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Pores Formation in Porcine Sperm Incubated in Streptolysin O and Its Post Thawing Viability after Trehalose Treatment

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ABSTRACT

Background: Streptolysin O (SLO), a pore-forming protein in plasma membrane (PM), has been used to internalize a variety of molecules (DNA and RNA) in cells. In sperm, however, SLO has only been used to release acrosomal contents. Its possible use as biotechnology in the cryopreservation of pig semen. However, porcine sperm are very sensitive to the freeze-thaw process. The study aimed to evaluate the pore formation in the PM, the addition of trehalose and the post cryopreservation viability of porcine spermatozoa using SLO.

Methods: Research period was spring 2017- summer 2018. Thirty ejaculates from five mature boars were used. Semen was incubated in SLO 0.6 IU/ml and trehalose (added at 100, 200 and 400 µM). Semen diluted in commercial diluent as control group. Presence of pores was checked by scanning electronic microscopy. To evaluate sperm membrane integrity and functional status the Coomassie stain with HOST test and the Chlortetracycline test were used.

Result: It was found that SLO could form pores in the sperm cell membrane The addiction of 200 µM trehalose to the freezing medium have different effects on the quality of boar sperm, showing highest motility and viability during the cooling process.

Key words: Boar semen, Cryopreservation, Permeabilization, Streptolysin O, Trehalose.

INTRODUCTION

Conservation of sperm for a long period is one of the reproductive tools with greater potential in the area of medicine and animal production. Semen cryopreservation is a very useful technology for storing gametes and achieving massive distribution of genes allowing the amplification of desirable reproductive traits (Toker *et al*., 2016). Different freezing methods have been studied for conserving mammalian spermatozoa. However, according to protocols, results are still deficient due to damage by cold shock, oxidative stress and cryocapacitation, which increases cell death (Chutia *et al*., 2014). Freezing of porcine semen is an active area of research where many attempts have been made to understand and avoid harmful conditions during cryopreservation. As a result, the formation of ice (Córdova *et al*., 2001), oxidative stress (Agarwal *et al*., 2012) and cryocapacitation had been studied (Atroshchenko and Bragina, 2011). Many protocols (with cryoprotectants that do not penetrate the sperm membrane) to avoid or decrease these damages have been developed and tested. Even though such improvements are not negligible, more additional studies are needed (Athurupana and Funahashi, 2016).

Trehalose is a non-permeating cryoprotectant with dehydration-protection property, which minimizes the probability of formation of the critical gel phase, ice crystals and loss of cell integrity after rehydration during frozen-thawed procedure. Because this attributes, trehalose it has been used like cryoprotectant in diverse preservation and storage of biological samples protocols (Malo *et al*., 2010; Men *et al*., 2013; El-Sheshtawy *et al*., 2015). On the other hand, membranes permeabilization has been widely used in different mammalian cells to introduce extrinsic molecules into the intracellular compartment (Brito *et al*., 2008; Pocognoni *et al*., 2013; Sim *et al*., 2013). For this purpose, some cholesterol-dependent cytolysins (CDC) have been used. Some CDCs have been tested on sperm, such as Streptolysin O in mice and humans (Johnson *et al*., 1999; Yunes *et al*., 2000) and Perfringolysine O (PFO) in humans (Pocognoni *et al*., 2013). Results of these studies show that SLO is a molecule that shows potential for biomedical studies of the sperm cell and possess a high potential for the genetic and/or structural modification of this gamete. In this sense, trehalose has properties that make interesting its use as an internal cryoprotectant of the sperm cell. Therefore, the present study was undertaken to assess the viability of the use of Streptolysin O and trehalose into sperm cells and determine their effect on sperm cell functionality.

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MATERIALS AND METHODS

Semen collection and semen processing

Research period was spring 2017- summer 2018. The experiment was conducted in the Laboratory of Biology of Animal Reproduction, School of Veterinary Medicine and Animal Science, University of Veracruz, Veracruz city, Mexico. Thirty ejaculates from five mature boars were used. Semen sperm-rich fraction (80-120 ml) was collected by the gloved hand technique (Bottini-Luzardo *et al*., 2012). After collection, the quality of the fresh semen samples was evaluated. Sperm concentration analysis was performed by counting cells in a Neubauer chamber as described by Gutiérrez-Pérez *et al*. (2009). After counting, semen was diluted (1:1) using a commercial long-term boar semen diluent (Vitacem LD 40g/1L H_2O format, from Megapor®, Spain) and finally storage for 24 h at 16°C until use.

