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Effect of sperm treatment on efficiency of EGFP-expressing porcine embryos produced by ICSI-SMGT

F.A. García-Vázquez [a,*], E. García-Roselló [b], A. Gutiérrez-Adán [c], J. Gadea [a]

[a] *Department of Physiology, Faculty of Veterinary Science, University of Murcia, Murcia 30100, Spain*
[b] *Department of Medicine and Animal Surgery, Faculty of Veterinary Science, University of CEU-Cardenal Herrera, Valencia (Moncada) 46113, Spain*
[c] *Department of Animal Reproduction, INIA, Madrid 28040, Spain*

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Abstract

Intracytoplasmic sperm injection–sperm-mediated gene transfer (ICSI-SMGT) is a useful tool for the production of transgenic mice but is still rather inefficient in farm animals. In the current study, we evaluated the effect of the sperm treatments on the efficiency for producing enhanced green fluorescent protein (EGFP)-expressing pig embryos by ICSI-SMGT. Four different sperm treatments were assayed: (1) fresh (control), (2) frozen-thawing (FT), (3) quick freezing without cryoprotectant agents (QF), and (4) Triton X-100 treatment (TX-100). First, we evaluated the DNA-binding ability and the viability of sperm under the different treatments coincubated with exogenous DNA (EGFP) by flow cytometry. Second, we evaluated the embryo production rate and the efficiency in transgene expression in embryos after using these spermatozoa to fertilize oocytes by ICSI. Sperm treatment significantly increased DNA-binding capacity but reduced sperm viability compared with that of the control group. Treatments damaging the spermatozoa's membranes (QF and TX-100) resulted in a greater capacity of sperm binding exogenous DNA than that after FT treatment ($P < 0.01$). Similar rates of EGFP-expressing embryos were obtained from the control, FT, and TX-100 groups ($37.04 \pm 3.52\%$, $43.54 \pm 5.41\%$, and $29.03 \pm 8.29\%$, respectively), but were significantly higher in the QF group ($80.43 \pm 5.91\%$). These results demonstrate that the integrity of the sperm plasma membrane plays a critical role in DNA interaction, and altered plasma membranes facilitate interactions between an injected exogenous DNA and the sperm chromatin. However, severe sperm treatments such as QF and TX-100 may damage the sperm nucleus, induce DNA fragmentation, and/or lead to chromosomal breakage with a detrimental effect on further embryonic development.

Keywords: Gene transfer technique; ICSI; Oocyte; Spermatozoa; Sperm-mediated gene transfer

1. Introduction

In recent years, transgenic pigs have become an important tool in biomedical research, including the production of biomolecules in the mammary gland, the development of transgenic animals to improve productivity, in research on xenotransplantation, and as models for human diseases [1–3]. All these applications depend on the output of transgene(s) expression, and many different strategies such as pronuclear microinjection, vector virus, nucleus transfer, and stem cells have been developed to generate transgenic animals [4,5]. DNA pronuclear microinjection has been the most popular system to generate transgenic animals, and transgenic mice, sheep, pigs, and cattle have been successfully produced using this technique. However, besides being

* Corresponding author. Tel.: +34 968 364309; fax: +34 968 364147.
E-mail address: fagarcia@um.es (F.A. García-Vázquez).

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expensive, this technique is still inefficient when used to generate transgenic farm animals (0.5% to 4% of transgenic rate) [4,5].

Sperm-mediated gene transfer (SMGT) is based on the ability of sperm to bind, internalize, and transport exogenous DNA into an oocyte during fertilization [6–10]. The foreign DNA can be either integrated into the spermatozoa chromosomal DNA or simply transferred to the egg by the spermatozoa and later integrated into the zygote's genome [11]. Rabbit sperm cells were reported to spontaneously take up and transfer DNA into an oocyte during fertilization resulting in the genetic modification of the 2-cell-stage embryos [12]. In 1989, the birth of live transgenic mice was reported after epididymal sperm cells transferred exogenous plasmid DNA into an oocyte during fertilization [6]. Sperm-mediated gene transfer has been used more or less successfully in the production of transgenic embryos and animals in a large number of species [13]. Although transgenic animals have been obtained using SMGT, its efficiency is still low, mainly due to the spermatozoa's low uptake of exogenous DNA, thereby reducing the number of fertilized oocytes with transfected spermatozoa [14]. In addition, interspecies and intraspecies success variability is still an unsolved problem associated with this technology.

The determination of the DNA binding to the sperm cells is a key point in this technique. Radiolabeled measurements [15–17], fluorescent microscopy [14], and immunohistochemistry techniques [18] have been used to measure DNA binding. Flow cytometry seems to be a very valuable tool for evaluating DNA binding and viability, so it is possible to evaluate the kinetics of the binding process. This methodology has been validated with fluorescent microscope observation and the use of multispectral imaging flow cytometry (ImageStream; Amnis Corporation, Seattle, WA), a combination of quantitative image analysis and flow cytometry (unpublished data).

The resultant DNA-carrying sperm are then used to fertilize eggs, via in vitro fertilization (IVF) or artificial insemination (AI) or, in the case of aquatic animals, via waterborne (natural) fertilization. Other studies have used "augmentation" techniques, such as electroporation or liposomes, to "force" sperm to capture transgenes [19]. More recent studies have introduced transgenes directly into the reproductive tract of male animals in what is known as testis-mediated gene transfer (TMGT) [20,21].

