Improved expression of green fluorescent protein in cattle embryos produced by ICSI-mediated gene transfer with spermatozoa treated with streptolysin-O

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ABSTRACT

The ICSI-sperm mediated gene transfer (ICSI-SMGT) has been used to produce transgenic mice with high efficiency; however, the efficiency of this technique in farm animals is still less than desirable. Pretreatment of sperm with membrane destabilizing agents can improve the efficiency of ICSI in cattle. The objective of the present study was to evaluate streptolysin-O (SLO) as a novel treatment to permeabilize the bovine sperm membrane and assess its effect on efficiency of generating transgenic embryos by ICSI-SMGT. First, there was evaluation of the plasma membrane integrity (SYBR/PI), acrosome membrane integrity (PNA/FITC), DNA damage (TUNEL) and binding capacity of exogenous DNA (Nick Translation) in bull sperm treated with SLO. Subsequently, there was assessment of embryonic development and the efficiency in generating transgenic embryos with enhanced expression of the gene for green fluorescent protein (EGFP). Results indicate that SLO efficiently permeabilizes the plasma and acrosome membranes of bull spermatozoa and increases binding of exogenous DNA mostly to the post-acrosomal region and tail without greatly affecting the integrity of the DNA. Furthermore, treatment of bull sperma-tozoa with SLO prior to the injection of oocytes by ICSI-SMGT significantly increased the rate of embryo expression of the EGFP gene. Future experiments are still needed to determine the effect of this treatment on the development and transgene expression in fetuses and animals produced by ICSI-SMGT.

1. Introduction

Transgenesis is a very useful biotechnological approach with diverse applications in the pharmaceutical industry, human

medicine and even the agricultural sector (Lavitrano et al., 2013).

Nevertheless, producing transgenic cattle is difficult and costly due, in part, to the low efficiency of transgenesis techniques and the long period of time required to assess the transgene and produce the desired recombinant protein (Monzani et al., 2016).

Different methods have been considered for production of transgenic cattle, including pronuclear microinjection (Loskutoff et al., 1986), lentiviral vectors (Hofmann et al., 2004), somatic cell nuclear transfer (SCNT) (Wang et al., 2015), embryonic stem cells (Malaver-Ortega et al., 2012) and more recently the CRISPR/Cas9 system (Heo et al., 2015). Currently, one of the most used methods to produce transgenic cattle is SCNT, due to the relatively greater rates of transgenic embryo generation (Monzani et al., 2016). The subsequent viability of the clones, however, limits the overall efficiency in transgenesis (Cibelli et al., 2002).

One method proposed several years ago to be simple, economical and rapid for large-scale transgenesis in farm animals is sperm-mediated gene transfer (SMGT). This technique is based on the ability of spermatozoa to bind, internalize and transport exogenous DNA molecules within the oocyte during fertilization to enable the generation of transgenic embryos and/or offspring (Lavitrano et al., 1989; Lavitrano et al., 2013). First transgenic mice generated by SMGT were reported in 1989 (Lavitrano et al., 1989). Later, the same authors reported that SMGT could be used to produce transgenic farm animals, establishing transgenic pigs lines capable of efficiently expressing the gene for human decay-accelerating factor (Lavitrano et al., 2002). Despite these promising findings, efficiency of this technique continues to be less than desirable, in part to lack of efficient binding and integration of exogenous DNA in the sperm nucleus, which prevents a larger number of transfected spermatozoa from fertilizing the oocytes (Alderson et al., 2006; Anzar and Buhr, 2006; Eghbalsaied et al., 2013).

Transfection methods such as lipofection, electroporation, protamine sulfate and restriction enzyme-mediated integration (REMI) have been addressed as a strategy to increase the rate of exogenous DNA uptake in bull spermatozoa, the results however, continue to be inconsistent, irreproducible and in many cases they reduce the quality of sperm (Alderson et al., 2006; Eghbalsaied et al., 2013; Cavalcanti et al., 2016; Arias et al., 2017).