Analysis of sperm motility and viability

Mass motility was evaluated by visual estimation of the percentage of spermatozoa showing progressive motility between 0 and 100% (Maxwel and Evans, 1990). Samples exhibiting values ≥70% progressive motility were selected for the study (Dziekońska and Strzezek, 2011). Viability and morphology were assessed utilizing Eosin-Nigrosin staining (Sigma-Aldrich, St. Louis MO, USA) (Björndahl *et al*., 2003).

Treatments

After 24 h in storage (16°C), each sample (in fractions of 2 ml [1 x 10^{-8}/ml]) received the following treatments.

T_1: Incubation in a SLO (Sigma-Aldrich, St. Louis MO, USA) solution (0.6 UI/ml) and trehalose (Sigma-Aldrich, St. Louis MO, USA) at 100 µM/ml for 5 minutes at 37°C.

T_2: Incubation in SLO (0.6 UI/ml) and trehalose at 200 µM/ml for 5 minutes.

T_3: Incubation in SLO (0.6 UI/ml) and trehalose at 400 µM/ml for 5 minutes.

T_4: Control group (no treatment just diluted semen).

The incubation in SLO was intended to permeabilize the sperm membrane (Valdés *et al*., 2015).

Pores and post-thawing viability evaluation

The observation of pores was done by scanning electronic microscopy (JEOL-JSM-5410-LV, USA) (Metkar *et al*., 2015). At the same time, trehalose was added during the incubation to evaluate post-thawing viability. Cryopreservation was made after pore sealing in accordance to Fawcett *et al*. (1998) using the two-step freezing protocol proposed by Westerndorf (Gutiérrez-Pérez *et al*., 2009). For the control group, the diluent contained 20% egg yolk and 230.8 mM glucose (as a replacement for trehalose). After 15 days of cryopreservation, straws were thawed for 30s in a water bath at 37°C. Then straws were held over liquid nitrogen vapors (4 cm) for 20 min before plunging them into liquid nitrogen. This procedure allowed to reach the optimal rate reported for boar sperm freezing of -30°C/min. Straw contents were placed in tepid assay tubes, previously supplemented with extender in a 1:6 v/v thawed semen/extender proportion. Samples were maintained 10 min at 37°C before evaluating the effect of temperature on motility.

Membrane integrity evaluation

Hypo-osmotic swelling test (HOST) in combination with Coomassie Bright Blue (CBB) (Sigma-Aldrich, St. Louis MO, USA) were used to evaluate the integrity of the membrane (Oliveira *et al*., 2013). Both tests were carried out in at least three replicates by the same researcher on a 400X magnification with a minimum of 200 cell counts per reading.

Sperm functional status

The functional status of spermatozoa was assessed with Chlortetracycline (CTC) (Sigma-Aldrich, St. Louis MO, USA), a fluorescent antibiotic (Álvarez-Guerrero *et al*., 2016). This assay allows observing changes in the sperm plasma membrane-associated with the capacitation status of the sperm. The sperms were stained during 30s and then fixed with 22 µl of 0.2% glutaraldehyde. Finally, 10 µl of the fixed sperm solution was placed on a glass slide with an equal amount of DABCO® (Sigma-Aldrich, St. Louis MO, USA) and then covered with a coverslip. Samples were evaluated using an epi-fluorescence microscope (Leica DM 020-518500/LS) with filter blue at 405-455 nm, 400X magnification.

Statistical analysis

Data in each treatment were compared using the non-parametric module of STATISTICA V10.0. Kruskal-Wallis H test was performed to determine effects among treatments and to test if a group of data came from the same population.

RESULTS AND DISCUSSION

The analysis of scanning electron microscopy of a sperm sample incubated with SLO allows the observation of structures on the spermatic surface (Fig 1). Vesicular and circular forms are seen in the head and tail. Fig 2 shows the

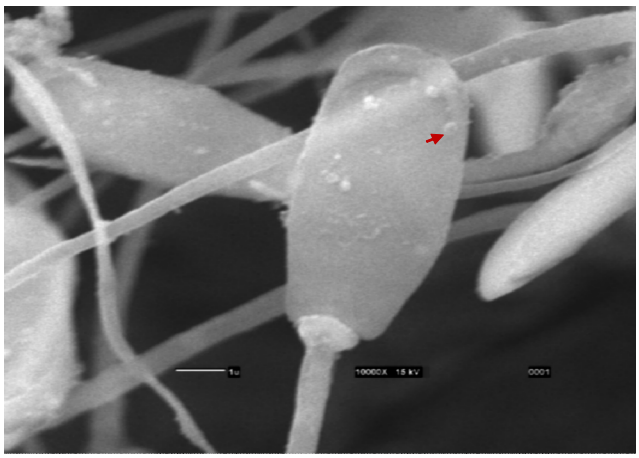

Fig 1: Photograph of a porcine sperm cell, obtained by scanning electron microscopy 10000x. The → indicate the sites of formation of structures similar to those formed in cells treated with Streptolysin O.