Another innovation related to SMGT technique has been the use of intracytoplasmic sperm injection (ICSI) to deliver transgene-containing sperm cells directly into the egg, a process known as the ICSI-mediated method [22]. In pigs, ICSI is a technique with potential application in diverse fields of animal production and biomedicine. The combination of the ICSI-mediated method and in vitro–matured (IVM) oocytes would both greatly reduce the cost and streamline the procedure and would facilitate an expansion of the practical value of transgenic pigs, increasing their availability [23]. Efficient ICSI-SMGT makes feasible the use of sperm with plasma membranes damaged by physical (freezing and thawing) or chemical methods (by using a detergent like Triton X-100). Disruption of the sperm membrane allows DNA constructs to associate with submembrane structures, and this is a key step for successful DNA insertion into oocytes [22]. Also, it has been reported in mice that in ICSI-mediated transgenesis, the mechanism of transgene integration depends on the sperm treatment used before the injection [24]. However, the efficiency to produce transgenic pigs by ICSI-SMGT is still low [19,23,25] because the rates of blastocyst formation and live newborn are greatly reduced when zygotes are generated by ICSI [26].

The current study was conducted to evaluate the effect of the sperm treatments on the efficiency for producing EGFP-expressing pig embryos by ICSI-SMGT. Four different sperm treatments were assayed: (1) fresh (control), (2) frozen-thawing (FT), (3) quick freezing without cryoprotectant agents (QF), and (4) Triton X-100 treatment (TX-100). First, we evaluated the DNA-binding ability and the viability of sperm under the different treatments coincubated with exogenous DNA (EGFP) by flow cytometry. Second, we evaluated the embryo production rate and the efficiency in transgene expression in embryos after using these spermatozoa to fertilize oocytes by ICSI.

2. Materials and methods

2.1. Media and chemicals

Unless otherwise indicated, all chemicals and reagents were purchased from Sigma-Aldrich Química S.A. (Madrid, Spain). The medium used for oocyte maturation was NCSU-37 [27] supplemented with 0.57 mM cysteine, 1 mM dibutyryl cAMP, 5 μg/mL insulin, 50 μM β-mercaptoethanol, 10 IU/mL eCG (Foligon; Intervet International B.V., Boxmeer, Holland), 10 IU/mL human chorionic gonadotropin (hCG; VeterinCorion; Divasa Farmavic, Barcelona, Spain), and 10% porcine follicular fluid (v/v).

The basic medium used for ICSI was TALP medium [28], consisting of 114.06 mM NaCl, 3.2 mM KCl, 8 mM Ca-lactate·$5H_2O$, 0.5 mM $MgCl_2 \cdot 6H_2O$, 0.35 mM NaH_2PO_4, 25.07 mM $NaHCO_3$, 10 mL/L Na-lactate,

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1.1 mM Na-pyruvate, 5 mM glucose, 2 mM caffeine, 3 mg/mL bovine serum albumin (BSA) (A-9647), 1 mg/mL polyvinyl alcohol (PVA), and 0.17 mM kanamycin sulfate.

The embryo culture medium was NCSU-23 containing 0.4% BSA (A-8022), 75 μg/mL potassium penicillin G, and 50 μg/mL streptomycin sulfate.

Swine Fertilization Medium (SFM) [17] was used for processing the spermatozoa (SFM: 1 L contains 11.25 g glucose, 10 g sodium citrate $2H_20$, 4.7 g EDTA $2H_2O$, 3.25 g citric acid H_2O, 6.5 g Trizma supplemented with 6 mg/mL BSA [SFM/BSA]).

2.2. Oocyte collection and in vitro maturation

Within 30 min of slaughtering, ovaries from prepubertal gilts were transported to the laboratory in saline (0.9% w/v NaCl) containing 100 μg/mL kanamycin sulfate at 37 °C and then washed once in 0.04% (w/v) cetrimide solution and twice in saline. cumulus cell–oocyte complexes (COCs) were collected from non-atretic follicles (3 to 6 mm diameter), washed twice in 35-mm plastic Petri dishes containing Dulbecco's phosphate-buffered saline (DPBS) supplemented with 4 mg/mL PVA and 0.0005 g/L red phenol, and washed twice more in maturation medium previously equilibrated for at least 3 h at 38.5 °C under 5% CO_2 in 100% humidified air. Only COC harvested within 2 h of slaughter [29] with a homogeneous cytoplasm and a complete and dense cumulus oophorus were matured. Groups of 50 COCs were cultured in 500 μL maturation medium for 20 to 22 h at 38.5 °C under 5% CO_2 in air. Once cultured, the COCs were washed twice, transferred to fresh maturation medium without hormonal supplements or dibutyryl-cAMP, and cultured for a further 20 to 22 h [30].

2.3. Transgene construction and labelling

The enhanced green fluorescent protein (EGFP) plasmid construction (pEGFPN1, 5.4 kb; Clontech Laboratories, Inc., Palo Alto, CA, USA) used for our experiments contained the human citomegalovirus (CMV) immediate early promoter and the enhanced GFP gene. This construction was linearized with AflII (gene from Anabaena flos-aquae) prior to use. The transgene was purified using an Elu-Quit DNA Purification Kit (Schleicher & Schuell, Dassel, Germany) following the manufacturer's instructions. DNA was resuspended in TE (10 mM Tris, 0.1 mM EDTA, pH 8).

Lineal plasmid was labeled with fluorescein-12-dUTP (Roche, Mannheim, Germany). DNA labeling was carried out by means of random primer. Briefly, mold DNA (linearized plasmid) was denatured by application of 95 °C for 5 min; nucleotides (1 mM of dATP, dCTP, and dCTP; 0.65 mM of dTTP; and 0.35 mM of the marked nucleotide) were added later as well as 5x buffer and 1 U/μL Klenow (Promega, Madrid, Spain). Subsequently, the mixture was incubated for an hour at 37 °C, and the reaction was stopped by adding 2 μL 0.2 M EDTA (pH 8). Marked DNA precipitated out with cold ethanol and was finally resuspended in TE microinjection buffer. Verification of plasmid marking was determined in an agarose and dying gel with ethidium bromide. The incorporation of the labeled nucleotide to synthesized DNA diminishes its electrophoretic mobility in relation to nonmarked DNA [31].

