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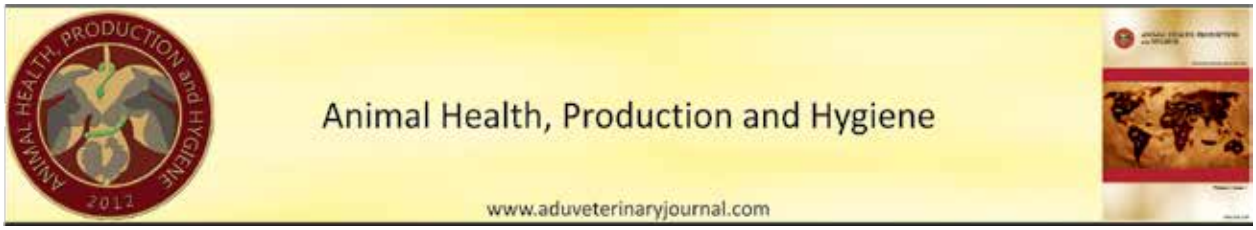

Short Paper

High Trehalose Concentration in Tris-Based Egg Yolk Extender has Detrimental Effect on Post-Thaw Semen Quality in Nili-Ravi Buffalo Bull

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A B S T R A C T

Background/Aim: The use of artificial insemination with cryopreserved semen is potential tool for breed improvement in buffalo production. However, the fertility with the use of cryopreserved semen is approximately 40% in buffaloes. It is the general opinion that a substantial number (50%) of sperm damaged or dead during freezing and thawing process, is the potential cause of low fertility. The aim of the present study was to determine the effect of high trehalose concentration in Tris-based egg yolk extender on post-thawed semen quality in buffalo bull. **Material and Method**: Semen ejaculates (n=04) collected from two mature buffalo bulls were extended with E1 (Tris-citric acid-fructose (TCF) with 7% glycerol), E2 (TCF plus 236 mM trehalose) or E3 (E2 plus 7% glycerol). All the extenders contained egg yolk (20%, v/v). After dilution, samples were cooled to 4°C and equilibrated for 2 hrs at 4°C. Samples were packed in 0.5 ml straws, frozen and stored in liquid nitrogen. Frozen straws were then thawed at 37°C for 20 sec. Motility was observed after dilution, equilibration and post-thawing. Plasma membrane integrity and percent live sperm were determined after thawing by using hypo-osmotic swelling assay and eosin-nigrosin staining, respectively. **Results and Conclusion**: The results showed that the motility was decreased ($p<0.05$) after dilution, equilibration and post-thawing in E2 and E3 compared to E1. Similarly, significanty ($p<0.05$) low percentages of live sperm and plasma membrane integrity were observed in extenders E2 and E3. It is concluded that high concentration of trehalose in Tris-based egg yolk extender has detrimental effect on sperm quality during cooling, freezing and thawing process in buffalo bulls.

Keywords: Buffalo semen, Trehalose, Glycerol, Post-thaw semen quality.

Tris-Yumurta Sarısı Sulandırıcısına Yüksek Düzeyde Trehaloz İlavesi Nili-Ravi Manda Spermalarında Çözüm Sonu Parametreleri Olumsuz Etkiler

Ö Z E T

Özbilgi/Amaç: Dondurulmuş sperma ile suni tohumlamanın manda yetiştiriciliği ve üretiminde kullanımı önemli bir yöntemdir. Buna karşın, mandalarda dondurulmuş sperma ile fertilite oranı yaklaşık %40 dolayındadır. Fertilitedeki düşüş genel olarak dondurma ve çözdürme sırasında spermanın %50'sinin hasar görmesine yorumlanmıştır. Bu çalışmada yüksek trehaloz yoğunluklarının mandalarda çözüm sonu sperma kalitesi üzerine etkisinin belirlenmesi amaçlanmıştır. **Materyal ve Metot:** Toplam iki baş erişkin manda boğasından alınan sperma (n=04) örnekleri E1 (Tris-sitrik asit-fruktoz+%7 gliserol (TCF), E2 (TCF+236mM trehaloz) veya E3 (E2+%7 glycerol) sulandırıcılarıyla sulandırıldı. Bu üç sulandırıcı da %20 (v/v) oranında yumurta sarısı içermekteydi. Daha sonra örnekler 4C°'ye kadar soğutuldu ve bu sıcaklıkta iki saat süre ile ekilibre edildi. Sperma örnekleri 0,5 ml lik payetlere çekildi ve sıvı azot içerisinde dondurularak saklandı. Payetler 37C°'de 20 saniyede çözdürüldü. Motilite değerleri sulandırma, ekilibrasyon ve çözüm sonrasında belirlendi. Plazma membran bütünlüğü ve canlı spermatozoon oranları, sırasıyla, hipoozmotik şişme testi ve eosin-nigrosin boyama yöntemiyle belirlendi. **Bulgular ve Sonuç**: Sulandırma, ekilibrasyon ve çözüm sonu motilite değerleri E2 ve E3 sulandırıcılarında E1'e göre daha düşük olarak belirlendi ($P<0.05$). Benzer şekilde, E2 ve E3 sulandırıcılarında dondurulan örneklerde sağlam membranlı canlı spermatozoon oranlarının da daha düşük olduğu görüldü. Sonuç olarak, yüksek trehaloz yoğunluklarının manda spermalarında çözüm sonu parametreleri olumsuz etkilediği kanısına varılmıştır.

