Comparison of different methods for exogenous DNA uptake by bovine spermatozoa

Comparação de diferentes métodos de incorporação de DNA exógeno pelo espermatozoide bovino

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Abstract

Although genetic manipulation of farm animals is of great interest for animal production and the pharmaceutical industry, its efficiency remains far from satisfactory. Pronuclear injection, which is the most widely used technique for such modification, mainly in mice, remains limited for this species. Some alternatives have been developed such as sperm mediated gene transfer, in which the spermatozoa are used as vectors for DNA delivery during *in vitro* fertilization. Mature sperm cells are able to spontaneously bind exogenous DNA molecules which may be internalized into sperm nuclei. Given the potential of sperm mediated gene transfer for livestock animals transgenesis, the aim of this study was to evaluate four methods of DNA uptake for sperm mediated gene transfer in bovine: incubation with DNA, plasma membrane alteration induced by calcium ionophore followed by incubation with DNA, electroporation and lipofection. Spermatozoa not exposed to exogenous DNA were used as control group. Cleavage, blastocyst and hatching rates were recorded at 72 hours post insemination (hpi), days 9 and 12 of embryo culture, respectively. Exogenous DNA-positive embryos were evaluated by PCR. No effect of treatment was observed on cleavage, blastocyst and hatching rates. In addition, percentage of DNA positive blastocysts did not differ among experimental groups. In spite of the low number of positive embryos, our results show that all treatments presented similar efficiencies for DNA delivery during *in vitro* fertilization. In conclusion, although the development rates were similar and constant in all groups, other factors such as exogenous DNA sequence, size and concentration should be considered to improve sperm mediated gene transfer.

Keywords: Spermatozoa. Cattle. Animal transgenesis. *In vitro* fertilization. Embryos.

Resumo

Apesar da manipulação genética de animais domésticos ser de grande interesse para a produção animal e para a indústria farmacêutica, a sua eficiência ainda é insatisfatória. A injeção pronuclear, a técnica mais utilizada para tal propósito, principalmente em camundongos, ainda apresenta limitações para esta espécie. Algumas alternativas têm sido desenvolvidas como o uso de espermatozoides como vetores para transferência gênica, na qual a célula espermática tem habilidade espontânea de se ligar à molécula de DNA e internalizá-la. Dado o potencial da transferência gênica mediada por espermatozoide para animais domésticos transgênicos, o objetivo do presente trabalho foi a avaliação de quatro métodos de incorporação de DNA para a transferência gênica mediada por espermatozoides na espécie bovina: incubação com DNA, alteração da membrana plasmática induzida por cálcio ionóforo seguida por incubação com o DNA exógeno, eletroporação e lipofecção. Espermatozoides não expostos ao DNA exógeno foram usados como grupo controle. Os índices de clivagem, blastocisto e eclosão foram avaliados, respectivamente, as 72 horas após a inseminação dos oócitos, bem como, aos 9 e 12 dias de cultivo embrionário. Os embriões positivos para o DNA exógeno foram avaliados por PCR. Nenhum efeito de tratamento foi observado nos índices de clivagem, blastocisto e eclosão. Além disso, a porcentagem de blastocistos positivos para o DNA exógeno não diferiu entre os grupos experimentais. Apesar do baixo número de embriões positivos para DNA exógeno, os resultados obtidos mostram que todos os tratamentos apresentaram eficiências similares. A conclusão obtida foi que, apesar de os índices de desenvolvimento embrionário terem sido similares e constante em todos os grupos experimentais, outros fatores como a sequência, o tamanho e a concentração do DNA exógeno devem ser avaliados para melhorar a transferência gênica mediada por espermatozoides.

Palavras-chave: Espermatozoides. Bovino. Transgenia animal. Produção *in vitro*. Embriões.

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Introduction

Introducing exogenous DNA in farm animal cells, particularly in the germ line, is of great value for agricultural production, development of xenotransplant techniques and generation of bioreactors for the pharmaceutical industry (NIEMANN; KUES; CARNWATH, 2005; KUES; NIEMANN, 2011). However, the methods generally used for this purpose present low efficiency, are laborious and expensive (EYESTONE, 1994; ROBL et al., 2007) in these species.