2.4. Semen collection and preparation of sperm for DNA uptake

Fresh semen was routinely collected from mature fertile boars (''Lo Navarro'' S.A., Murcia, Spain) using the manual method and a dummy [32]. The sperm-rich fraction was collected in a prewarmed thermo flask, and the gel fraction was held on a gauze tissue covering the thermo opening.

Semen was prepared according to the previously described methods [17]. Briefly, after collection of the sperm-rich fraction of ejaculate, the semen was diluted 1:1 v/v in SFM medium without BSA, and it was transported to the lab at 37 °C. Once in the lab, it was diluted again in medium SFM (37 °C) in 1:10 v/v proportion and centrifuged at 800 × g for 10 min at 25 °C. Supernatant was removed by aspiration and the pellet resuspended in SFM (with 6 mg/mL BSA) at 25 °C, centrifuged again at 800 × g for 10 min at 25 °C, supernatant was discarded, and the pellet was resuspended in 1 mL SFM with BSA at 25 °C. Motility was evaluated again once spermatozoa processing had finished (only samples with motility over 65% and a progressive motility no less than 2.5 using an arbitrary scale from 0 to 5 were used) [33], and final concentration was calculated using a calibrated photometer (Spermacue, Minitüb, Tiefenbach, Germany). Finally, concentration of sperm suspension was adjusted to 10^8 sperm cells/mL.

Spermatozoa were incubated with linearized EGFP transgene (10^8 cells/mL and 5 μg DNA/mL) in a final volume of 25 μL SFM with BSA at 16 °C.

2.5. Sperm treatment

2.5.1. Frozen-thawed spermatozoa

Semen samples were processed using a freezing procedure previously described [34] and were preserved

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in 0.5 mL straws in liquid nitrogen until use. Thawing was done by immersing the straws in a circulating water bath at 50 °C for 12 sec [35]; immediately after thawing, the semen was diluted 1:5 v/v in SFM medium at 37 °C. Diluted sample was washed twice by centrifugation (10 min, 800 × g at 25 °C) to eliminate freezing medium and cellular debris. Finally, pellet was recovered and resuspended in SFM/BSA medium.

2.5.2. Membrane disruption by quick freezing

To prompt structural alteration of sperm membranes, spermatozoa were subject to a quick and repeated process of freezing/thawing without cryoprotectant agents. We introduced a suspension of fresh spermatozoa into a liquid nitrogen bath for 20 sec, followed by immediate thawing in a water bath at 37 °C. This process was repeated three times. After the process, it was confirmed that all sperm cells had serious alterations of the structure of their membrane by microscopic observation.

2.5.3. Treatment with TX-100 detergent

The treatment with a solution of TX-100 was developed according to the protocol previously described [22]. One hundred microliters of TX-100 at 0.5% (v/v) in saline solution was added to 900 μL fresh sperm suspension. After incubation at room temperature for 10 min, sample was centrifuged twice (3 min at 10,000 × g). Pellet was recovered and resuspended in SFM/BSA medium.

2.6. Flow cytometric analyses

Flow cytometric analyses were performed on a Coulter Epics XL cytometer (Beckman Coulter Inc., Miami, FL, USA). A 15-mW argon ion laser operating at 488 nm excited the fluorophores. Data from 10,000 events per sample were collected in list mode, and three measures per sample were recorded. Flow cytometric data were analyzed using the program Expo32ADC (Beckman Coulter Inc.) using a gate in forward and side scatter to exclude eventual remaining debris and aggregates from the analysis.

Spermatozoa were incubated with transgene labeled with fluorescein (FITC-DNA) and simultaneously with propidium iodide (PI) for evaluating DNA binding capacities and cellular viability during the incubation time (0, 15, 30, 60, 90, and 120 min). Fluorescence was measured using an FL-1 sensor, a 525-nm band-pass filter to detect FITC-DNA, an FL-2 sensor, and a 575-nm band-pass filter to detect PI. Cells were classified into four categories: (1) living spermatozoa without DNA bound (no sign of fluorescence), (2) living spermatozoa with DNA bound (only green fluorescence), (3) dead spermatozoa with DNA bound (red and green fluorescence), and (4) dead spermatozoa without DNA bound (red fluorescence).

2.7. Intracytoplasmic sperm injection

The treated spermatozoa (fresh, frozen-thawed, quick frozen, or TX-100) were incubated with transgene plasmid Enhanced Green Fluorescent Protein (pEGFP) DNA at 16 °C for 30 min and then prewarmed at 38 °C for 5 min before ICSI.