Perry et al. (1999) suggested combining intracytoplasmic sperm injection (ICSI) and SMGT to destabilize the sperm membrane with chemical or physical treatments prior to sperm injection (Perry et al., 1999). The use of membrane destabilizing agents, however, is thought to damage the sperm DNA, thus reducing the embryonic developmental potential (Yanagimachi, 2005; Hoseini Pajooh et al., 2016; Canel et al., 2018). This is why recent studies have focused on developing less invasive transfection methods for sperm cells (Moisyadi et al., 2009; Sim et al., 2013; Sanchez-Villalba et al., 2018). Sim et al. (2013) demonstrated that ICSI-SMGT efficiency in mice can be improved when the sperm membrane is permeabilized with streptolysin-O (SLO). SLO forms stable pores in the sperm membrane and allows for exogenous DNA uptake without greatly affecting the sperm nuclear DNA, a problem that occurs with other compounds (Johnson et al., 1999). The objectives of the present study, therefore, were to establish cattle sperm incubation conditions to permeabilize the plasma and acrosome membranes using SLO and assess the effects of SLO treatment on embryonic development and the generation of transgenic embryos by ICSI-SMGT.

2. Materials and methods

Unless otherwise indicated, all the reagents used were acquired from Sigma-Aldrich (St Louis, MO, USA).

2.1. Exogenous DNA and labeling

The plasmid used was pCX-EGFP, kindly supplied by Dr. Masaru Okabe of the University of Osaka, Osaka, Japan. The plasmid was labeled with fluorescein isothiocyanate (FlTC)-12-dUTP (Thermo Fisher Scientific, Inc., MA, USA) using the Nick Translation System (Thermo Fisher Scientific, Inc., MA, USA) according to the manufacturer's instructions to identify the presence and location of the plasmid in spermatozoa with flow cytometry and confocal microscopy, respectively.

2.2. Preparation of spermatozoa for incubation with exogenous DNA

Cryopreserved semen from a commercial bull with proven fertility both *in vivo* and *in vitro* was used (Alta Genetics Inc., Alberta, Canada). The frozen sperm were thawed in a water bath for 1 min at 38.5 °C and selected by Percoll gradient (Parrish et al., 1995). Selected cryopreserved spermatozoa were washed in Ca^{2+} and Mg^{2+} free Hank´s balanced salt solution (HBSS) and incubated with 5, 10 and 20 U/ml SLO. A control group without treatment was also included. Subsequently, 500 ng of exogenous DNA was added to 1×10^6 sperm and incubated for 30 min at 37 °C.

2.3. Evaluation of exogenous DNA binding in spermatozoa

To assess the capacity of exogenous DNA to bind to spermatozoa and the effect of this treatment on cell viability, the sperm subjected to each treatment were incubated with DNA marked with fluorescein isothiocyanate (DNA-FITC) and propidium iodide (PI) for 30 min and immediately evaluated by flow cytometry (FACS CANTO II, Becton Dickinson, Mountain View, USA). Later, the fluorescence signal was used to determine the amount of exogenous DNA bound to spermatozoa.

2.4. Detection of exogenous DNA

Spermatozoa subjected to each treatment were incubated with FITC-labeled DNA and evaluated by confocal microscopy

(Olympus FluoView1000) using a laser excitation spectrum of 488 nm and an emission spectrum of 530 nm. The images were taken at a magnification of 100×. The images were analyzed using the software FV10-ASW version 2010 (Nikon Instruments, New York, NY, USA). Three samples were selected at random from each treatment.

2.5. Sperm quality analysis by flow cytometry

Plasma membrane integrity was assessed using the LIVE/DEAD Sperm Viability Kit (Molecular Probes, Eugene, OR, USA) and acrosome integrity of spermatozoa was evaluated using PNA-FITC and PI (L7381) according to the protocols described previously by Arias et al. (2017).

Sperm DNA damage was evaluated by the TUNEL assay using the In-Situ Cell Death Detection Kit with Fluorescein (Roche Biochemical, Indianapolis, IN, USA), according to the manufacturer's instructions.

Before the analyses, spermatozoa were centrifuged at 1200 rpm for 5 min, the supernatant was removed, and spermatozoa were resuspended in 400 μL of PBS and evaluated using flow cytometry (10,000 events per treatment with three biological replicates).