The use of spermatozoa as a vector of exogenous DNA was described as a simple and cheaper alternative for livestock transgenic technology (LAVITRANO et al., 2002). The foreign DNA can be either integrated into the spermatozoa chromosomal DNA or simply carried to the egg by the spermatozoa and integrated later in the zygote's genome (WALL, 1999). In 1989, Arezzo described that sperm cells spontaneously bind to exogenous DNA molecules and transfer them to the oocyte during *in vitro* fertilization (IVF), generating transgenic offspring. However, the efficiency of this technique remains low and inconsistent. Efficiencies of <1% required microinjection and transfer of thousands of embryos to produce a few transgenic offspring (WALL, 2002; ROBL et al., 2007).

Several protocols have been used to improve the production of transgenic embryos using sperm mediated gene transfer (SMGT). In 1991, Gagné, Pothier e Sirard described exogenous DNA uptake by bovine spermatozoa after electroporation. Electroporation is a well established *in vitro* technique and is used to introduce DNA or other molecules into cells. High electric field pulses cause a transient increase in the permeability of cell membranes, leading to pore formation and augmented permeability to ions, molecules and even macromolecules as exogenous DNA (CHEN et al., 2006).

Rottmann et al. (1996) obtained genetically modified mice offspring after treatment of sperm cells with artificial vesicles called liposomes. These cationic lipids interact with the negatively-charged nucleic acid molecules, protecting the DNA from proteases and DNases. The positive outer surface of the liposome can then associate with the negative-charged cell membrane allowing the internalization and further delivery of exogenous materials to the cells, including transgenes (SMITH, 2002; BALL; SABEUR; ALLEN, 2008; MIAO, 2012).

Plasma membrane modification induced by Calcium ionophore (CaI) to produce genetically modified animals is rarely reported in literature (SIMÕES et al., 2012). This is perhaps because CaI leads to some morphological and functional changes in sperm plasma membrane (capacitation and induced acrosome reaction) that could result in decreased oocyte fertilization (ZHANG; MUZS; BOYLE, 1991). However, Perry et al. (1999) demonstrated that sperm with membrane disruption are able to support full embryo development. In addition, Pereira et al. (2000) reported that bull and goat sperm cells treated with CaI showed a significantly improved percentage of live spermatozoa with true acrosome reaction. This information can be of great value for sperm exogenous DNA uptake since during the acrosome reaction several changes occur in membrane fluidity which may facilitate the entry of the transgene. In order to improve the efficiency of SMGT in bovine, the objective of the present study was to evaluate four methods of sperm DNA uptake: electroporation, lipofection, plasma membrane modification induced by CaI followed by incubation with DNA and incubation; and their effects on embryo development *in vitro*.

Material and Methods

A total of 1513 bovine oocytes were subjected to *in vitro* production (IVP) of embryos (mean of 302.6 oocytes per group distributed in 10 replicates). Unless otherwise indicated, all chemicals were obtained from Sigma Chemical (St. Louis, MO, USA). Tissue culture media (TCM), Hepes and Bicarbonate and fetal calf serum (FCS) were obtained from Gibco (Grand Island, NY, USA). Recombinant plasmid DNA (pEYFP-NUC) was obtained from Clontech, BD Biosciences (USA). All experiments were performed using 500ng of recombinant plasmid DNA as exogenous DNA, which was 4.8 kb in size and linearized with *Stu* I at 37ºC for 2 h. These experiments were approved by bioethics committee of the School of Veterinary Medicine and Animal Sciences, University of São Paulo (protocol number 400/2003).

Sperm preparation and exogenous DNA uptake

Frozen-thawed semen was obtained from one bull. The thawed semen (37ºC in water bath / 30 sec) was centrifuged at 600 x g for 30 min in Percoll gradient (45% and 90%) to purify viable sperm cells. The pellet was washed in Sperm-TALP [19] followed by centrifugation at 200 x g for 5 min. The pellet was resuspended in Sperm-TALP to a 5×10^6 spermatozoa/mL final concentration for each of the experimental groups: Control (spermatozoa not exposed to exogenous DNA), electroporation (E) lipofection (L), plasma membrane modification induced by calcium ionophore followed by incubation with DNA (CaI), and incubation (I).

Electroporation

A concentration of 5×10^6 spermatozoa/mL was added to 1mL of TCM199 and 500ng of exogenous DNA. Electroporation was accomplished with one pulse of 12msec, 500 V and 25 µF (Cellject Pro Hybaid, Franklin, MA, USA).

Lipofection

Effectene® (Qiagen, Canada) reagent was used for lipofection following manufacture recommendation. Briefly, 10 uL of liposome were incubated with 500ng of exogenous DNA for 10 min at room temperature. Sperm cells were added to the liposome-exogenous DNA mix and incubated for 2 hours at 39ºC, 5% CO_2 in air (v/v) and high humidity.