Cumulus cell–oocyte complexes cultured for 44 h in maturation medium were mechanically stripped of cumulus cells by gentle aspiration with an automatic pipette. Denuded oocytes were washed twice in supplemented DPBS medium and transferred to ICSI drops. ICSI was conducted on a heated plate at 200× magnification using an inverted microscope (Nikon Diaphot 300, Tokyo, Japan) with attached micromanipulators (TransferMan NK; Eppendorf, Hamburg, Germany). The ICSI medium used was DPBS supplemented with 10% FCS (v/v). Prior to ICSI, oocytes were placed on a lid of Petri dish (1 oocyte per 4-μL drop of DPBS/FCS). In total, 10 to 15 microdrops were placed on each lid surrounding central sperm drops containing 4 μL DPBS/FCS and 1 μL sperm suspension. The microdrops were covered with mineral oil. ICSI was performed as we have previously described [36,37]. Briefly, one single sperm was aspirated with the tail first. Thereafter, the injection pipette was moved into the drop containing the oocytes to be injected. A single oocyte was fixed by the holding pipette, positioning the polar body at 6 or 12 o'clock position. The injection pipette was pushed through the zona pellucida and subsequently through the oolemma into the cytoplasm at 3 o'clock position. A small amount of ooplasm was aspirated into the injection pipette in order to ensure oocyte membrane penetration. Subsequently, the immobilized spermatozoon was released into the cytoplasm.

2.8. Microscopic evaluation of the embryos

To assess the embryo development, the number of 2-cell-stage embryos and blastocysts were evaluated at 48 and 144 h after fertilization, respectively, under a stereomicroscope. Embryos were then placed on a slide, air-dried, and fixed in absolute ethanol for 24 h. After staining with Hoechst 33342 (10 mg/mL in DPBS), cell nuclei were counted under an epifluorescence microscope.

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2.9. Analysis of EGFP expression in embryos

The expression of EGPF in embryos was evaluated 144 h after fertilization. We examined embryos for expression of GFP by epifluorescence microscopy (Diaphot 300, Nikon) with fluorescein isothiocyanate filters (excitation range of 395 to 470 nm and emission spectrum of 509 nm). This enabled the clear identification of nonfluorescent (non–GFP-expressing) and fluorescent (GFP-expressing) embryos, which were scored accordingly.

2.10. Statistical analysis

Data are presented as means ± SEM and analyzed by one-way ANOVA considering the specific sperm treatment as the main variable. When ANOVA revealed a significant effect, values were compared by the least significant difference pairwise multiple comparison post hoc test (Tukey). Differences were considered statistically significant at $P < 0.05$.

Cleavage and blastocyst rate and protein expression data (categorical data) were modeled according to the binomial model of parameters by arcsine transformation of the data and were analyzed by one-way ANOVA.

3. Results

3.1. Effect of sperm treatment on DNA-spermatozoa interaction

Percentage of total DNA-bound spermatozoa, dead and live cells, and dead DNA-bound spermatozoa were recorded and analyzed. Sperm treatment significantly increased DNA-binding capacity and reduced the sperm viability compared with that of control after 120 min (Table 1). The treatments that induce an immediate alteration of the membrane structure (QF and TX-100) were associated with a higher degree of DNA binding (97.52 ± 0.74% and 90.93 ± 2.61%, respectively) and significantly greater capacity to bind to exogenous DNA than that of the less aggressive treatment, the FT (Table 1). So, control group reached 30.30 ± 1.94% of DNA-bound spermatozoa, whereas FT group reached 61.36 ± 2.86% (Table 1). The DNA-binding capacity was inversely related to the sperm viability (Fig. 1; $r = 0.95$, $P < 0.01$).

Exogenous DNA mainly bound to spermatozoa with reduced viability in all the experimental groups (Table 1). In consequence, only a low percentage of living spermatozoa was bound to DNA (mean value lower than 4% in all treatments). A remarkably higher percentage of live cells bound to DNA were detected when FT spermatozoa were used than that in the other treatments ($P < 0.001$, Table 1). For FT spermatozoa, the percentage of DNA-bound live spermatozoa increased significantly after 15 min of coincubation and decreased later with the incubation time (Fig. 2). However, no differences were detected during the incubation time for the other experimental groups.

3.2. Evaluation of in vitro–produced EGFP-expressing embryos by ICSI

3.2.1. Evaluation of in vitro–produced EGFP-expressing embryos by ICSI using fresh spermatozoa

In a first study, a total of 167 IVM oocytes were injected with spermatozoa incubated or not (control) with EGFP to evaluate whether the exogenous DNA affects embryo development. The cleavage rate for the DNA group was 44% (64 of 144), and 16 of them reached the blastocyst stage (25%) with an average number of cells per blastocyst of 22.71 ± 3.64 (Table 2). From the obtained embryos, 14 (21.8%) of them expressed the fluorescent green protein (from 2-cell embryos to blastocyst stage).

The incubation of the spermatozoa with DNA did not affect the embryo output, where there were similar rates for cleavage and blastocysts and the same number of cells per blastocyst than in a control ICSI procedure

Table 1
Values (percentage) for sperm viability and DNA binding to the spermatozoa after 120 min incubation measured by flow cytometry after different sperm treatments.

Sperm treatment	Dead spermatozoa, %	DNA-bound spermatozoa, %	DNA-bound and dead spermatozoa, %	DNA-bound and live spermatozoa, %
Control	29.59 ± 01.88[a]	30.30 ± 1.94[a]	29.06 ± 1.91[a]	1.24 ± 0.09[a]
FT	61.63 ± 2.84[b]	61.36 ± 2.86[b]	57.48 ± 2.65[b]	3.87 ± 0.92[b]
QF	96.70 ± 0.26[c]	97.52 ± 0.74[c]	95.28 ± 0.66[c]	2.24 ± 0.28[a]
TX-100	97.54 ± 0.42[c]	90.93 ± 2.61[c]	89.84 ± 2.64[c]	1.08 ± 0.16[a]

[a,b,c]Different letters in the same column indicate significant differences.