2.6. ICSI procedure, oocyte activation and embryo culture

The ICSI procedure was performed with an inverted microscope as previously described (Zambrano et al., 2016). Injected oocytes were activated chemically by incubation with 5 μM ionomycin (Calbiochem, San Diego, CA, USA) for 5 min followed by incubation for 5 h in KSOM medium (EmbryoMax, Millipore Corp.) supplemented with 10 μg/mL cycloheximide (CHX). Embryos were cultured in KSOM medium. Cleavage rate was recorded on culture day 3 (ICSI = day 0), at which point the embryos were supplemented with 5% FBS. The blastocyst formation rate was recorded on day 8.

2.7. Determination of EGFP fluorescence in embryos

After 4 and 7 days of in vitro culture, embryos were briefly exposed to light with an excitation filter of 488 nm and an emission filter of 530 nm to determine the percentage of EGFP gene expression by epifluorescence microscopy (Nikon Instruments Inc., New York, NY, USA).

2.8. Statistical analysis

For statistical analyses, the SPSS software (SPSS Inc; version 20) was used. Differences among treatments were analyzed using one-way ANOVA after arcsine transformation of the proportional data. When ANOVA revealed a significant effect, values were compared by post hoc test (Scheffe's) to identify differences between groups. For all analyses a difference of $P < 0.05$ was considered significant. The data are presented as means ± standard deviation.

3. Results

3.1. Effect of sperm treatment with different concentrations of SLO on plasma and acrosome membrane integrity

Incubation of bull sperm with SLO significantly decreased the viability particularly with the greatest concentrations (~2%; 10 and 20 U/ml, respectively) compared to the untreated control (65.4%; Fig. 1A). Similarly, acrosome membrane damage was greater
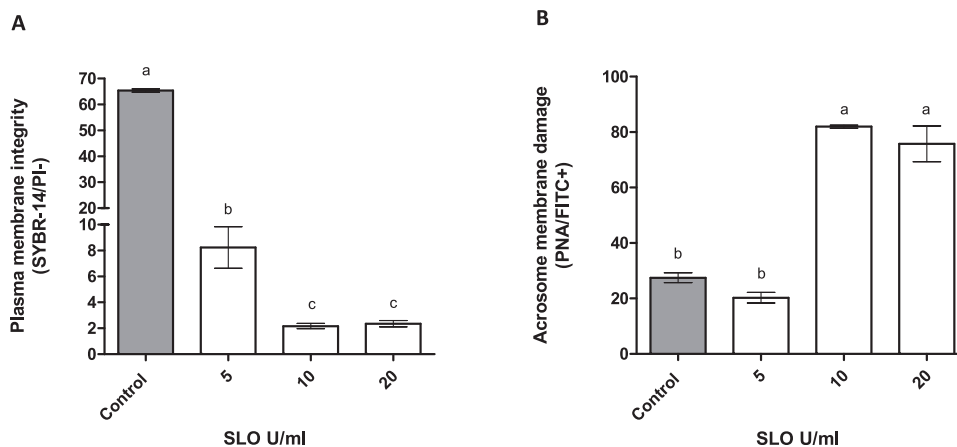

Fig. 1. Effect of bull sperm incubation with different concentrations of streptolysin (SLO U/ml).
Panel A: Plasma membrane integrity (%); Panel B: Acrosome membrane damage [a,b,c] on the bars indicate differences (P < 0.05).

Table 1

Effect of SLO treatment and sperm incubation with (+) or without (-) exogenous DNA for 30 min on the plasma and acrosome membrane integrity and sperm DNA damage assessed by flow cytometry.

Sperm treatments	Exogenous DNA incubation	Plasma membrane integrity (%)	Acrosome membrane damage (%)	DNA damage (%)
		(SYBR14 + /PI-)	(PNA/FITC+)	(TUNEL +)
Control	–	65.40 ± 0.60[a]	27.46 ± 1.75[a]	1.73 ± 0.37[a]
Control	+	64.16 ± 2.02[a]	25.33 ± 5.50[a]	1.43 ± 0.40[a]
SLO	–	2.16 ± 0.20[b]	81.96 ± 0.50[b]	3.73 ± 1.02[b]
SLO	+	1.66 ± 0.57[b]	81.66 ± 0.57[b]	4.06 ± 0.70[b]

[a,b]in the same column indicate differences ($P < 0.05$).

in spermatozoa incubated with 10 (82.0%) and 20 (75.8%) U/ml of SLO compared to the untreated control (27.5%, Fig. 1B).

