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# Spermatic quality after thawing of pig semen treated with Streptolisin and Trehalosa

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## Abstract

As it is known boar semen, is more sensitive to the frozen-thawed process. The aim of this study was to improve the boar semen viability after the frozen – thawed process, this through the trehalose expositiotion in the cytoplasm of the boar sperm, by the formation of pores by the streptolysin–O (SLO) toxin. First, the cells were exposed to different concentrations of SLO (0.2 U/ml, 0.6 U/ml, 1U/ml and 5U /ml) to determinate the SLO concentration, that allows to maintain the sperm viability. The cells treated with 0.6 U/ml concentration of SLO showed the best membrane integrity percent (57.44% ± 2.26). Besides, the percentage of living sperm with intact acrosome, was higher in the cells treated with 0.6 U/ml SLO (63.72 % ± 1.75); therefore this SLO concentration was selected to iexpose to the trehalose the spermatic samples, wich would be cryopreserved. After the thawing, the treatment with 100 mM trehalose showed 40.61±3.08% the sperm with intact acrosome, while the control samples 13.61±3.08% ($P<0.05$). However, there were not differences ($P>0.05$) between 100 mM trehalose treatment (7.83±0.76) and the control (9.89±0.76). In spite of the last result, the presences of trehalose on of the cryopreserved boar sperm, allowed to conserve a higer percentage of alive sperm. The incubation with SLO and trehalose (100 mM) of the boar sperm, increased the acrosome integrity after to the frozen-thawed process and maintained the viability. But is necessary more studies for determines, the effects over fertility.

**Keywords:** Trehalose, Streptolysin O, Frozen-thawed, Viability, Acrosome

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## Introduction

Currently, cryopreservation is the only procedure that exists to preserve sperms for long time period. There are some advantages in the use of frozen semen compared with the use of fresh semen, for example: a) the establishment of semen banks; b) dispose of genetic material, even if the animal no longer exists; c) overcome time limitations of the viability of diluted fresh semen (Grobfeld et al., 2008). These advantages allow to use frozen semen widely in several species, but in pigs is limited. The lower costs and good results with

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liquid semen, are some of the reasons of the limited use of frozen semen. On this respect, there is a reduction of the rates of fertility and prolificacy, when frozen semen is utilized, in comparison with the use of fresh sperm. It has been suggested that lower fecundity rates, is associated with molecular changes in plasmatic membrane of the spermatozoa head and the production of reactive species oxygen, leading to instability of the membrane and a similar to the capacitation state after thawing (Breininger et al., 2005). Also, it is well known that diluent, the kind of cryoprotector and the frozen-defrosting method, are important factors on the success of the semen cryopreservation. But nevertheless, it is not yet clear which part of the process affects mainly the spermatozoids (Saravia et al., 2007). The protocol commonly used for the freezing of boar semen, (Westerdof et al., 1975) consists of a two-step dilution method, using a diluent based on egg yolk. The first step consists of diluting semen with an extensor without glycerol and cooled at 5°C, the second step is to mix it with a diluent that contains a 9% concentration of glycerol, resulting in a final concentration of 4.5%. However, several studies has demonstrated that glycerol, despite its effect cryoprotector, is metabolically toxic for the spermatozoids (Fahy, 1986). In this regard, it has been proposed that glycerol and other penetrating cryoproectants are osmotically active and, it changes the contents of water, causing stress when it comes in or out of the cell (Kheirolomoom et al., 2005). Likewise, the beneficial effects of sugars as mammal spermatozoid cryoprotectants, have already been reported (Garde et al., 2009). The egg yolk with diluted lactose is the most used media for freezing boar semen (Westerdof et al., 1975). However, recently, there have been other studies conducted to examine the effects of other sugars (Gutierrez-Perez et al., 2009). Trehalose and sacarose are disaccharides, which apparently protects the cells, because of the increment of tonicity and the stability of the plasmatic membrane. This may be due to the direct interaction with the polar head of the phospholipids membrane (Crowe et al., 1987). Disaccharides are effective in the stabilization of cellular membrane bilayer and the spermatic metabolism can be kept better in diluents containing such sugars (Aisen et al., 2005). The lactose, sucrose, rafinose, trehalose and dextrose, are sugars cannot spread through plasmatic membrane; when used in diluents. They generate osmotic pressure, induce dehydration and ultimatly reduce the formation of intracellular ice. These sugars interact with membrane phospholipids increasing the spermatic survival in the freezing process (Aisen et al., 2002). In the last decade trehalose has been included in the sperm cryopreservation, in some species such as goats, sheep and cattle. The addition of this disaccharide in diluents improves protection, which has been registered as motility parameters, recovery rates, heat resistance and acrosome integrity (Aboagla and Terada, 2003). This sugar increases membrane fluidity which leads sperms to a higher resistance to damage post-defrosting (Woelders et al., 1997). On the other hand, the addition of trehalose for freezing boar semen, improved significantly sperms viability, evaluated in vitro, achieving higher survival and fertility rates (Malo et al., 2010). On the other hand, the streptolisine (SLO) is a citolytic toxine produced by streptococcus. The SLO sticks to cholesterol in the citoplasmatic membrane of animals' cells (Palmer et al., 1998) resulting in reversible pores in the structure. This particularity has been extensively useful for the introduction of extrinsic molecules like proteins, DNA and RNA inside cellular compartment (Brito et al., 2008). In the case of sperm, SLO has been used to study the physiological mechanism that regulates some process like spermatic capacitation, acrosome reaction and motility (Azamar et al., 2007). Despite several reports that demonstrates the utility of pores formation using SLO in mammal cells, this technology has not yet been used in mammals' sperms to improve its resistance in cryopreservation by introducing cryopreservative substances such as trehalose.