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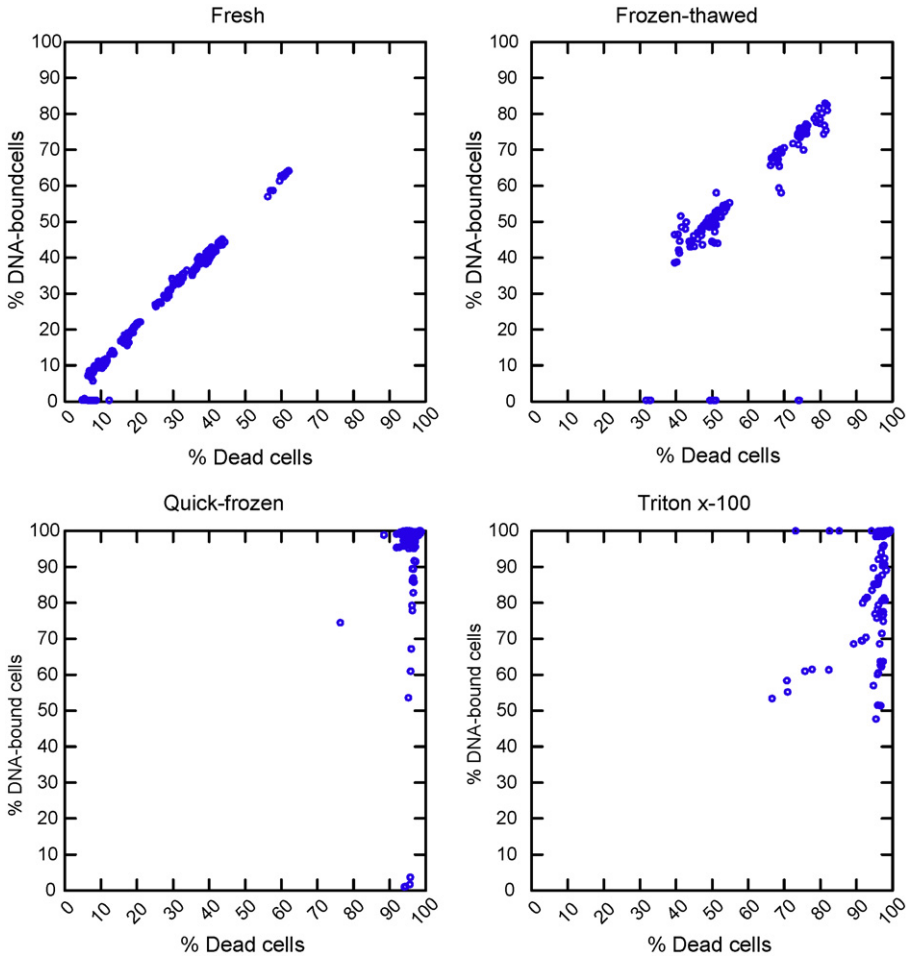

Fig. 1. Regression plots for dead cells (%) and DNA-bound spermatozoa (%) in boar spermatozoa throughout 120 min of incubation evaluated by flow cytometry after different sperm treatments. Pearson correlation coefficient (r) = 0.95, $P < 0.01$.

when the sperm was not incubated in presence of exogenous DNA (Table 2).

3.2.2. *Production of EGFP-expressing embryos by ICSI using physical (FT and QF) and chemical (TX-100) methods for sperm membrane damage*

In total, 758 IVM porcine oocytes were injected with spermatozoa incubated with EGFP plasmid. Cleavage rates were similar in control, FT, and QF groups being around 50%, but cleavage rate was lower in TX-100 group (32.29 ± 4.80%, $P < 0.02$) (Table 3). Blastocyst formation rate tended to be lower in the QF than in fresh and FT groups and any blastocyst formation in TX-100 was produced ($P = 0.12$). The number of cells/blastocyst was similar among groups (Table 3) ($P = 0.49$).

The rate of EGFP-expressing embryos that expressed partially or totally the fluorescent green protein obtained in this experiment for fresh, FT, and TX-100 did not show any differences between experimental groups (37.04 ± 3.52%, 43.54 ± 5.41%, and 29.03 ± 8.29%), but was significantly higher in the QF group with 80.43 ± 5.91% of embryos expressing the EGFP protein (Table 3). Most of these EGFP-expressing embryos were

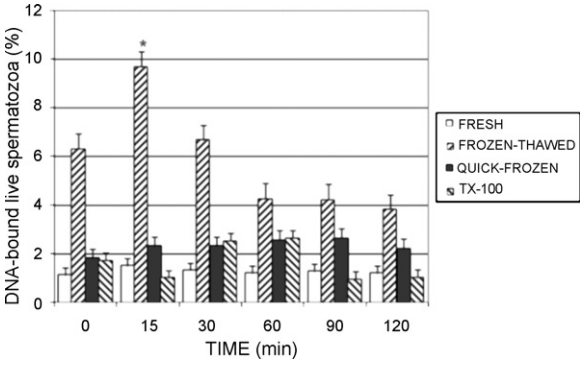

Fig. 2. Kinetics of DNA binding between live spermatozoa and exogenous DNA throughout 120 min incubation evaluated by flow cytometry. *Indicates significant differences among treatments.

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Table 2
In vitro embryonic development of oocytes injected with spermatozoa incubated (+pEGFP) or not (–pEGFP = control) with DNA and expression of EGFP protein in the embryos.

Sperm treatment	pEGFP incubation	Number of oocytes	Cleavage rate, %	Blastocyst rate, %*	Number of cells per blastocyst	Percentage EGFP total expression, %*
Fresh	–	23	60.00 ± 10.00	20.00 ± 10.69	22.00 ± 3.00	–
Fresh	+	144	44.44 ± 4.16	25.00 ± 5.46	22.71 ± 3.64	21.87 ± 5.21
P value			0.15	0.69	0.93	

* Related to cleaved embryos.