effect of trehalose on sperm cell membrane integrity after post-thawing, in which shows a significant difference (P<0.05) between cells with intact and damaged membrane. Fig 5 indicates the effect of the trehalose concentration on the acrosomal reaction state; there is a significant difference (P<0.05) in treatments between the percentage of no capacitated spermatozoa with intact acrosome and the percentage of cells capacitated with acrosomal reaction. During capacitation, sperm undergo a change in the motility pattern called hyperactivation and become capable to go through a physiological exocytotic process known as acrosome reaction (Puga *et al*., 2018), both required (and important) for successful fertilization (Darszon *et al*., 2011). They are indicators of the viability of frozen-thawed sperm. Numerous research studies have been carried out using the CDC as a tool of reversible permeabilization in different cell types (Fawcett *et al.,* 1998; Brito *et al.,* 2008), including the sperm cell (Pocognoni *et al*., 2013; Sim *et al*., 2013); however, there is little information available on SLO permeabilization in porcine spermatozoa (Sim *et al.,* 2013). In the present study, 0.6 IU of SLO was used to permeabilize the plasma membrane of the spermatozoa, in the presence of 200 µM of trehalose. Works in mouse and porcine spermatozoa, concluded that the optimum and safe (without loss cell viability) permeabilization, was achieved at 0.6 IU/ml of SLO (Johnson *et al*., 1999; Valdés *et al*., 2015).

The evaluation of the plasma membrane/functional integrity of cells with the HOST test indicates that the 200 µM treatment obtained the highest percentage of sperm quality (Fig 2). It has been shown that 100 µM treahalose used externally improves the viability and parameters of *in vitro* fertilization (Malo *et al*., 2010). It has also been observed in bovine semen that the addition of 50-100 µM of trehalose in the freezing medium improves its viability (El-Sheshtawy *et al*., 2015). The addition of trehalose in the freezing medium possess an antioxidant activity, enhancing the viability and fertility of semen (Perumal *et al*., 2015; Iqbal *et al*., 2016).

For the specific case of the acrosome status (damaged), there was a significant difference in each treatment (Fig 3). The treatment using 200 µM presented the lower percentage of damage with 35% (Fig 3). Our results are in contrast with those by Gutiérrez-Pérez *et al*. (2009), where they reported 10.9% acrosomal integrity in pigs and bulls respectively. Nonetheless, these studies used higher concentrations of trehalose and only freezing diluent was used without a previous cell permeabilization. This disaccharide can provide a greater stability and produce less damage when the cell is defrosted, which in turn influences the percentage of cells with integral acrosome and good motility (Fig 4). Disaccharides such as trehalose, sucrose and maltose have been reported to reduce the number of dead spermatozoa and/or damaged acrosome rates (Yildiz *et al*., 2000; Hu *et al*., 2010; Ahmad *et al*., 2014).

The capacitation status and acrosomal reaction of the spermatozoa had similar results to those of sperm with an

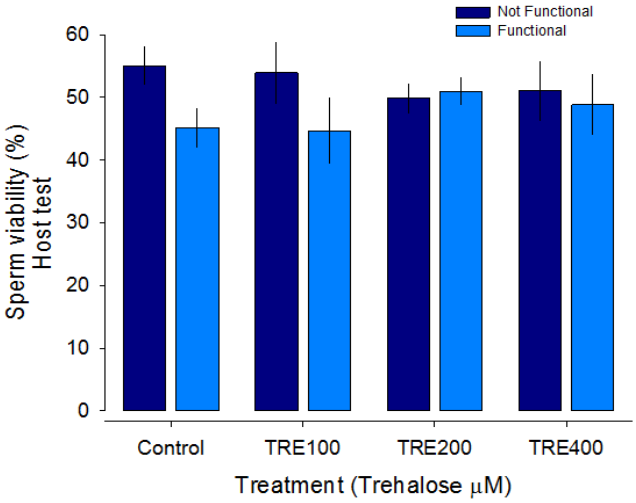

Fig 2: Effect of Trehalose concentration on the functional membrane state.