## Materials and Methods

### Chemicals

All the chemical products used in this experiment were of analytical grade and bought from Sigma-Aldrich Co. (St. Louis, MO, EE.UU.). The extender used to dilute the semen was purchased from IMV USA 870 XT-R ®.

### Biological materials

Eighteen ejaculates from three different boars (9 for each experiment) were used. The boars were three years old at the time of the experiment and the ejeculates were taken once a week. All procedures were performed taking care of animal welfare.

### Methods

#### Semen preparation and evaluation

The ejaculates were collected by the hand-gloved technique. The including criteria was: ejaculates with at least 80% of progressive motility and lees than 20% of abnormalities. For its transportation to the lab, the ejaculates were diluted in a 1:1 relation of a commercial diluent (IMV USA 870 XT-R ®). At its arrival to the lab, the spermatic concentration was calculated in a neubauer chamber.

### Experiments

Experiment No. 1 Pore formation with Streptolisine O (SLO)

**Treatments**

The ejaculates samples were exposed to different SLO concentrations

Control treatment= 0.0 of SLO
1st treatment= 0.2 U of SLO
2nd treatment= 0.6 U of SLO
3rd treatment=1.0 U of SLO
4th treatment= 5.0 U of SLO

**Pore formation and sealed procedure**

Cells were permeabilized using the protocol described by Fawcet et al. (1998).

**Semen Evaluation**

After permeabilization and sealing of pore, was evaluated the spermatic motility, spermatic viability, membrane integrity and functional spermatic cell state, using techniques described below. The assessment of sperm viability was performed with eosin nigrosine staining (EN), membrane integrity were evaluated with a hipoosmotic test (HOST) in combination with Coomassie brilliant blue (CBB). Spermatozoids samples were taken, the ones that were washed by centrifugation, to remove the diluent. The combination of HOST and Coomassie Brilliant Blue Stain is done searching for a better assessment of the results providing information about functional state of the flagellum and acrosomal integrity (Gutierrez et al., 2009). The Interpretation the HOST test, is considered positive (functional membrane) when flagellums are founded bent, curled or wound whip.