Table 3
In vitro embryonic development and EGFP expression in porcine embryos produced by ICSI–mediated gene transfer under different sperm treatments.

Sperm treatment	pEGFP incubation	Number of oocytes	Percentage cleavage rate, % (n)	Percentage blastocyst rate, %* (n)	Number of cells per blastocyst	Percentage EGFP total expression, %* (n)	Percentage mosaicism, %
Control	+	378	50.00 ± 2.58[a] (189/378)	14.29 ± 2.55 (27/189)	38.59 ± 6.30	37.04 ± 3.52[a] (70/189)	82.50 ± 6.08
FT	+	183	46.45 ± 3.70[a] (85/183)	12.94 ± 3.66 (11/85)	40.91 ± 8.49	43.54 ± 5.41[a] (37/85)	91.67 ± 8.33
QF	+	101	45.54 ± 4.98[a] (46/101)	8.70 ± 4.20 (4/46)	22.40 ± 1.17	80.43 ± 5.91[b] (37/46)	75.68 ± 7.15
TX-100	+	96	32.29 ± 4.80[b] (31/96)	0	–	29.03 ± 8.29[a] (9/31)	–
P value			<0.02	0.12	0.49	<0.01	0.45

[a,b]Different letters in the same column indicate significant differences.
* Related to cleaved embryos.

mosaic (ranging from 70% to 90%) where only a few of the blastomeres expressed the protein (EGFP) (Table 3). For example, in Fig. 3, a 2-cell embryo expressing only EGFP in one blastomere is shown.

When we analyzed embryo development and EGFP expression after the use of different sperm treated, we confirmed the previous results. The effect of the aggressive sperm treatments like QF and TX-100 tended to reduce the embryo development with higher proportion of embryos in early stages of development (Table 4, P = 0.10) and lower percentage of blastocyst (Table 4, P = 0.11), but without significant differences between these two treatments. Similarly, the QF treatment induced the higher percentages of expression of EGFP in all the types of embryos (Table 5). However, these differences were of statistical significance level only for the 2- to 8-cell embryos. The percentage of expression for each treatment was similar in all the types of embryos (Table 5, P > 0.05).

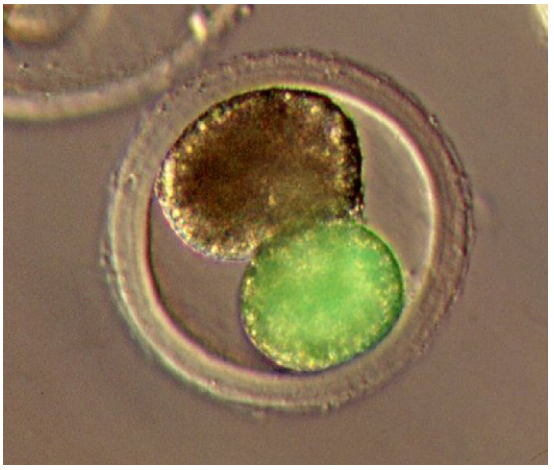

Fig. 3. Fluorescent 2-cell embryo was produced by injecting spermatozoa that had been coincubated with EGFP transgene. Mosaic EGFP expression. Embryo is shown under bright and blue light (488 nm) combination (original magnification ×200).

With the objective to evaluate whether the EGFP expression inhibited the embryo development, we compared the degree of embryo development between EGFP-expressing embryos (n = 140) and no EGFP-expressing embryos (n = 191) produced in this experiment with the different sperm treatments. Results showed that there was not any difference among both groups (EGFP expression and no EGFP expression embryos), with the same number of 2- to 8-cell embryos, >8-cell morulae, and blastocyst formation.

In Fig. 4, porcine embryos in different stages of development are shown expressing EGFP.

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Table 4
In vitro embryonic development in porcine embryos produced by ICSI-mediated gene transfer under different sperm treatment.

Sperm treatment	Number of embryos	Percentage embryos 2 to 8 cells, % (n)	Percentage embryos >8 cell-morulae, % (n)	Percentage blastocysts, % (n)
Control	172	69.77 (120)	14.53 (25)	15.70 (27)
FT	85	70.59 (60)	16.47 (14)	12.94 (11)
QF	46	80.43 (37)	10.87 (5)	8.70 (4)
TX-100	28	89.29 (25)	10.71 (3)	0 (0)
P value		0.10	0.79	0.11

Table 5
EGFP expression in porcine embryos produced by ICSI-mediated gene transfer under different sperm treatment.

Sperm treatment	Number of embryos	Percentage embryos 2 to 8 cells, % (n)	Percentage embryos >8 cell-morulae, % (n)	Percentage blastocysts, % (n)
Control	172	30.83[a] (37/120)	36.00 (9/25)	51.85 (14/27)
FT	85	40[a] (24/60)	42.86 (6/14)	36.36 (4/11)
QF	46	83.78[b] (31/37)	80.00 (4/5)	50.00 (2/4)
TX-100	28	32.00[a] (8/25)	33.33 (1/3)	–
P value		<0.01	0.35	0.69

[a,b]Different letters in the same column indicate significant differences.

