation.*

INTRODUCTION

Artificial insemination (AI) has been playing a vital role in improving the production of milk, meat, wool, leather, and hair. The success of AI relies on semen collection, preservation, storage and its utilization (Leboeuf et al., 2000). Various protocols have been developed and are being used for semen cryopreservation in cattle and buffalo at the moment. In most of the species, cryo-survival rate of spermtoza is still not optimum. Previously, irreversible damages to spermatozoa caused by cryopreservation have been appraised (Medeiros et al., 2002). The factors which are liable for sperm damage during semen dilution, freezing, and thawing are mainly the temperature change, ice formation, toxicity of cryoprotectants, alterations in sperm membrane and osmotic stress (Watson, 2000). Additionally, lipid peroxidation (LPO) and reactive oxygen species also contribute to reduced sperm motility and fertility rate (Leboeuf et al., 2000) through disrupting the plasma membrane of spermatzoa. The addition of sugars in semen extenders provides energy to spermatozoa and maintain osmotic pressure of the diluents (Aboagla and Terada, 2003). During cryopreservation, about 50% of the spermatozoa are damaged due to exertion of chemical and mechanical stresses, which is reflected in poor quality of post-thaw sperm characteristics. Besides, loss of viable spermatozoa leads to poor fertility rate (45%) as compared to fresh semen (Akhter et al., 2010).

Trehalose is a non-permeable, non-reducing disaccharide, consisting of two glucose moie-

ties joined by an alpha-1, 1 glucosidic bond which prevents spermatozoa from the deleterious effects of dehydration (Aboagla and Terada, 2003). Several studies have been conducted on trehalose supplementation in semen extenders of cattle (El-Sheshtawy et al., 2015), goat (Atessahin et al., 2008), ram (Bucak et al., 2007), and buffalo (Reddy et al., 2010) to improve post-thaw semen characteristics. Different concentrations of trehalose (25-400 mM) have been used in several species to assess its effect on post-thaw semen characteristics such as in goat (Khalili et al., 2009), ram (Jafaroghli et al., 2011) and bulls (Hu et al., 2010; Ahmad and Aksoy, 2012). Results revealed an improved plasma membrane integrity (PMI) of spermatozoa while in buffalo bull semen, it reduced cryo-capacitation and maintained acrosomal integrity (Reddy et al., 2010). In buffaloes, various studies on trehalose have been conducted using tris-based extender (Badr et al., 2014; Iqbal et al., 2016), its use in lactose extender has not been reported yet. The present study was, hence, designed to: 1) study the influence of various concentrations of trehalose on buffalo bull spermatozoa extended in lactose-egg yolk- glycerol extender (LEYGE) and 2) compare the fertility rate between LEYGE supplemented with trehalose and traditionally used tris-citric-acid-fructose extender.

MATERIALS AND METHODS

This study was conducted in two phases. In the first phase, semen was extended in lactose egg yolk glycerol extender supplemented with trehalose at 0.0, 30.0, 50.0, and 70.0 mM concentrations and evaluated for post-thaw sperm characteristics (motility, viability, acrosomal integrity, PMI, DNA integrity and LPO). In the second phase, semen doses of LEYGE (supplemented with 70.0 mM trehalose) and traditionally used tris-citric-acid-fructose extender were used to inseminate multiparous buffaloes (n = 50/group) in heat for fertility trial in September/October. Semen collection, evaluation, processing, and cryo-preservation were carried out at Semen Production Unit (SPU), Qadirabad, District Sahiwal, Pakistan. Post-thaw semen evaluation was performed in post-graduate laboratory of the Department of Physiology, University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan. The fertility trial was executed in Sheikhupura District at artificial insemination (AI) Centers of Livestock and Dairy Development Department, Government of the Punjab, Pakistan. The study was approved in full by the Office of Research, Innovation, and Commercialization (ORIC) of UVAS, Lahore, Pakistan vide 20-133.

Extenders preparation

The LEYGE was prepared as described by Mughal et al. (2013). Briefly, a 100 gm of D-Lactose monohydrate was dissolved in distilled water to attain a final volume of 1000 mL. After vortexing, the osmotic pressure was maintained at ≥300 mOsm/kg. At the time of semen collection, benzyl penicillin and streptomycin sulphate (1000 IU/mL and 1000 µg/mL, respectively.) At each collection, LEYGE was divided into four parts, maintained at 37°C, and trehalose was added in three extenders at 30.0, 50.0 and 70.0 Mm, respectively, whereas the fourth extender was kept as the control group having no trehalose (0.0) in it.