Anahtar kelimeler: Manda sperması, Trehaloz, Gliserol, Çözüm sonu sperma kalitesi.

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Introduction

The use of artificial insemination with cryopreserved semen is potential tool for breed improvement in buffalo production. However, the fertility with the use of cryopreserved semen is approximately 40% in buffaloes. It is the general opinion that a substantial number (50%) of sperm damaged or dead during freezing and thawing process, is the potential cause of low fertility (Watson, 2000). The major site of cryodamage is sperm plasma membrane during freeze-thawing process. So the addition of cryoprotectants to extenders is essential in order to reduce the damaging effect of freeze-thawing process. Sugars have several functions in sperm extender, including providing energy substrate for the sperm cell during incubation, maintaining the osmotic pressure of the diluent, and acting as a cryoprotectant (Watson, 1979). Trehalose, a non-reducing disaccharide, has a protective role against osmotic effect and forms specific interactions with membrane phospholipids, rendering hypertonic media, causing cellular osmotic dehydration before freezing, and hence decreasing the amount of cell injury by ice crystallization (Ahmad and Aksoy, 2012). The beneficial effects of the addition of trehalose to the extender on the post-thaw viability of mammalian sperm have been reported in many studies (Molinia et al., 1994; Khalili et al. 2009; Reddy et al., 2010; Jafaroghli et al. 2011).

In contrast to these proofs in support of the advantageous effects of trehalose, some other studies showed its minor or no effect on cryosurvival rate of sperm (Chen et al., 1993; Liu et al., 1998). Similarly, there are conflicting reports on the high trehalose concentrations in semen extenders. In one hand, the high trehalose concentration enhanced the post-thawing semen parameters of goat sperm (Aboagla and Terada, 2003), whereas, on the other hand high trehalose concentrations in semen extenders showed deleterious effect on ram sperm during cooling process (Aisen et al., 2002). These promising but somewhat conflicting results warrant more detailed investigation on the effects of trehalose on the survival of bull sperm during freezing-thawing process. The present study was therefore conducted to examine the influence of high trehalose concentration in the presence or absence of glycerol in Tris-based extender on the viability of frozen-thawed buffalo bull sperm, evaluated in terms of motility, plasma membrane integrity and vitality.

Materials and methods

Preparation of semen extenders

Three extenders; E1 (199.7 mM Tris, 63.76 mM citric acid, 55.37 mM fructose and 7% glycerol), E2 (Tris-citric acid-fructose plus 236 mM trehalose) and E3 (extender E2 plus 7% glycerol) were prepared. All three extenders contained egg yolk (20%, v/v), penicillin (1000 IU/mL) and streptomycin (100 ug/mL) and were adjusted at pH 7.0.

Semen collection

Semen from two Nili-Ravi buffalo bulls was collected at weekly interval with the help of artificial vagina (42°C) during the months of May and June, 2009. The bulls were maintained at Department of Theriogenology, University of Veterinary and Animal Sciences, Lahore, Pakistan. Ejaculate (n=04) with motility more than 60% were used for further processing. Semen was kept at 37°C for 15 min before further processing.

Semen processing

Semen sample was divided into three aliquots and extended with E1, E2 and E3 (1:10) at 37°C. Motility was evaluated immediately after dilution. The extended semen was cooled to 4°C in 2 hrs and then equilibrated for 2 h at 4°C. Motility was evaluated after equilibration as well. After equilibration semen was filled into French straws (0.5; IMV, L'Aigle, France). The straws were then frozen in vapors 4.5 cm above the level of liquid nitrogen (-120°C) for seven min and then plunged into liquid nitrogen (-196°C) for storage (Hu et al., 2010).