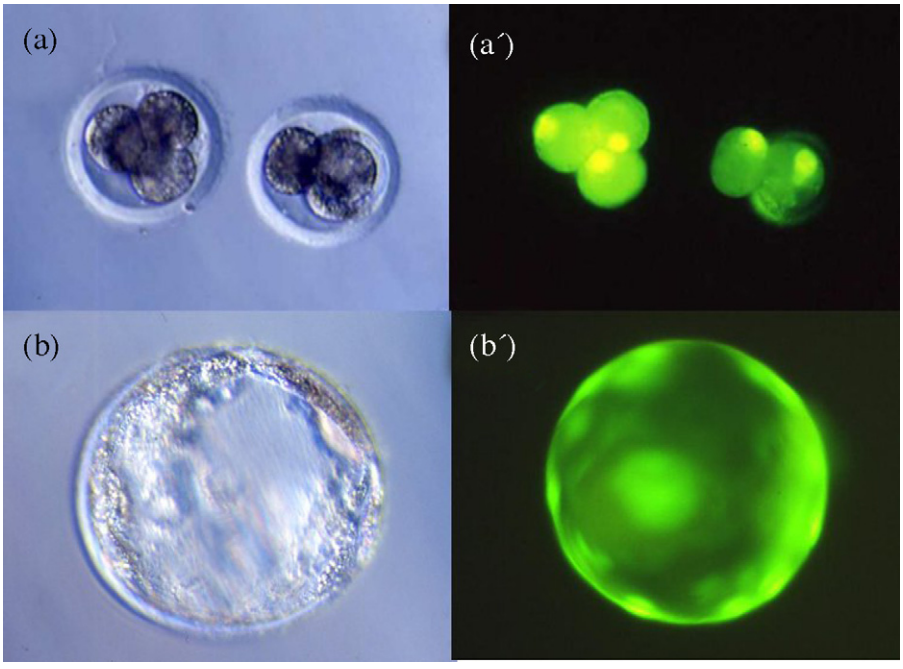

Fig. 4. Fluorescent embryos in different development stages were produced by injecting spermatozoa that had been coincubated with EGFP transgene. (a) Two- and four-cell embryos under bright light; (a′) the same embryos shown under blue light (original magnification ×100). (b) Blastocyst under bright light; (b′) the same embryo shown under blue light (original magnification ×200).

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4. Discussion

Sperm-mediated gene transfer provides important new perspectives in the field of animal transgenesis. In pigs, ICSI efficiency is very limited [26], and the application of this transgenic technique is reduced to a small number of experiments [19,23,25,38]. However, in the mouse, ICSI-SMGT has been shown to be a valuable tool for the production of transgenic animals [22,24,39].

Our objective in this study was to evaluate whether the sperm treatment could affect the transgenic efficiency by ICSI-SMGT. First, we detected that the sperm treatment significantly affected the spermatozoa DNA-binding capacity and viability. Frozen-thawed spermatozoa presented a lower viability and a greater DNA-binding capacity than that of fresh spermatozoa. Similarly, the transfection is more effective in frozen bull than in fresh spermatozoa (49% vs. 29% [14]), probably because cryopreservation induces changes in the plasma membrane that could facilitate the DNA-binding and sperm internalization. Moreover, it has been shown that cryopreserved spermatozoa have modified membranes similar to the modifications induced during the capacitation denoted as ''capacitation like'' [40]. Therefore, it is in this early capacitation phase when the binding of exogenous DNA to live sperm could take place [17]. On the other hand, when spermatozoa are subjected to an abrupt process that induces an intense disruption of the membrane (QF or TX-100 treatments in our study), we observed a drastic decrease in viability and a high increase in DNA-binding capacity that surpasses 90% of the cells.

In our study, most of the exogenous DNA is bound to dead cells or to cells with severe membrane alterations (PI stained), the percentage of DNA bound to fresh semen being close to 30% after 2 h of coincubation. This accords with other authors employing a non-radioactive method [18,41], but lower than the data reported when radiolabeled measurements are done [15–17]. It has been previously reported that there is a window of opportunity in which the exogenous DNA binds to spermatozoa, and it coincides with the early stage of capacitation [17]. Maybe under our experimental conditions, the sperm capacitation occurs very quickly, so the DNA binding can occur, but the acrosome reaction takes place too early and thus the spermatozoa die. In this case, changes in the sperm membrane functionality might modulate the time of DNA binding; the populations of viable sperm attached to the DNA may determine the success or failure in the production of transgenic animals by this method. On the other hand, it is possible that the DNA-binding induces the alteration and death of the cell by endonuclease activation in an apoptotic-like process. The apoptosis of the spermatozoa could be a natural phenomenon to prevent the transmission of exogenous DNA to the following generation [14]. It has been suggested that mature spermatozoa contain nucleases that when induced by internalization of foreign DNA might cause degradation of both sperm chromosomal DNA and added exogenous DNA [42–44]. Such DNA degradation might decrease the possibility of a sperm carrying exogenous DNA and participating in fertilization [45]. In addition is possibility that minimal amounts of seminal fluid, which can antagonize DNA binding, still present in the sperm preparations. We also have to consider that while the centrifugation process is efficient enough to eliminate most of the seminal plasma, it cannot remove traces of plasma proteins. However, there are two pieces of evidence suggesting that the likely inhibitory effect of these traces is not biologically important. First, according to another report [24] and our own experimental data [46], the inhibitory effect of seminal plasma on DNA binding is shown with a high percentage of seminal plasma in the media. Second, our experiments carried out with epididymal spermatozoa (no contact with seminal plasma) show the same results: most of the DNA binding occurs to damaged or altered membrane spermatozoa, and the DNA-binding process in epididymal spermatozoa occurs at a similar rate as that in ejaculated spermatozoa [47]. We therefore assume that the presence of seminal plasma is not of reliable value.