3.2. Effect of SLO treatment and exogenous DNA incubation of cryopreserved spermatozoa on plasma and acrosome membrane integrity and sperm DNA damage

Data included in Table 1 indicate there were no differences in plasma membrane integrity when spermatozoa were incubated with or without exogenous DNA in either the control group or the group treated with SLO. A significant decrease, however, was observed ($P < 0.05$) in the percentage of spermatozoa treated with SLO. Spermatozoa treated with SLO had greater damage in the plasma membrane (~98%) compared to the untreated control (~35%; Table 1).

There was a similar pattern detected with evaluation of acrosome membrane integrity in spermatozoa stained with PNA/FITC (Table 1). When spermatozoa were incubated with and without exogenous DNA, no differences were detected in the acrosome membrane integrity; however, there was a greater damage when SLO was used (81%) than in the control group (26%; $P < 0.05$).

Analysis of sperm DNA damage using TUNEL indicated there were no differences when exogenous DNA was added to either the control sample or the group treated with SLO; however, the spermatozoa treated with SLO had greater damage compared to the control group (Table 1).

3.3. Effects of sperm treatment with SLO on the ability to bind exogenous DNA

Both the spermatozoa incubated with exogenous DNA (control) and those treated with SLO prior to incubation with exogenous DNA had a greater capacity to bind exogenous DNA molecules (> 99%; Table 2). As in the previous viability assessment, spermatozoa treated with SLO were less viable than those of the control group ($P < 0.05$).

3.4. Location and amount of exogenous DNA in spermatozoa treated with SLO

To determine the location of the exogenous DNA in spermatozoa, cryopreserved spermatozoa were incubated with 500 ng of DNA labeled with FITC for 30 min in the presence and absence of SLO (10 U/ml). Confocal microscopy analysis allowed for identification of the positive fluorescent signal in the post-acrosomal region and tail of spermatozoa (Fig. 2).

A flow cytometry-based evaluation of the amount of exogenous DNA present in spermatozoa revealed a significant increase in exogenous DNA ($P < 0.05$; Fig. 2) in spermatozoa treated with SLO (14,672 MFI) compared to the control (327 MFI) (Fig. 3).

3.5. In vitro embryonic development and EGFP gene expression in embryos generated by ICSI-SMGT

Of 497 MII-oocytes that were injected *in vitro*, there were no differences in the cleavage in the groups of embryos produced by SLO-treated spermatozoa with or without exogenous DNA treatment compared to the control group (Table 3). The blastocyst rate, however, was significantly less in spermatozoa incubated with exogenous DNA (16.8% and 15.5% for control and SLO-treated group, respectively) compared to those not incubated with DNA ($P < 0.05$). Nevertheless, the efficiency rates of EGFP gene expression in the cleavage and blastocysts increased significantly when the spermatozoa were permeabilized with SLO, unlike the control group (Table 3, $P < 0.05$).
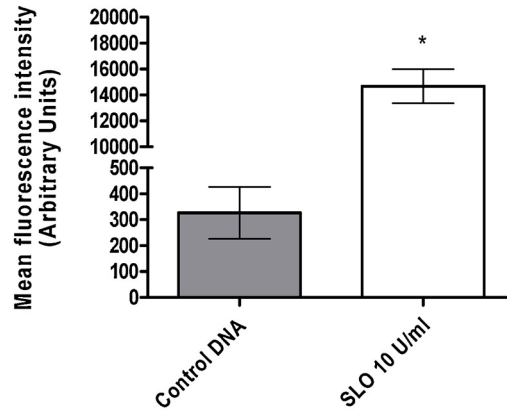
Table 2

Sperm viability and exogenous DNA binding with or without SLO after 30 min of incubation.

Sperm treatment	% Bound spermatozoa		
	Total	Dead	Live
Control	99.3 ± 0.08	28.4 ± 0.72	70.9 ± 0.79[*]
SLO	99.1 ± 0.34	92.3 ± 1.81[*]	6.8 ± 2.16

[*] in the same column indicate differences ($P < 0.05$).

