

## Cell Viability, Acrosomal Status and DNA Integrity in Porcine Sperm Permeabilized with Streptolysin O

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### Authors' contributions

*This work was carried out in collaboration between all authors. Authors CMV and MBM designed the study, wrote the protocol and interpreted the data. Author MBD anchored the field study, gathered the initial data and performed preliminary data analysis. While authors APC, BAH and DRS managed the literature searches and produced the initial draft. All authors read and approved the final manuscript.*

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### ABSTRACT

The aim of this study is to determine the use of streptolysin O (SLO), to open pores on the plasmatic membrane of the swine sperm, to see the effect on the viability, functional status and integrity of nuclear chromatin. 30 ejaculates were used; immediately after collection, semen was diluted in a solution of BTS (2:1, v/v preheated to 35°C). Two aliquots of semen samples were assigned to each group, of which: Group 1. 0.3 IU/ml SLO, Group 2. 0.6 IU/ml, Group 3 1.2 IU/ml, and Group 4 Control (PBS). All groups were incubated for 5 minutes at 37°C, in SLO; after this

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period, the samples were incubated in 5% fetal bovine serum (FBS) for 15 minutes at 37°C. Each treatment were evaluated before and after incubation with FBS (to close the pores), in duplicate. Sperm viability, acrosome status and integrity of the nuclear chromatin were evaluated with eosin nigrosine (NE), chlortetracycline (CTC) and acridine orange (AO) stains, respectively. The data obtained was analyzed by comparing proportions in each treatment using non-parametric module of STATISTICA V10.0 program. The Kruskal Wallis H test was used for the analysis. In assessing sperm viability each of the treatments were compared in between, and the resulting differences were also compared with the control group (iv). There was no difference in the proportion of viable sperm, using the concentration of 0.6 IU/ml, before and after sealing of pores (83.15±9.10 vs 91.52±4.83) (p>0.05). And mathematical trend indicates that, after sealing the pores, a higher sperm proportion were viable in both treatments. However, no statistical differences were found (P>0.05). Also no changes were found in patterns of acrosomal status and DNA integrity, suggesting that the SLO executes its permeabilizing effect at the level of sperm membrane.

**Keywords:** *Streptolysin O; pores; sperm; integrity.*

## 1. INTRODUCTION

The Streptolysin O (SLO) is a toxin produced by *Streptococcus pyogenes*, it's a protein with a molecular weight of 60 kDa; belonging to group A of cytolytic toxins β-haemolytica, known as oxygen-labile and produced by a gram positive bacteria. Besides its pathogenic interest, SLO has been reported as an ideal tool for cell pores forming [1]. The SLO is toxic for eukaryotic cells, because of its affinity for cholesterol, but in controlled doses, has proven to be useful for live cell permeabilization [2,3]. In the case of sperm, the SLO has been used to study the mechanisms that regulate some physiological processes of the sperm as capacitation, the acrosome reaction and motility [4,5]. The SLO binds to cholesterol in the cytoplasmic membranes of animal cells [6], and subsequently participates in reversible pore formation, in the plasma membrane [6,7,8]. The poration with SLO has been used extensively to introduce external molecules, such as proteins, DNA, and RNA, into the intracellular compartment [9,10,11]. Fawcett et al. [3] conducted a research with SLO (0.2 IU / ml) to form pores in the cells membrane of mouse myocyte and introduce fluorescent macromolecules [3]. Other work with mouse sperm, shown that these cells remain viable in SLO medium with concentrations up to 0.6 IU / ml for short periods of time, showing greater viability than cells treated with lower concentrations and a longer exposure [4]. Other studies mention the use of SLO as a former of pores in the membrane, to introduce proteins in different cell types of the immune system; Moreover, after the sealing of pores, such cells showed similar viability rates to those of control cells that were not treated with SLO [9]. In a more recent work, Bo-Woong et al. [12] used SLO a dose of 5 IU/

ml as a medium for the introduction of exogenous DNA into mouse sperm and showed that there was an increase in the rate of transgenic embryo production and later litters than with intracytoplasmic sperm injection. The sperm cell is a complex and a good biological model to several studies; the opening of the pores can modify their structure and, therefore, serve as a vehicle for cryopreservation studies and biotechnology. The aim of this study is to determine the effect of streptolysin on cell viability, functional status and integrity of nuclear chromatin of swine sperm.