Tris-citric-acid-fructose extender was prepared as: 24.2 g of Tris (Hydroxymethyl) aminomethane, (Research Organics Inc, Ohio, U.S.A.), 13.4 g of citric acid monohydrate (Riedel-de Haën, Germany) and 10.0 g of D (-) Fructose (Riedel-de Haën, Germany) were mixed in bi-distilled water to achieve 730 mL final volume. This solution was pasteurized at 65ºC for 30 minutes and then cooled down to 38ºC to add 200 mL egg yolk and 70 mL glycerol (Riedel-de Haën, Germany) and 1.0 g benzyl penicillin (Sinobiotic, Shanxi Shuguang Pharmaceutical Co, China). Extender was well mixed at 37ºC using a magnetic stirrer and stored overnight in a refrigerator at 4ºC and kept at 37°C before use.

Semen collection and processing

The semen of Nili Ravi buffalo bull (n = 4) was collected at a weekly interval for 7 weeks. Two ejaculates were collected from each bull at each collection. Ejaculates with >70% motile spermatozoa were chosen for further processing. Semen collection, evaluation,

processing, and storage were carried out as described earlier (Mughal et al., 2013). At each collection, equal volumes of ejaculates were pooled to eliminate individual differences and divided into five equal aliquots. Three aliquots were diluted with LEYGE supplemented with trehalose at 30.0, 50.0, 70.0 mM concentration, the fourth aliquot was diluted in LEYGE without trehalose as a control, whereas, the fifth aliquot was used to process semen doses extended in tris-citric-acid-fructose extender for fertility trial. A total spermatozoa concentration of 20×10^6 was maintained in each of the 0.5 mL straws.

Post thaw spermatozoa characteristics

The semen doses with trehalose in LEYGE, and control group were evaluated for various post-thaw attributes (motility, viability, acrosomal integrity, PMI, DNA integrity, and LPO) after thawing at 37°C for 30 seconds. motility rate of spermatozoa was assessed under a phase-contrast microscope as described, whereas viability rate was evaluated through vital staining (Khan and Ijaz, 2008). Assessment of acrosomal integrity and PMI was carried out using by counting spermatozoa having normal acrosomal ridge and hypo-osmotic swelling test, repectively to assess spermatozoa (Adeel et al., 2009).

Acridine orange staining technique was used to evaluate DNA integrity of spermatozoa under a fluorescent microscope (Labomed Lx 400, USA) as explained by Tejada et al., (1984). Heads of spermatozoa with green fluorescence were counted as having an intact DNA, whereas, heads with red fluorescence were counted as having a damaged DNA. The LPO was determined using thiobarbituric acid assay as described (Wadood et al., 2015). The absorbance of organic layer was estimated at 532 nm and results were expressed as nanomole of malondialdehyde.

Fertility trial

Frozen thawed semn samples were utilized for coductng in vivo fertility trial. Semen doses extended in LEYGE (supplemented with 70.0 mM trehalose) and traditionally used tris-citric-acid-fructose extender were used as treatment and control groups (n = 50 per group), respectively to inseminate the buffaloes in heat under field conditions to assess the fertility rate. Fifty buffaloes in each group were inseminated with semen doses, prepared under the same conditions by the same AI technicians to avoid any variation in the and were kept under the same environment throughout the trial. All the buffaloes were in their 2nd to 4th lactation ad were pluriparus. Thawing of semen straws was performed for 30 seconds at 37°C. The AI was done using an AI gun and the pregnancy test was carried out at day 50 ± 10 post-insemination through rectal palpation. Fertility rate attained at single insemination was recorded.

Statistical analysis

It was executed by using Statistical Package for Social Science (Version 13, SPSS Inc., USA). The results are presented as mean ±SE. The data were analyzed using a one-way analysis of variance (ANOVA). The difference in groups was compared by Duncan's Multiple Range Test. The difference in fertility rate was analyzed using chi-square. The fertility rate has been given along with a 95% confidence interval *i.e.*, $P \leq 0.05$.

RESULTS AND DISCUSSIONS

The results on post-thaw seminal attributes revealed that sperm motility, viability, acrosomal integrity, PMI and DNA integrity were significantly ($P \leq 0.05$) greater in LEYGE supplemented with 70.0 mM trehalose as compared to other groups (Table 1).

Table 1. Post thaw spermatozoa characteristics after supplementing lactose egg yolk extender with various concentrations of trehalose

Trehalose Concentration (mM)	Spermatozoa characteristic (%)					LPO (nm)
	Motility	Viability	Acrosomal Integrity	PMI	DNA Integrity	
0.0	49.3±2.4b	58.0±3.3b	69.9±2.1c	59.7±3.3c	96.0±0.2b	66.2±8.8b
30.0	48.3± 1.7b	59.8±2.9b	74.6±2.3a	60.3±2.3b	97.6±0.1b	66.7±11.0b
50.0	48.3± 1.5b	65.8±3.3a	73.8±1.9b	58.0±2.1c	98.0±0.3a	81.2±12.3a
70.0	51.0± 2.5a	63.2±2.3a	75.1±2.4a	62.0±2.5a	98.4±0.1a	57.33±9.1c

Values are represented as Mean ± S.E

Dfferent letters (a-c) within a column indicate indicate significan at $P \leq 0.05$.