**Functional Status**

Capacitation and status spermatic, usually is valued with a fluorescent antibiotic like: chlortetracycline (CTC). CTC is used to detect $Ca^2$ present in the membrane surface (Silva and Gadella, 2006). Slides were observed under fluorescence microscope (200X) with a blue filter. Hundred sperms were counted and the percentage was determined by the patterns of fluorescein in the head of the sperm with the classification used

- F pattern: uniform fluorescence in the head, indicating no capacitation of the sperms and with the intact acrosome.
- B pattern: with a free band of fluorescein in the post acrosome zone, indicating capacitated sperms with intact acrosome
- RA pattern: Fluorescein free head or with a small band of fluorescein in the equatorial region, indicating acrosome reaction

**Experiment No. 2 Trehalose exposition and cryo-preservation**

Once the treatment of SLO toxin, that permeabilized and kept the viability of boar semen, were obtained. Two treatments of Trehalose were applied to nine ejaculates obtained from three different boars.

Treatment 1: 100mM Solution of Trehalose
Treatment 2: 200mM of Trehalose
(Erougle et al., 2003).

Trehalose was added at the same time as the SLO toxin, to its incorporation into the sperm intracellular media. For the reconstitution of the plasmatic membrane, sperms were washed in PBS and further briefly suspended (2 min) in 5% of BSA/PBS. Immediately sperms were processed for their cryopreservation.

Aliquot samples were taken to value sperm viability before freezing process, using the same techniques described above in this assay, for permeabilization and pore sealing.

**Cryopreservation and thawing**

For the control group, sperms were frozen using the technique used by Westerdof (Gutierrez et al., 2009).

For the sperm permeabilized and treated with trehalose, the freezing diluent used was the same as reported bu Hu et al. (2009), but replaced yolk and glycerol with trehalosa.

**Thawing**

It was performed in a water bath at 37C° for 30 seconds and then immediately transferred into a test tube with BTS in a 1:6 volume for 10 minutes at 37°C (Gutierrez et al., 2009).

**Statistic analysis**

For the analysis of the results, the statistical package SAS (Statistical Analysis Systems 2003, SAS/STAT User's Guide, Release 9.0, Inst. Inc, Cary NC, USA), was used. An ANOVA was used for statistical evaluation. A one classification criteria ($Y_{ij} = \mu + \gamma_i + \xi_{ij}$), covariance of the data for each variable was tested: variance components (VC), compound symmetry (CS), autoregressive (AR1) and non structurated (NS), the best one was selected according to information criteria from Akaike (AIC) and Bayesian criteria from Swarz (BIC), as the minimum obtained value.

Saphiro-Wilks and Bartlett test were used to prove the normality and homogeneity of variance model. In case, the variables that do not fit in normal distribution patterns were tested by using Kruskall-Wallis test for more than two independent mean.

In multiple comparison analysis of means, Tukey Test was used. Tukey test (Adjusted Tukey, SAS) was also used when lower square means (LSMEANS) were compared.

## Results

The effects of exposure of the spermatic cells to different SLO doses is detailed in Table 1. The analysis of progressive motility percentage indicates that there is a difference (P<0.05) between treatments and control, however, there was no difference between the sperm treated with SLO (P>0.05). In the membrane integrity, the concentration of 5 U/ml got the lower value 39.27%. On the other hand, the percentage of capacitated sperm and acrosome reaction showed that the percentage of capacitated sperm remained constant in all groups (P = 0.34). Similarly, the number of sperm with acrosome reaction was lower for all treatments (P = 0.29). In Figure 1, the results obtained from the HOST/CBB test showed that percentage of sperm live with acrosome was higher in the treated with 0.6 U / ml SLO, However, no was difference in treatments (P <0.05). According to the results obtained, the concentration of 0.6 U / ml of SLO, that used to permeabilize the sperm and the introducing of the trehalose, showed a higher number of spermatozoa alive after the sealing process of the pores.

Linear regression analysis between different characteristics of spermatic viability showed a significant effect of SLO on permeabilization percentage, membrane integrity, and acrosome intact and dead (Table 2).