## 2. MATERIALS AND METHODS

### 2.1 Reagents

Unless otherwise stated, all of the chemicals used in the experiments were analytical grade and were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). The basic medium used to semen dilution, was Beltsville Thawing Solution (BTS): composed of 205 Mm glucose, 20.4 Mm sodium citrate, 10.0 Mm KCl, 15.0 Mm NaHCO<sub>3</sub>, and 3.6 Mm EDTA, pH 7.2, and 290–300 mOsmol/kg) supplemented with kanamycin sulfate (0.05 Mm).

### 2.2 Biological Material

The work took place from February to July 2014. Thirty ejaculates, which were analyzed before use, to check that met inclusion criteria. Procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of Veracruz, Mexico. Two-four years old clinically healthy boars without pathologies associated to the male reproductive tract and with a proven history of fertility after

conventional AI with fresh extended semen were selected. In addition, boars followed a routine schedule of vaccination, de-worming and vitamins (mainly ADE) appropriate to the species. Boars were housed in individual pens, fed twice daily with 1.5 kg of concentrate that contained 15% of crude protein and 1.2 Mcal of metabolizable energy.

## 2.3 Method

### 2.3.1 Semen preparation and evaluation

#### 2.3.1.1 Collection

Semen was collected by the gloved hand technique. During the collection, semen was filtered through sterile gauze and only the sperm-rich fraction was used. Semen samples with 70% progressive motility and 80% normal sperm morphology and intact acrosome status (assessed by phase contrast microscopy, the sperm samples were fixed in 2% buffered glutaraldehyde).

#### 2.3.1.2 Motility evaluation

To evaluate mass motility, a semen drop was placed in a glass slide at 37°C, and was observed under microscope (Olympus CX41) 100 x magnification, for one minute. The estimate is based on intensity of waves, sorting have values 0-5; samples with less than 3 degrees, were not considered for this study. For individual motility analysis, a drop of diluted semen in saline, was placed on a glass slide at 37°C, covered with a microscope was observed under microscope at 400 x magnification; was evaluated for progressively motile spermatozoa (scale 0-100%) [13].

#### 2.3.1.3 Morphology

The morphology was evaluated with the technique of chinese ink, which is prepared by placing a volume 1: 8 v/v semen-ink and it was observed with a microscope at 400 x counting 200 cells per replicate [14].

#### 2.3.1.4 Dilution

Immediately after the collection, the semen was diluted in a BTS solution (2: 1, v / v) preheated to 35°C. Subsequently, the semen was transported in a container between 20-22°C, to the Laboratory of Biology of Animal Reproduction FMVZ UV.

### 2.3.2 Sample preparation and groups description

Two aliquots with semen samples were assigned to each group with: Group 1. 0.3 IU/ml SLO, Group 2. 0.6 IU/ml, Group 3 1.2 IU/ml, and Group 4 Control (FBS). All samples were incubated for 5 minutes at 37°C, in SLO; after this period, the samples were incubated in fetal bovine serum (FBS) at 5% for 15 minutes at 37°C.

#### 2.3.2.1 Cell permeabilization

Cells were permeabilized, using the protocol described by Fawcett et al. [3]. For the membrane permeabilization, three treatments were used with Streptolysin O (SLO) lyophilized Sigma Aldrich brand. The lyophilized streptolysin was dissolved in cold distilled water to 60 IU / ml and activated with 4 mM DTT at for 2 h at 37°C and aliquots were stored at -20°C until use. 100 ul of semen was added to each treatment and incubated for five minutes at 37°C, washed twice by centrifugation at 1200 rpm for 3 minutes, the supernatant removed and resuspended in 1 ml PBS. Concentrations for the treatment with SLO were 0.3, 0.6 and 1.2 IU / ml.