Trehalose is a non-permeant disaccharide, found in various animals and plant tissues. It protects spermatozoa during osmotic changes and creates specific interaction with plasma membrane phospholipids to minimize the cell injuries caused by ice crystals before freezing (Bucak et al., 2007). The spermatozoa motility rate in 70.0 mM trehalose supplemented group (51.0 ± 2.54%) is comparable to those for buffalo bull semen supplemented with 50.0 mM of trehalose (51.25 ± 1.25%) in tris-based extender (Badr et al., 2010). However, other studies conducted by Uysal and Bucak (Uysal and Bucak, 2009) and Bucak et al. (Bucak et al., 2007) on ram semen supplemented with 50.0 mM of trehalose have reported a greater motility rate of 68.0 ± 2.9 and 59.0 ± 2.9%, respectively. The nature of extender used during dilution of semen has vital effects on post-thaw motility of spermatozoa. Another report supports this statement, as a significant drop in post-thaw motility rate was observed at 0 hr for buffalo semen extended in Andromed supplemented with 25.0 mM and 75.0 mM of trehalose (Piri et al., 2014). After using various concentrations of trehalose in this study, it is found that 70 mM trehalose concentration seems an optimal inclusion level of trehalose in LEYGE in terms of sperm post-thaw motility.

Spermatozoa viability also improved significantly (P>0.05) with the supplementation of trehalose at supplementation level of 70.0 mM (63.2 ± 2.3%). Our findings are in accordance with another study that reports 66.0 ± 4.4% viability of ram spermatozoa with the same concentration of trehalose while decreased viability rate compared to that of control group using tris-based extender (Bucak et al., 2007). These results are also in agreement with earlier findings in cattle bull semen using the same concentration of trehalose, which declined significantly as the concentration of trehalose was increased up to 100.0 mM and 200.0 mM (El-Sheshtawy et al., 2015). It is speculated that higher concentrations of trehalose may exert deleterious effects on spermatozoa viability rate. However, the chemical nature of extender, presence of antioxidants in seminal plasma, specie difference, and methods of handling semen might be some of the key factors responsible for these changes.

Spermatozoa with normal acrosome are of prime importance for successful acrosome reaction and fertilization (Bailey et al., 2000). In the present study, the addition of trehalose improved acrosomal integrity, and an increase (P≤0.05) was noticed for 70.0 mM trehalose being 75.1 ± 2.4%. As compared to our results, lower values in rams supplemented with 50.0 and 100.0mM of trehalose using tris extender have been reported being 50.4±0.68 and 38.5 ± 3.0% of spermatozoa with damaged acrosomes, respectively (Bucak et al., 2007; Tonieto et al., 2010). Another study (Iqbal et al., 2016) has revealed a 10.0% improved acrosomal integrity by using 30.0 mM trehalose supplementation in buffalo bull semen extended in tris-citric-acid-fructose extender compared to control group. However, further increase in trehalose concentration in groups supplemented with 45.0 or 60.0 mM of trehalose reduced acrosomal integrity rate. The results of the present study using trehalose supplementation support the finding of work conducted in rams also (Panyaboriban et al., 2015). Trehalose supplemented group showing improved acrosome integrity (5.52%) compared to the control group in the present study is a feature that needs to be assessed using lactose egg yolk glycerol extender in other species.

The plasma membrane is the exterior structure and protective barrier of spermatozoa. if damaged during semen cryopreservation, it exerts detrimental effects on spermatozoa capacitation, acrosome reaction, and sperm-oocyte fusion (Giraud et al., 2000). Structural changes in plasma membranes disturb spermatozoa viability, longevity, and fertility rate (Iqbal et al., 2016). Spermatozoa PMI being higher at 70 mM (62.0 ± 2.5%; P≤0.05)) in LEYGE supplemented by trehalose also depicted significant effects These findings are higher from those reported in rams which were 22.9 ± 1.8 and 49.0 ± 8.9% by supplementing 100.0 mM and 50.0 mM of trehalose, respectively (Bucak et al., 2007; Tonieto et al., 2010). Results of the present study are comparable to those in rams which had 66.1 ± 2.8% PMI of spermatozoa when supplemented with 50.0 mM of trehalose (Uysal and Bucak, 2009). However, while working on different Iberian red deer and stallions, other researchers have reported no effect of trehalose addition

(Fernández - Santos et al., 2007; Squires et al., 2004). On the other hand, a substantial improvement in PMI of buffalo bull semen has been reported elsewhere, though, their reported integrity rate was lower compared to our study (Iqbal et al., 2016). This variation in results may be due to the extender difference in both studies. It is speculated that trehalose renders the plasma membrane less prone to changes during water efflux by, by forming a hydrogen bond between the sugar hydroxyl and phospholipid polar group to substitute the water molecules under cryopreservation (Giraud et al., 2000).