The viability results evaluated by the HOST/CBB test are shown in Table 3. It was observed that the treatments with 100mM and 200mM of trehalose significantly (P<0.05) decreased live with acrosome and increased dead with acrosome. The orthogonal polynomials technique a linear effect (P=0.0001) and quadratic effect (P=0.0001) on live with acrosome and dead with acrosome sperm.

Table 4 shows that the percentage of sperms with membrane integrity was significantly low in 200 mM of trehalose while percentage motility was significantly low in the same group.

The percentage of live sperms with the acrosome intact is shown in Table 5. Spermatozoa alive with acrosome was significantly low in 200 mM of trehalose while dead sperms with acrosome were significantly high in the treatment groups. The integrity of membrane was significantly low in the treatment groups.

The linear (P = 0.0001) and quadratic (P = 0.0002) effects of the percentage of spermatozoa with intact acrosome indicate that they increased and decreased according to trehalose concentration.

## Discussion

Trehalose has been applied in cryopreservation processes in various cell types such as erythrocytes, liposomes, bacteria, larvae and sperm (Linch et al., 2011). In this work, it was found that the intracellular presence of trehalose at a concentration of 100 mM preserved 27% greater with acrosomal integrity after thawing than the control group. These results are lower than those previously reported (Gutierrez et al., 2009). The test results HOST/BBC show that trehalose at 100 mM was able to keep sperm alive with intact acrosome in the absence of other cryoprotectant.

Thus it can be inferred that trehalose intracellularly is able to stabilize biological membranes and proteins. These results support those reported in pig sperm (Malo et al, 2010). In bovine sperm, Sitaula et al. (2009) using trehalose (200 mM) in lyophilized cells showed a 40% survival.

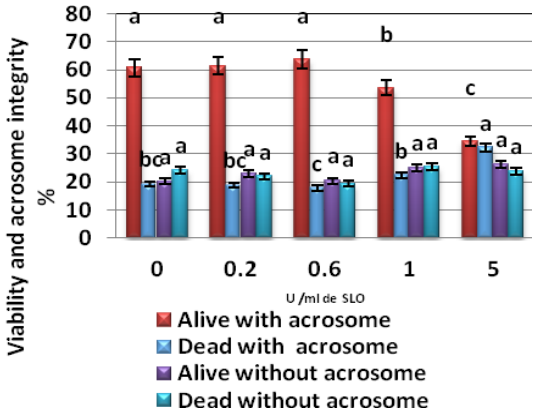


**Fig. 1: Effects of different concentrations. The bars represent means of minimum squares + standar error of the mean (SEM).** Means with different; Means with different superscripts in the bars are different (P<0.05); Tukey tests values: Living with intact acrosome and dead with intact acrosome; Kruskall – Wallis test: Living without acrosome and dead without acrosome.

**Table 1: Least square means and standard error of the percentage of motility, permeabilization, membrane integrity, capacitation and the acrosomal state before pore sealling of the SLO**

| Treatment U /ml SLO | Ejaculates (n) | Motility Percentage* | Permeabilization (%)* | Intact Membrane* (%) | Capacitated* | Acrosomal Reaction** |
|---|---|---|---|---|---|---|
| 0 | 9 | 59.27 $^{b}$ | 41.44 $^{b}$ | 52.55 $^{a}$ | 6.66 | 2.66 |
| 0.2 | 9 | 65.86 $^{a}$ | 57.11 $^{a}$ | 56.44 $^{a}$ | 6.88 | 3.22 |
| 0.6 | 9 | 63.44 $^{ab}$ | 59.94 $^{a}$ | 57.44 $^{a}$ | 7.22 | 2.66 |
| 1 | 9 | 60.61 $^{ab}$ | 63.94 $^{a}$ | 48.77 $^{a}$ | 8.11 | 3.66 |
| 5 | 9 | 60.57 $^{ab}$ | 65.55 $^{a}$ | 39.27 $^{b}$ | 8.11 | 4.11 |
| | SEM | 1.32 | 2.30 | 2.26 | 0.62 | 39.4 |

Different letters per column indicate difference (P<0.05); Standard error of the mean (SEM); *Tukey test values; **Kruskall – Wallis tests values.