#### 2.3.2.2 Sealed method

For closing the pores, Fetal Bovine Serum (FBS) at 5% in PBS was used, to restore the integrity of cell membrane. The incubation was performed at 37°C for 15 minutes [3].

### 2.3.3 Cell evaluation

Each experimental group was evaluated before and after incubation with FBS, this was done in duplicate. The sperm viability was evaluated by eosin nigrosine stain (NE), the acrosomal status of sperm by chlortetracycline technique (CTC), and the integrity of nuclear chromatin was evaluated by fluorescence technique with acridine orange (NA).

#### 2.3.3.1 Sperm viability

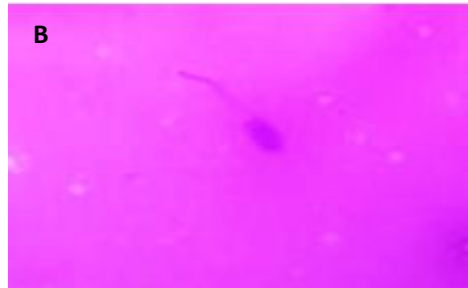
The assessment of sperm viability was performed with eosin nigrosine staining (EN) (HYSEL<sup>®</sup>). Using a 1:8 dilution of the semen sample and dye, the sample mixture was incubated for 5 minutes at 37°C and a smear was made on a slide. The slides were observed under phase contrast microscope at 400x, several fields were reviewed to count 200 cells, which showed the following pattern of coloration.

- Cells without eosinophilia or white color = live sperm
- Cells with eosinophilia or sperm purple color = dead [14,15].

### Images of Eosin Nigrosine staining (EN)



A. Cells whether eosinophilia or white color = live sperm

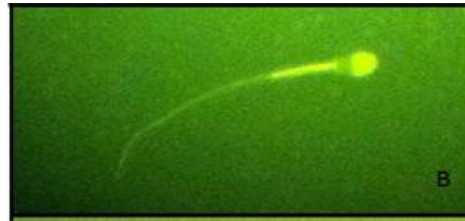


B. Cells with eosinophilia or sperm color purple = dead

### Images of Acrosomal Status by CTC Test



A. Uniform fluorescence over the whole head= Intact

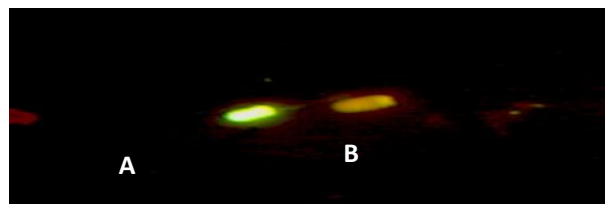


B. Fluorescence-free band in the post-acrosomal region= Capacitated



C. Dull fluorescence over the entire head=: reacted

### Images of sperm DNA integrity evaluated by Acridine Orange (OA)



A. Green sperm: intact DNA  
B. Orange sperm or red Sperm: denatured DNA

2.3.3.2 Acrosomal status

To determine the status of capacitation and acrosome reaction, It was used the technique described in 1993 by Das Gupta et al, which must presented the following fluorescence patterns: [16].

- i) Uniform fluorescence over the whole head: Un-Capacitated and -acrosome-reacted.
- ii) Fluorescence-free band in the post-acrosomal region: Capacitated.
- iii) Dull fluorescence over the entire head: capacitated and acrosome-reacted.

2.3.3.3 DNA integrity assesement

DNA integrity was assessed by fluorescence technique with acridine orange (OA). It was used as described by Tejada (1984). Covered the slide and immediately observed under the fluorescence microscope (Leica DM 020-518500 / LS) with filter blue excitation 405-455 nm, 400x, reviewed several fields to count 200 cells which must present the following fluorescence patterns:

- Green sperm = intact DNA.
- Orange sperm or red Sperm = denatured DNA [17].