A

B

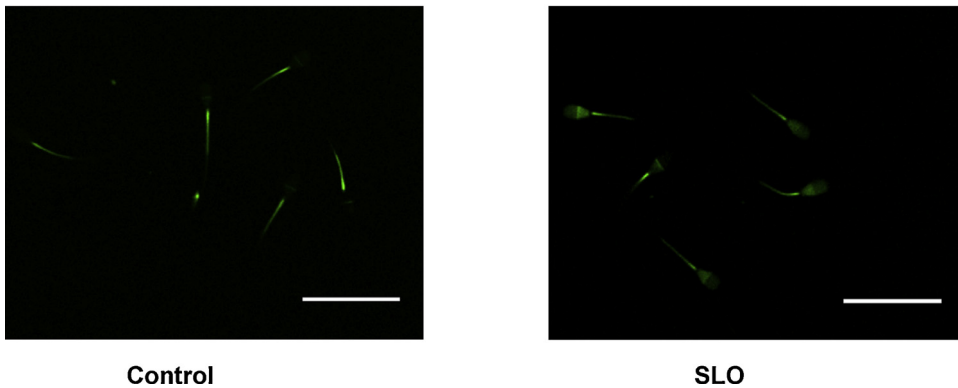Control   SLO

Fig. 2. FITC-labelled DNA bound to sperm treated with SLO.
Bull sperm treated or not treated with SLO were incubated for 30 min with exogenous DNA. Panel A shows the mean fluorescence intensity (MFI) of flow cytometry data and Panel B shows representative confocal images depicting DNA binding location; Original magnification 100x, scale bar 20 μm; [*] on the bars indicate differences ($P < 0.05$).

4. Discussion

The ICSI-SMGT technology is valuable for production of transgenic animals. Even with the success of this technique in mice (Perry et al., 1999; Sim et al., 2013), its efficiency continues to be less than desirable in farm animals such as cattle (Arias et al., 2015; Salamone et al., 2017). In cattle, it has been reported that the exogenous DNA uptake can be improved with the use of cryopreserved rather than fresh spermatozoa (Anzar and Buhr, 2006).

Cryopreservation induces changes in the plasma membrane due to low temperatures and osmotic effects (Holt and North, 1984; Yeste, 2015). This is why it has been suggested that sperm membrane alterations caused by the cryopreservation process would promote exogenous DNA uptake, thereby improving the production of transgenic embryos (Anzar and Buhr, 2006). The results described to date, however, continue to be controversial and irreproducible, and the efficiency of the technique has not improved, limiting the use of ICSI-SMGT to produce transgenic animals (Hoelker et al., 2007; Bevacqua et al., 2010; Eghbalsaied et al., 2013; Arias et al., 2017).

A novel approach supported by studies in several species suggests that gene transference and expression efficiency can be improved in ICSI-SMGT when physical and/or chemical damage to spermatozoa membrane increases. Some of these treatments, however, markedly increase exogenous DNA uptake, affecting sperm DNA integrity, subsequent embryonic development and transgene expression (Garcia-Vazquez et al., 2009; Sanchez-Villalba et al., 2018). It, therefore, is important to maintain a balance between the amount of sperm membrane damage and the different treatments to ensure both optimal uptake of exogenous DNA and proper embryonic development (Kurome et al., 2007; Garcia-Vazquez et al., 2009).