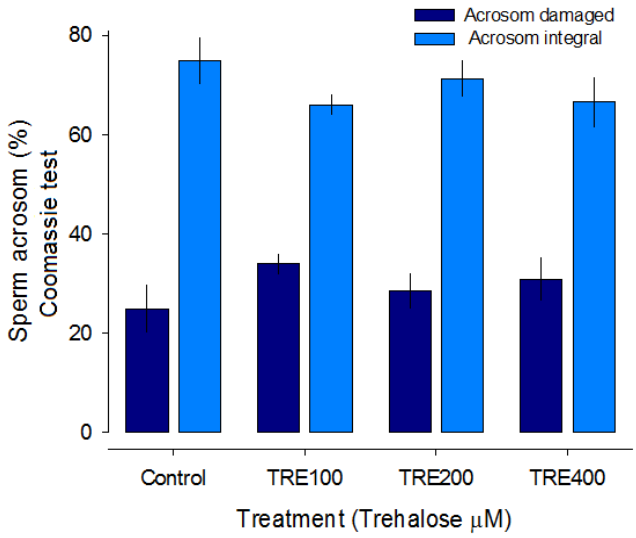

Fig 3: Effect of Trehalose on the acrosome status.

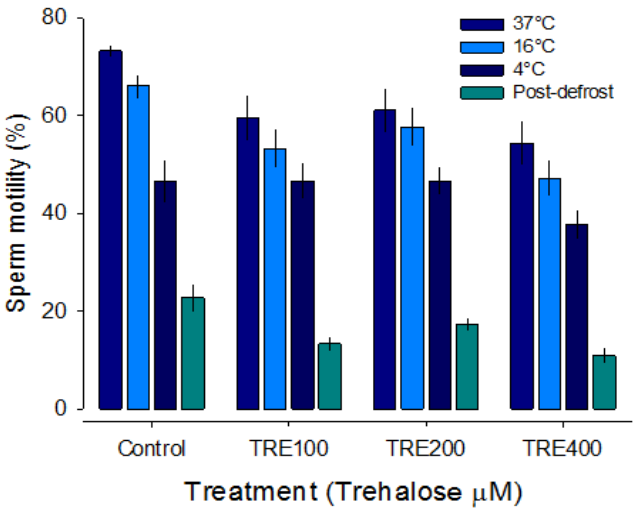

Fig 4: Effect of Trehalose concentration on sperm motility.

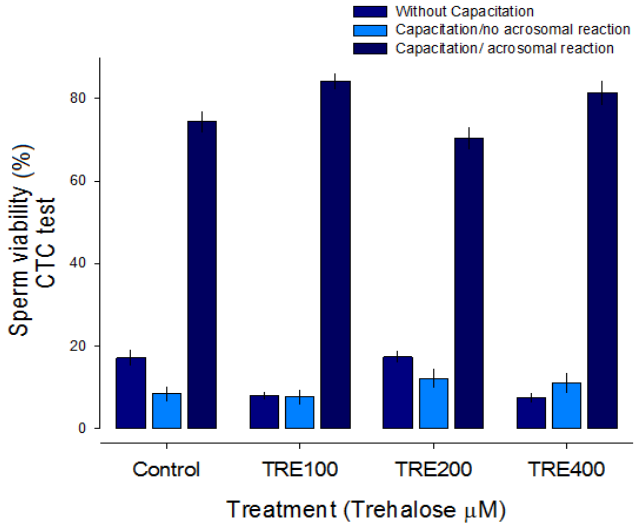

Fig 5: Effect of Trehalose concentration on sperm capacitation status and acrosomal reaction.

intact acrosome. The treatment at 200 µM showed the highest percentage of spermatozoa without capacitation with 16.8% and 11.1% of sperms capacited but without acrosomal reaction (Fig 5). Silva *et al*. (2015) reported a 37.2% of cells with intact acrosome. They noted the relevance of using a defreezing curve in other to allow trehalose to get better results. This is a variation of the feeezing process, similar to the method utilized in the present study. The use of different extenders, the combined use of lactose, trehalose and glycerol can provide the best quality of sperm and suggests that there may be important differences between animal species (Karunakaran *et al*., 2018).

CONCLUSION

The use of cryoprotectants is an important parameter influencing sperm cell viability during frozen-thawed process. The addition of trehalose to the freezing medium have different effects on the quality of boar sperm. A suitable dose decreases the effects of low temperatures on sperm quality during freezing preservation compared with a control group. The T2 group has almost the same sperm motility than the control group and longer survival time than the other groups. Moreover, this research shows that SLO was able to form pores in plasmatic membrane of the porcine sperm cell.

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