2.3.4 Statistical analysis

The data obtained were analyzed using the non-parametric module V10.0 STATISTICA program; the parameters evaluated include: the percentage of the acrosomal status CTC technique, the percentage of plasma membrane integrity by eosin nigrosin technique and the percentage of the integrity of the nuclear chromatin and acridine orange technique.

3. RESULTS

3.1 Cell Viability

Fig. 1 shows the percentage of live sperm in each of the treatments and there was no statistically significant differences (P>0.05), among the groups. The percentage of live spermatozoa increased in all treatments after incubation with 5% FBS; however this increase was not significant (P>0.05). Moreover, as shown in Table 1, the value of the lowest sperm viability was 83.15±9.10 with the SLO 0.6 IU / ml treatment, before incubation with FBS, while the highest value (91.52±4.83) was found in the

same concentration of SLO but after being incubated with SFB to seal the pores; the differences were not significant (P>0.05).

3.2 Acrosomal Status

A not significant (P>0.05) decrease in the percentage of un- capacitated and -acrosome reacted spermatozoa only in 1.2 IU / ml of SLO treatment group in comparison to control before and after incubation with FBS was observed. The differences among the groups are shown in Fig. 2. The highest value (83.68%) was for control treatment before incubation with FBS and the lowest (79.42%) was in the 1.2 IU / ml of SLO treatment group, after sealing of pores; However, these results are not significant (P<0.05) for any of the treatments due to the standard deviations are > 4.7% (Table 2).

Table 1. Mean±SD of of live spermatozoa (%) before and after incubation with 5% FBS

Treatment / FBS incubation	Before FBS open pore	After FBS close pore
Control	84.46±10.31	87.33±6.43
0.3 UI/ml SLO	85.92±8.61	88.14±6.72
0.6 UI/ml SLO	83.15±10.9	91.52±4.83
1.2 UI/ml SLO	87.5±6.83	90.22±5.28

Table 2. Mean±SD non capacitated and acrosome reacted spermatozoa (%) before and after incubation with 5% FBS

Treatment / FBS incubation	Before FBS open pore	After FBS close pore
Control	83.68±4.76	83.07±9.17
0.3 UI/ml SLO	80.34±7.8	81.44±7.66
0.6 UI/ml SLO	83.67±5.6	83.66±7.37
1.2 UI/ml SLO	81.71±8.18	79.42±5.03

3.3 DNA Integrity Assesement

Fig. 3 shows the effect of treatment on the integrity of the nuclear chromatin of spermatozoa. There was no statistically significant difference (P<0.05) between treatments before or after incubation with FBS. The percentages of cells with chromatin integrity remained > 98% in all treatments (Table 3) before and after sealing of pores. Although standard deviations were lower than in other techniques, but still no significant (P<0.05) difference was observed between control and treatment groups.