Sim et al. (2013) previously described the highly desirable rate of transgenic embryo development in mice by permeabilizing the sperm membrane with SLO. One of the most important toxins of *Streptococcus pyogenes* is SLO and its actions are through the binding of cholesterol in the plasma membrane of mammalian cells creating stable pores (Bhakdi et al., 1985; Atanassoff et al., 2014). There has been extensive use of SLO to introduce different exogenous molecules such as DNA, RNA and proteins within cell compartments
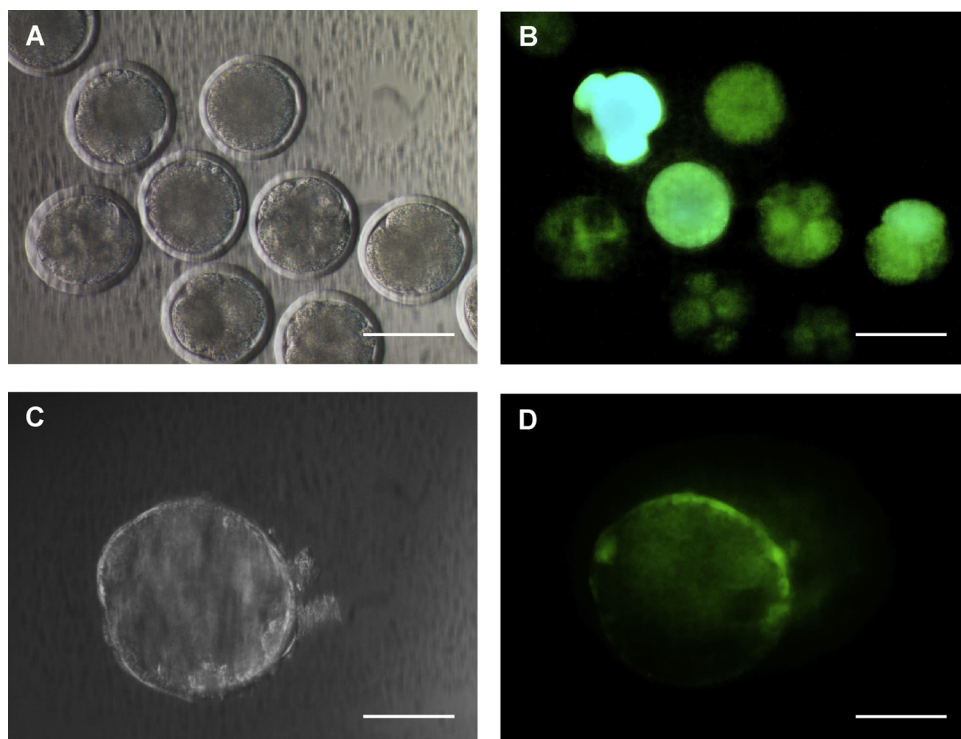
Fig. 3. EGFP gene expression in bull embryos at different developmental stages; Embryos were generated by injecting SLO-treated sperm incubated with EGFP plasmid; A and C: Cleaved and blastocyst embryos under bright-field; B and D: The same embryos expressing the EGFP gene shown under blue light; Original magnification 200x, scale bar 30 μm.

Table 3
Effect of sperm treatment with SLO and incubation with exogenous DNA on the *in vitro* embryonic development and EGFP-gene expression of cattle embryos produced by ICSI-SMGT.

Sperm treatment	Exogenous DNA incubation	No. of injected oocytes	Embryonic development n (%)		EGFP expression in embryos n (%)	
			Cleavage	Blastocysts/ injected oocyte	Transgenic/Cleaved	Transgenic/ total blastocysts
Control	–	125	104 (83.2)	28 (22.4)[b]	–	–
Control	+	125	100 (80.0)	21 (16.8)[c]	10 (10.0)	2 (9.5)
SLO	–	125	106 (84.8)	36 (28.8)[a]	–	–
SLO	+	122	102 (83.6)	19 (15.5)[c]	40 (39.2)[a]	10 (52.6)[a]

Cleavage and transgenic gene expression assessment of EGFP in embryos was recorded at 72 h of culture and blastocyst and transgenic blastocyst rates were recorded at 192 h; SLO: Streptolysin-O (10 U/ml) and Control: sperm without SLO treatment; [a,b,c] In the same column indicate differences ($P < 0.05$).

(Walev et al., 2001; Brito et al., 2008; Sim et al., 2013); however, its use in ICSI-SMGT in cattle has not been previously described.

To study the effect of SLO as a membrane permeabilizing treatment, the plasma and acrosome membrane integrity in spermatozoa treated with different concentrations of SLO were initially evaluated and assessments as to what is the most desirable treatment with sperm incubated or not incubated with exogenous DNA. The result of these experiments confirmed that SLO can efficiently permeabilize the plasma and acrosome membrane in bull sperm.