**Table 2: Linear regression model with the variables**

| Variable (%) | Model | $R^2$ | P |
|---|---|---|---|
| Permeation | $\hat{y} = 53.8 + 2.7$ | 0.22 | 0.001 |
| Membrane integrity | $\hat{y} = 55.2 - 3.2$ | 0.42 | 0.0001 |
| Alive with acrosome | $\hat{y} = 62.3 - 5.5$ | 0.77 | 0.0001 |
| Dead with acrosome | $\hat{y} = 36.6 + 5.5$ | 0.75 | 0.0001 |
| Capacitated | $\hat{y} = 7.07 + 0.2$ | 0.05 | 0.123 |
| Motility | $\hat{y} = 62.47 - 0.3$ | 0.025 | 0.295 |

**Table 4: Membrane integrity and sperm motility treated with trehalosa**

| Treatment in mM of trehalose | Ejaculates (n) | Sperm with integral membrane (%)* | Percent Motility** |
|---|---|---|---|
| 0 | 9 | 74.66[a] | 81.55[a] |
| 100 | 9 | 59.33[b] | 58.46[b] |
| 200 | 9 | 59.77[b] | 36.25[c] |
| E.E.M. | | 1.73 | 1.22 |
| | Polynomials orthogonal | | |
| Linear Effect | 0.0001 | 0.001 | |
| Cuadratic Effect | 0.001 | 0.732 | |

Different letters per column indicate difference (P<0.05); Standard error of the mean (SEM); *Values subject to Tukey's test; **Values subject to the Kruskal – Wallis.

Eroglu et al. (2003) found that the concentration of 150 mM and 500 mM increased the survival rate upto 66%. However, in another study by the same author, but with mouse oocytes reached the conclusion that the presence of trehalose at a concentration of 100 mM and 150 mM intracellularly, provides protection against stress associated with the process of freezing and does not alter cell division and blastocyst development (Eroglu et al., 2003). In this assay, the motility percentage of sperms treated with trehalose (100 mM and 200 mM) at defrosting, were low and that's why viability was not evaluated subjectively. The low movement might be in between other things due to an increased viscosity of the cytoplasm caused by trehalose (Jain and Roy, 2009).

Trehalose has a significant cryoprotectant effect and a study in human oocytes confirmed that concentration of 150 mM and 500 mM increased the survival rate - 60°C (Eroglu et al., 2002).

The higher number of dead cells, obtained in this assay, even in control group, might be due to the effect of the continuous centrifugations, performed during the permeabilization and pore sealing process by SLO. Also, during this process reactive oxygen species are generated, and they cause an irreversible damage to the cell membrane by the lipid peroxidation (Membrillo et al., 2003).

The regression analysis showed that only 22% of the cell permea-bilization, is due to the presence of SLO, which differs markedly with the assay performed by Johnson et al. (1999). In another assay, Yunes et al. (2000) reported 100% of permeabilized of the sperms, which is far less than the percentage found in this assay, and could be possible due to a different handling procedure. In addition to the exposure time, SLO is a toxin that binds to cholesterol in the plasma membrane of the cell (Hu et al., 2009). The boar semen has a low rate molar proportion of phospholipids (Cordova et al., 2001) compared to human. In this assay, the largest number of permeabilized sperms was achieved with the concentration of 5 U/ml of SLO, the regression analysis shows that as the concentration of SLO, in the medium rises, the spermatic and the viability decreases, which is similar to the results obtained by Fawcett et al. (1998).