Table 1. Effect of high trehalose concentration in extender on post-thawing semen parameters in buffalo bull
Tablo 1. Yüksek konsantrasyondaki trehaloz'un farklı sulandırıcılarda bulunan boğa spermlerinde çözdürme sonrası sperma parametreleri üzerine etkisi

Variables	E1	E2	E3
Motility after dilution (%)	68.3 ± 1.6^{a}	46.6 ± 8.8^{b}	55.0 ± 5.0^{ab}
Motility after equilibration (%)	61.6 ± 1.6^{a}	15 ± 2.8^{b}	30.0 ± 5.0^{c}
Post-thaw motility (%)	48.3 ± 1.6^{a}	3.3 ±1.6^{b}	10.0 ± 2.8^{c}
Live sperm (%)	49.3 ± 1.7^{a}	6.33 ± 2.3^{b}	14.33 ± 3.9^{c}
Plasma membrane integrity (%)	31.3 ± 3.2^{a}	6.0 ± 1.0^{b}	6.0 ± 2.0^{b}

Different superscripts within the same row are significantly different ($p<0.05$)

Post-thaw semen evaluation

Assessment of post-thaw motility

To evaluate sperm motility, a small drop (10 µl) of frozen-thawed semen was placed on a pre-warmed (37°C) slide, covered by a coverslip and examined under a microscope equipped with phase contrast optics (400×) and warm stage.

Sperm Vitality

A small drop of frozen-thawed semen was placed on a pre-warmed glass slide and mixed with a relatively larger drop of the 1% eosin-nigrosin stain by an applicator stick and a thin and uniform smear was made. After air-drying, the smear was observed under bright field microscope at 400× for unstained heads of sperm (live) and stained/partial stained heads of sperm (dead) as described previously (Ahmed et al., 2011).

Plasma Membrane Integrity

Plasma membrane integrity (PMI) of sperm was assessed using an HOS assay as described previously (Ahmad et al., 2011). Briefly, sodium citrate (0.735 g; Merck) and fructose (1.351 g; Merck) were dissolved in 100 mL distilled water to prepare a HOS solution (osmotic pressure ~ 190 mOsmol/kg), and maintained at 37°C for 5 minutes before use. Fifty micro liters of each frozen-thawed semen sample was mixed with 500 µl of HOS solution and incubated at 37°C for 30 minutes.

After incubation, a 10 µl semen sample was examined under a phase-contrast microscope at 400× magnification. One hundred sperm were assessed for their swelling ability in HOS. The swollen sperm characterized by coiling of the tail were considered to have an intact plasma membrane .

Statistical Analysis

The data were analyzed by one way analysis of variance (ANOVA), and Fisher protected least-significant difference (LSD) post-hoc test using SPSS software (version 10.0). Data are presented as means ± SEM. A probability of $P<0.05$ was considered to be statistically significant.

Results

Data for all the parameters (motility after dilution, equilibration, and post-thaw, percentages of live sperm and plasma membrane integrity) are given in Table. 1. The results revealed that the use of a Tris-based trehalose supplemented extenders E2 and E3 led to decreased ($p<0.05$) percentages of motility after dilution, equilibration and post-thawing when compared with E1. Similarly, significant ($p<0.05$) decrease was observed in the percentages of live sperm and plasma membrane integrity in E2 and E3 extenders compared to E1 extender.

Discussion

In the present study, deleterious effect of high (236 mM) trehalose concentrations was observed on pre-freezing and post-thawing semen quality in Nili-Ravi buffalo bulls. This is in confirmation with previous study in ram (Aisen et al., 2005) where 200 and 400 mOsm trehalose showed negative influence on post-thawing motility and plasma membrane integrity. Similar observations have been made in other studies in bull and ram that trehalose concentration more than 100 mM (Aisen et al., 2005; Uysal and Bucak, 2009) did not improve the post-thaw sperm quality. The mechanism of this negative influence of high trehalose concentration is not clearly understood; however, it can be postulated that high trehalose concentration made the media hypertonic and therefore increased the external pressure on sperm. As a result, the sperm shrinks down leading to decreased cell volume by compression of its content and an efflux of water. Enough shrinking might be at least responsible for altering cell architecture by membrane protein denaturation, lipid phase transitions and reducing membrane fluidity.

The beneficial effects of the trehalose on the post-thawing viability of sperm has been reported in bovine bull (Hu et al., 2010; Chhilar et al., 2012) buffalo bull (Reddy et al., 2010; Badr et al., 2010; Kumar et al., 2013) ram (Bucak et al., 2007; Khalili et al., 2009) and goat (Quan et al., 2012). All these studies suggested that a certain level/concentration (50-100 mM) of trehalose provide the beneficial effects during cryopreservation. In contrast Aboagla and Terada (2003) noted that high concentrations (375 mM) of trehalose to sperm extender provide the best protection with regard to post-thaw motility parameters, recovery rates, thermal resistance, and acrosome integrity in goat. Discrepancies may in part be due to the fact that the substitution of TCG with trehalose solution (375 mM trehalose with 20% egg yolk and 8% glycerol) was very different from that used in the present study.

In conclusion, it can be explained that 236 mM trehalose in semen extender exhibited toxic effect on sperm parameters during cooling, freezing and thawing process.

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