Embryo development is negatively affected by spermatozoa DNA damage caused by cryopreservation. The damage exceeding 8%, is irreparable and might result in impaired development of the embryo and early pregnancy loss. Additionally, oxidative stress on spermatozoa during cryopreservation also supports DNA damage and alters spermatozoa head DNA (Anzar et al., 2002). In bulls, DNA integrity rate of 97% to 99% with high fertility has been reported (Bochenek et al., 2001). In present study, spermatozoa head DNA integrity rate significantly ($P \leq 0.05$) increased with trehalose supplementation at 70mM concentration (98.4 ± 0.1). Results of this study are comparable to another in which supplementing 25.0, 50.0 and 100.0 mM of trehalose and documented 2.64 ± 0.69, 2.56 ± 0.56 and 1.83 ± 0.71% spermatozoa with damaged DNA in buffalo bull semen, respectively (Badr et al., 2010). Similar results in buffalo spermatozoa extended in tris-citric-acid-fructose extender with 30.0 mM trehalose concentration have been also reported (Iqbal et al., 2016). Our results are also in line with another report that adding of trehalose in freezing extender reduced cryodamage of the buffalo sperm (Reddy et al., 2010). Exact mechanism liable for DNA damage is not understood. However, high ROS production as a result of antioxidant imbalance in seminal plasma and high contents of unsaturated fatty acids in sperm plasma membrane are believed to affect nuclear membrane and sperm DNA (Aitken & Krausz, 2001).

Cryopreservation exerts cold shock and oxidative stress, trehalose addition in semen extender can improve antioxidant action to protect spermatozoa plasma membrane and decrease LPO (Aisen et al., 2005). The spermatozoan cryoprotective capacity varies with the concentration of trehalose supplementation in the extenders (Naing et al., 2010). In our study the LPO was significantly ($P \leq 0.05$) lower at 70 mM supplementation of trehalose being 57.33 ± 9.1 nM. This indicates that minimum ROS production took place at this concentration and, hence, stands for good semen quality (Chaudhari et al., 2008).

Based on the influence of various concentrations of trehalose on buffalo bull spermatozoa extended in LEYGE, 70mM was considered most optimal and was carried forward for fertility trial in the present study. In all the SPUs of Pakistan, tris-citric-acid-fructose extender is being used for AI purposes. Hence, a comparison of fertility rate was made between semen doses extended in LEYGE supplemented with 70.0 mM trehalose and traditionally used tris-citric-acid-fructose extender. The fertility rate of inseminated animals through rectal palpation at day 50 ± 10 post-insemination was 38% with routinely used tris-citric-acid-fructose extender and 54% with LEYGE supplemented with 70.0 mM of trehalose (Table 2) being significantly ($P \leq 0.05$) different.

Table 2. Comparison of pregnancy rate in groups inseminated with semen extended in tris citric acid fructose extender and LEYGE supplemented with trehalose

Group	Number of Inseminations	Pregnant (%)	Non-pregnant (%)	95% CI	Chi Square Value	P-value
1*	50	19 (38%)	31 (62%)	24.55-51.45	2.576	0.005
2**	50	27 (54%)	23 (46%)	40.19-67.81		

*Tris citric acid fructose extender
**LEYGE (supplemented with 70.0 mM trehalose)

Microscopic evaluation of cryopreserved semen using different protocols or advanced computer software is not an alternative for estimating fertility through AI. Fertility is dependent on various factors including accuracy of heat detection, time of insemination, and inseminators skill. During the present study, the fertility rate of the trehalose-supplemented group (70.0 mM) was statistically ($P \leq 0.05$) higher as compared to the traditionally used tris-citric-acid-fructose

extender group. The fertility rate of this study is comparatively lower than that of 69.2% in buffalo with tris-citric-acid-fructose extender (Anwar et al., 2008). However, current results for the trehalose-supplemented group are in accordance with the fertility rate (58.55%) reported in buffaloes extended in skimmed milk and better (45.8%) (Akhter et al., 2007). Many factors including milk production, species difference, body condition of the animal, heat detection, lactation state, semen quality, inseminator skills and time of insemination are critical to improving the pregnancy rate of buffalo under field conditions through artificial insemination (Anzar et al., 2003).

CONCLUSIONS

An optimal inclusion level for trehalose in LEYGE in respect to seminal attributes is 70mM. Furthermore, LEYGE supplemented with 70 mM trehalose presented higher fertility rate as compared to traditionally used tris-citric-acid-fructose extender under field conditions. The additive effect of trehalose and fructose needs to be studied in future with a larger sample size and over a longer period of time.

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