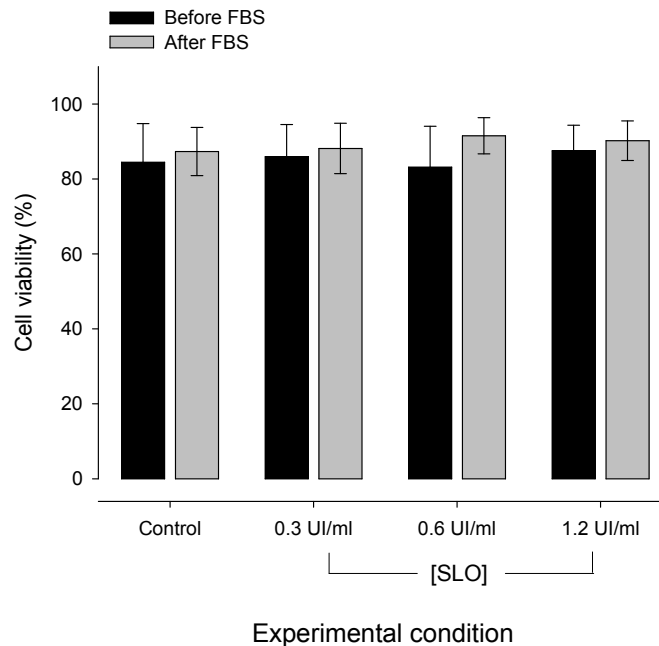
#### 4. DISCUSSION

In this study the effect of incubation with streptolysin O on sperm viability, indicated that there is no difference between treatments before and after sealing of the pores. The observed decrease in the percentage of viable sperm incubated with SLO for the permeabilization (Fig. 1), followed by an increase of the percentage of viable sperm after incubation with FBS for sealing pores, indicates SLO acts on the sperm plasma membrane, this allows the entry of eosin dye into the cell, similar to that described by Tartakoff [18], which states that certain dyes waterproof such as trypan blue and eosin, can be able to cross the plasma membrane of cells treated with toxins pores forming as the  $\alpha$ -toxin, and the digitonina SLO and that prolonged exposure to such dyes causes staining in intact cells; however, in this study no statistical differences between treatments were found before and after the sealing of pores, contrary to what was reported by Johnson et al. [4], that using the same technique at a dose of 0.6 SLO IU / ml achieved 80% permeabilization in mouse sperm. Moreover, Michaut et al. [21] and Yunes et al. [19,20] conducted a study in human sperm and achieved to permeabilize 100% cells. These differences can be associated to the cholesterol found in the membrane, which differs significantly

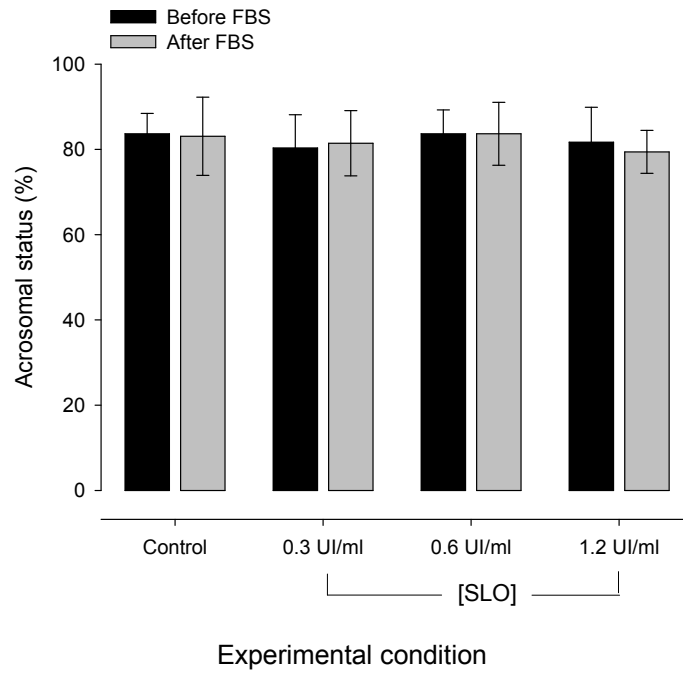
between species, being the lowest in pigs than in mouse or human and as mentioned above, the SLO is a toxin that acts by binding to cholesterol in plasma membranes, which have great concentration in these species. Fawcett et al. [3] found that the temperature, the dose and the period of exposure to the toxin, are factors that are associated with the effect of this on the membranes. Worked with mouse myocytes at a dose of 0.2 IU / ml at 37°C for 5 min, and a higher percentage of viable cells when higher exposure periods and higher doses of the toxin used was found; In our study the incubation temperature was 37°C and the time of 5 minutes, and it was found that the viability, status of acrosome integrity and integrity of nuclear chromatin, were maintained in high percentages in all treatments, similar to reported by these authors.