Because sperm membrane damage caused by SLO or exogenous DNA might contribute to the activation of endonucleases capable of degrading exogenous and/or sperm DNA, preventing the transmission to the next generation (Anzar and Buhr, 2006), sperm DNA integrity was further evaluated using the TUNEL assay. Results indicate the addition of exogenous DNA does not affect sperm DNA integrity. The DNA damage, however, was slightly increased as a result of treatment with SLO compared to the control, regardless of whether sperm were incubated with exogenous DNA. Nevertheless, the rates observed are within the normal parameters previously described by different studies in cattle (Feitosa et al., 2010; Goodla et al., 2014; Zambrano et al., 2016; Arias et al., 2017).

Subsequently, the effects of SLO on the binding capacity and uptake of exogenous DNA was evaluated. Results indicate that there were no differences in the percentage of spermatozoa that bind exogenous DNA in the SLO or control treatment groups. Differences, however, were observed in the amount of exogenous DNA bound to spermatozoa. Exogenous DNA uptake was greater when the

cryopreserved spermatozoa were permeabilized with SLO compared to the untreated control group, which is consistent with a previous study in mice that demonstrates greater exogenous DNA uptake after this treatment (Sim et al., 2013). To verify this finding, the binding site of exogenous DNA on different structures of cryopreserved spermatozoa was identified using confocal microscopy. The result of this experiment indicated that exogenous DNA binding was mainly located in the post-acrosomal region and the tail of spermatozoa, which is consistent with findings in previous studies with cattle and other species (Anzar and Buhr, 2006; Eghbalsaied et al., 2013; Sim et al., 2013; Arias et al., 2017).

After the effectiveness of SLO on exogenous DNA uptake and DNA integrity in cryopreserved spermatozoa was confirmed, the effects of SLO treatment in the presence or absence of exogenous DNA on development and EGFP gene expression of embryos produced by ICSI-SMGT were assessed. The results of these experiments indicate incubation of spermatozoa with exogenous DNA does not affect the early stages of embryonic development; nevertheless, the blastocyst rate was affected in both groups (control and SLO-treated) when incubated spermatozoa were injected with exogenous DNA. This effect could be related to the oxidation mechanism and release of hydrogen peroxide during the maturation of EGFP protein (Tsien, 1998), which is consistent with previous reports describing the sensitivity of cattle embryos to free radicals (Cheuqueman et al., 2015). By contrast, when evaluating the effect of SLO in the absence of exogenous DNA, blastocyst rate was greater compared to that with untreated spermatozoa, an effect that was also observed in the control group. These data are consistent with those from previous studies confirming that plasma and acrosome membrane destabilization positively affects the development of cattle embryos produced by ICSI (Zambrano et al., 2016, 2017). This may be due to the lack of coordination in the formation of both pronuclei when an oocyte is injected with an intact spermatozoon (Kasai et al., 1999; Roldan, 2006). Additionally, hydrolytic enzymes released from the acrosome to the oocyte´s cytosol generate a toxic and proportional effect to the size and content of the acrosome (Morozumi and Yanagimachi, 2005).

The evaluation of EGFP gene expression in cattle embryos produced by ICSI-SMGT confirm that the transgene is expressed from the initial stages of embryonic development (4- to 8-cell stages) similar to previously reported results using EGFP (Chan et al., 2000; Pereyra-Bonnet et al., 2008; Garcia-Vazquez et al., 2009). The greater rate of embryos expressing EGFP, however, was observed in SLO-treated spermatozoa compared to the control group. A similar effect was observed in the rate of blastocysts expressing the EGFP gene. These data are consistent with the results in mice previously described by Sim et al. (2013), demonstrating that there was a greater rate of embryos expressing EGFP gene (60%) with SLO-treated sperm.

In conclusion, the results of the present study confirm the capacity of SLO to permeabilize the plasma and acrosome membranes of bull spermatozoa and to increase the binding of exogenous DNA without greatly affecting the integrity of the DNA. Furthermore, SLO treatment of bull spermatozoa prior to injection of oocytes by ICSI-SMGT increased the rate of efficiency of embryo production in which there was expression of the EGFP gene. Future experiments are still required to determine the effect of this treatment on the development and transgene expression in fetuses and animals generated by ICSI-SMGT.

Conflict of interest

Authors declare to have no conflict of interest.

Acknowledgements

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