In the published literature, there is no assay reporting the sperm permeabilization and sealing with SLO in boar sperm cells, therefore, comparing our results with other kind of cells, we found that there was a huge difference of the percentage of permeabilized and satisfactorily sealed cells. For example, In rat myocytes, Fawcett et al. (1998) obtained 50% and 25% of permeabilized and satisfactorily sealed cells, with concentraions of 0.2 U/ml and 0.6 U/ml of SLO respectively. Similar results were also reported by Walev et al. (2001), in THP cells.

To determine that SLO permeabilization was limited to the plasma membrane of the boar esperm, the integrity of the acrosome was evaluated by the HOST/CBB and CTC tests to estimate the capacitation state of the sperm (Eougle et al., 2003). The bovine serum albumin promotes the sperm capacitation, acting as a cholesterol receptor and favouring the efflux of the plasma membrane, which results in a lower cholesterol: phospholypids proportion, that contributes an increasing of the membrane efflux, followed by an increase of the ionic permeability (Vadnais et al.,

**Table 3: Viable sperm and acrosome integrity**

| Treatament in mM of trehalose | Ejaculates (n) | Alive with acrosome* | Dead with acrosome* | Alive without acrosome** | Dead without acrosome** |
|---|---|---|---|---|---|
| 0 | 9 | 80.83[a] | 18.44[b] | 11.55 | 12.05 |
| 100 | 9 | 54.55[b] | 43.33[a] | 14.77 | 15.5 |
| 200 | 9 | 57.88[b] | 41.61[a] | 15.66 | 14.44 |
| E. E. M | | 2.29 | 2.13 | 23.81 | |
| | | Polynomials orthogonal of treatment | | | |
| Linear effect | | 0.0001 | 0.0001 | | |
| Cuadratic effect | | 0.0001 | 0.0001 | | |

Different letters per column indicate difference (P<0.05); Standard error of the mean (SEM); *Values subject to Tukey's test; **Values subject to the Kruskal – Wallis.

**Table 5: Least squares means and standard error of live sperm with intact acrosome, sperm with intact acrosome, membrane integrity and percentage motility at thawing**

| Treatament in mM of trehalose | Ejaculates (n) | Spermatozoa alive with acrosome * | Dead with acrosome* | Integral membrane* (%) | Motility** (%) |
|---|---|---|---|---|---|
| 0 | 9 | 9.89[a] | 13.61[b] | 19.77[a] | 23 |
| 100 | 9 | 7.83[ab] | 40.61[a] | 17.44[b] | 9.5 |
| 200 | 9 | 6.05[b] | 33.27[a] | 17.11[b] | 9.5 |
| E.E.M. | | 0.76 | 3.08 | 0.65 | 23.81 |
| Polynomials orthogonal of treatment | | | | | |
| Linear Effect | | 0.001 | 0.0001 | 0.008 | 0.008 |
| Quadratic Effect | | 0.884 | 0.0002 | 0.224 | 0.224 |

Different letters per column indicate difference (P<0.05); Standard error of the mean (SEM); *Values subject to Tukey's test; **Values subject to the Kruskal – Wallis.

2007). Our results showed that the use of BSA do not cause any side effect over sperm physiology. These results are higher than the ones observed in control group (59.27%). And, even though the number of permeabilized sperms was low, this results suggests that SLO, at this doses, wasn't toxic for the cells, since non permeabilized remained mobile.

Finally, one of the important effects of trehalose, before freezing, in the permeabilized sperms was the capacity of this sugar for the stabilization of the membrane and proteins, because a large part of this sperms in spite of some of them were dead, they still had the intact acrosome. The percentage of progressive motility in permeabilized sperms and treated with trehalose, was lower than the one found in fresh semen (control). The lower percentage of permeabilized-living sperms is due to the increase of the viscosity produced by trehalose or the hyperosmotic change due to sugar, because increased molarity of the media intervenes negatively with the sperm motility (Sitalua et al., 2009).

## Conclusions

1. The percentage of permeabilization produced by SLO depends on the used concentration
2. The viability decreases as the concentration of SLO increases
3. The intracellular presence of trehalose maintains the viability of the boar semen.

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