**Table 3. Mean±SD of spermatozoa with nuclear chromatin integrity (%) before and after incubation with 5% FBS**

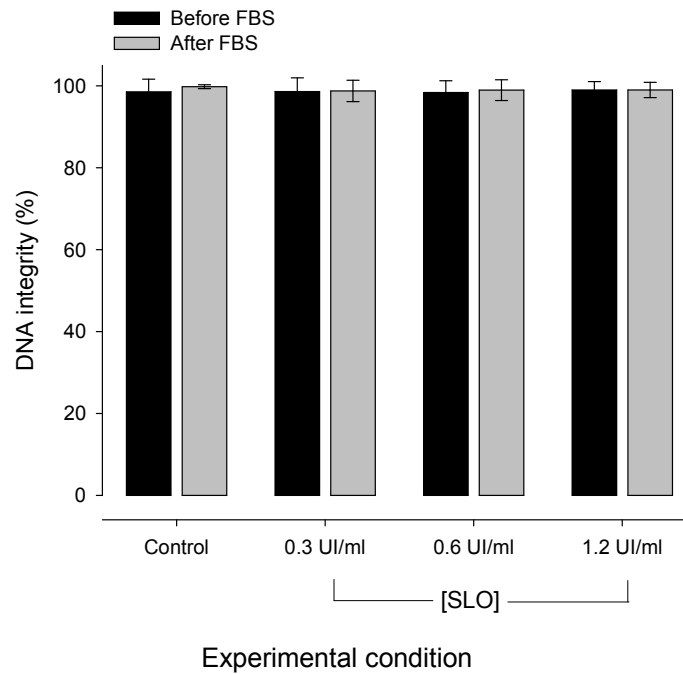
Treatment / FBS incubation	Before FBS open pore	After FBS close pore
Control	98.56±3.09	99.8±0.48
0.3 UI/ml SLO	98.61±3.35	98.76±2.6
0.6 UI/ml SLO	98.39±2.87	98.96±2.53
1.2 UI/ml SLO	98.99±2.04	99.01±1.87



**Fig. 1. Effect SLO concentrations before and after sealing of pores with FBS on sperm viability**



**Fig. 2. Effect of SLO concentrations before (open pore) and after sealing of pores (close pore) with FBS on the functional status of sperm**



**Fig. 3. Effect of SLO concentrations on the functional DNA integrity before and after sealing of pores with FBS**

To determine the effect of SLO on sperm physiology technique CTC, which indicates the state of capacitation and acrosome reaction was used to perform the study. No statistically significant differences between treatments before and after incubation with FBS for sealing; however, a decreasing trend is observed in the percentage of un-capacitated and acrosome reacted. This decrease can be attributed to the contact of the sperm and the seminal plasma and begins to develop such processes naturally [22].

In this study, porcine sperm permeabilization for 5 minutes with SLO 0.3, 0.6 and 1.2 IU / ml was found that the percentages of cells without capacitation and without acrosome reaction were of  $80.34 \pm 7.8$ ,  $83.67 \pm 5.6$  and  $81.71 \pm 8.18$  respectively, similar to those reported by Johnson et al. [4]; to permeabilize SLO mouse spermatozoa with 0.6 IU / ml for 5 minutes found that most of the cells remained with intact acrosome (75%) and that increasing the exposure time of 15 minutes the percentage decreased (44%). These results indicate that permeabilization with SLO performed in the plasma membrane and not in acrosomal membrane.

Until now there are no reports about the effect of the use upon the integrity of the nuclear chromatin of sperm cells SLO as permeabilizer; However, many authors report that individuals with spermatozoa exhibiting > 50% full chromatin are considered normally fertile. In our study, > 98% of spermatozoa were with intact chromatin in all treatments before and after incubation with FBS, which indicates that the SLO has no negative effect on the structural integrity of nuclear chromatin and permeabilized and sealed cells are able to fertilize.

## 5. CONCLUSION

In this work it was found that the SLO acts on the plasma membrane while the use of FBS as pore sealant does not affect sperm physiology. Finally no negative effect was observed in the use of the SLO in the structural integrity of DNA.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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