Results show that incubation of the spermatozoa with exogenous DNA does not affect further embryo development. Our embryo production output results are in accordance with data previously published [25,36,37,48]. The rates of blastocyst production by ICSI are very limited as previously shown for porcine (revised by Ref. 48), some causes being related to an inadequate oocyte cytoplasmic maturation [49,50] or lack of oocyte activation [36,37,51,52]. Improvements in ICSI and embryo culture procedures must be introduced to increase the final porcine ICSI performance [53].

The percentage of EGFP-expressing embryo production with fresh semen (ranging from 21.8% to 37%) was in accord with data reported in mouse [22], rat [54], rhesus monkey [55,56], and boar spermatozoa [19] using liposomes and different sperm treatments [25]. In our study, the expression of the EGFP in the embryos is detected from the first step of the embryo development (2 to 4 cells) in accordance with that of other reports [38].

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We observed a high level of mosaicism in EGFP-expressing embryos produced by ICSI, ranging from 70% to 90%. This mosaicism could be related to imbalanced DNA integration [57] or different patterns of EGFP expression in the blastomers. Nevertheless, the ICSI-SMGT method produces a smaller number of cases of mosaicism than does pronuclear microinjection [23]. According to another report [58], the DNA integration process in the embryos expressing GFP in 100% of their blastomeres probably occurred in the first cellular cycle, before the replication of the DNA, which takes place at the pronuclear stage. These embryos express EGFP in 50% of their blastomeres, and their transgene integration process can be explained in two ways: (1) after the replication of the DNA, in the first cellular cycle; or (2) before the replication of the DNA in the second cellular cycle, at the 2-cells stage. Similarly, an embryo expressing EGFP in 75% of its cells probably comes from a combination of numerous integration events.

In our study, sperm treatment affected the efficiency of the production of EGFP-expressing embryos. When FT sperm was used, we obtained 43.54% of embryos expressing the EGFP protein, and no differences were found compared with the control group. In previous studies, 50% of EGFP-expressing embryos were obtained with the use of FT pig spermatozoa [48,59], and when ICSI was performed using FT-sperm heads, the blastocyst formation and GFP expression tended to be higher than that with unfrozen sperm [25]. Accordingly, it was hypothesized that plasma membrane changes induced by cryopreservation facilitated the binding and internalization of exogenous DNA, leading to a higher transfection rate in FT spermatozoa [14]. These results demonstrate the possibility of using FT seminal samples in an ICSI-SMGT system with the same efficiency as that of fresh semen, with the advantage of the availability of cryopreserved samples from selected boars ready to be used where and when it is necessary. This fact would increase the versatility of the technique.

The transgenesis success has been increased when mouse spermatozoa with damaged membranes, such as those in the QF process, were used [22]. In pigs, the QF procedure without cryoprotectant agents markedly damaged the sperm membrane, which could facilitate the DNA integration increasing the EGFP-expressing embryo production compared with that of the control procedure (37.04% vs. 80.43%, $P < 0.01$). These results are in accord with another report using QF spermatozoa (81% expression) [60]. However, an excess of membrane damage could affect the nucleus structure or cause DNA fragmentation [44], which could be the reason of the observed tendency to obtain a lower percentage of blastocyst formation in the QF group. In this way, a significant reduction in oocyte activating capacity and a 10-fold increase in the incidence of structural chromosomal abnormalities in spermatozoa treated by unprotected freezing has been reported [61]. In our study, it is possible that sperm QF procedure is unable to induce suitable oocyte activation after ICSI, thus decreasing the embryo development results compared with that of the control group. To overcome the disadvantage of using frozen-thawed sperm for ICSI, the mentioned study showed that such compromised oocyte activation can be rescued by electric activation. Electric activation of oocytes is the most efficient method among those available and it has been frequently employed [62]. Nonetheless, the development of a more powerful activation method will be an important future challenge to meet in efficiently applying frozen-sperm ICSI in pigs [25]. Therefore, further investigations are needed to clarify the conditions required to ensure both uptake of transgene by sperm with a damaged cell membrane and sperm DNA integrity at the same time.

When we used TX-100 as a chemical agent to permeabilize membranes, we obtained lower rates of embryo development and EGFP-expressing embryos than that in the control group (29.03% vs. 37.04%, respectively). However, 64% transgenic mouse embryos has been obtained when TX-100 was used [22] and 59% of expression in pig embryos [63]. The high permeability of sperm membranes after TX-100 treatment could increase the amount of DNA binding to the sperm, thus causing high concentrations of transgene into the oocyte. However, high concentration of this chemical could become toxic causing disturbance during embryogenesis or a direct damage effect on the sperm nucleus structure. It is known that TX-100 is not a natural substance, and it could be toxic for the oocytes as well [64]. In fact, different natural products have been used, like lysolecithin, a natural cellular hydrolysis product of membrane and unlikely to be toxic to oocytes [64], resulting in better embryonic development compared with that for TX-100 [52]. Furthermore, membrane disruption is not only essential for the functioning of the ICSI-mediated method but also is crucial for determining the effect on embryonic development after ICSI carried out with such kind of sperm [23].

ICSI with sperm frozen using non-cryoprotectant solution gave rise to normal piglets [25], and use of bull sperm killed by QF process has led to birth [65]. These results together suggest that sperm do not necessarily need to be strictly intact to support normal

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embryonic development [66]. In contrast, when the membrane damage is too severe, the sperm nucleus is also likely to be damaged, which in turn may negatively influence embryonic development, especially in the later stages [44]. If disruption of the cell membrane is required for sperm to function as a vector of target genes, it is crucial in future studies to determine the suitable degree of damage that is sufficient and yet does not negatively affect later development, thus meeting the dual requirements for the ICSI-mediated method. Further studies are required to evaluate the effect on the development of ICSI embryos into fetuses or newborns.

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