Calcium ionophore (CaI)

Sperm cells were incubated in 1 mL of in vitro fertilization (IVF) medium supplemented with 250 nM of CaI (A23187) for 5 min, centrifuged at 200 g for 5 min, resuspended in Sperm-TALP and centrifuged again. The pellet was resuspended in 1 mL of IVF medium (IVF-Talp – PARRISH et al., 1988) without heparin containing 500 ng of exogenous DNA at 39ºC, 5% CO_2 in air (v/v) and high humidity for 1 h.

Incubation

Sperm cells were added to 1 mL of IVF medium without heparin and 500 ng of exogenous DNA for 1h at 39ºC, 5% CO_2 in air (v/v) and high humidity.

Oocyte in vitro maturation

Embryos were produced according to Yamada et al. (2007). Briefly, cumulus oocyte complexes (COCs) were obtained by aspirating follicles from abattoir ovaries. Groups of 15-30 COCs with homogeneous ooplasm surrounded with multilayer compacted cumulus cells were placed in 90 µL maturation medium (TCM 199 Bicarbonate supplemented with 10% (v/v) FCS, 22 µg/ml pyruvate, 50 µg/ml gentamycin, 0.5 µg/ml FSH (Folltropin-V; Bioniche Life Science Inc., Ontario, Canada), 50 µg/ml hCG (Chorulon, MSD-Animal Health, The Netherlands) and 1 µg/ml 17 beta estradiol), covered with mineral oil and cultured for 24 h at 38.5ºC under an atmosphere of 5% CO_2 in air and high humidity.

In vitro fertilization

For in vitro fertilization, COCs were washed three times in fertilization medium before being transferred in groups of 15-30 oocytes, onto plates containing 90 μL of fertilization medium IVF-Talp: modified tyrode stock solution supplemented with PHE (0.5 mM penicillamine; 0.25 mM hypotaurine and 25 μM epinephrine in 0.9% (w/v) NaCl), 50 μg/mL gentamycin and 0.3% essential fatty acid-free bovine serum albumin, under mineral oil. After treatments, spermatozoa from experimental and control groups were washed by centrifugation in Sperm-TALP and in IVF medium at 200 x g for 5 min. Oocytes were inseminated with 1 x 10^6 spermatozoa/mL (for all experimental and control groups). IVF was carried out at 38.5°C in a 5% CO_2 atmosphere in air and high humidity.

In vitro embryo culture (IVC)

At approximately 18 hpi, presumptive zygotes were partially denuded by gentle pipetting and washed three times in SOFaa medium (TERVI; WHITTINGHAM; ROWSON, 1972), supplemented with 5% (v/v) FCS, MEM nonessential amino acids and MEM essential amino acids solution before being transferred to 90 μL drop of culture medium (SOFaa) in the same plate used for IVM (co-culture system) under mineral oil, at 38.5°C in a 5% CO_2 atmosphere and high humidity. IVC was carried out for 12 days with IVF considered as day 0. Cleavage rate was recorded at 72 hpi and blastocyst and hatching rates were respectively recorded at days 9 and 12. Blastocysts were collected at day 12 of IVC and washed individually in PBS drops of 20 μL and frozen in microcentrifuge tubes. After extraction of DNA, polymerase chain reaction (PCR) was performed to assess the presence of exogenous DNA in day 12 embryos. The experiment was repeated 10 times including all treatments.

PCR

A 272bp plasmid DNA fragment was amplified with specific primers (IDT, IA, USA): 5'- GGCAAGCTG ACCCTGAAGTT – 3' (forward) and 5'- AAGTCGAT GCCCTTCAGCTC – 3' (reverse). Each amplification mixture (30μL) was prepared in tubes containing the frozen-thawed embryos, 1.25 mM of each dNTP (dATP, dCTP, dTTP and dGTP - Invitrogen, USA), 2UI of Taq DNA polimerase (Invitrogen, USA), 20pmol of each specific primer, 3 μL of 10x buffer (200 mM Tris-HCl – pH 8.0; 500 mM KCl – Invitrogen, USA) and 1.5 mM of $MgCl_2$ (Invitrogen, USA). PCR amplification was performed with a DNA thermal cycler (PTC-100 MJ Research) for 35 cycles of 95ºC for 1 min, annealing at 60ºC for 1min and extension at 72ºC for 1 min. PCR mixtures without sample were used as PCR-negative control.

PCR from control group embryos was carried out as negative control of treatments. The absence of false positive embryos was checked by PCR reactions of culture medium (SOFaa) and washing medium (PBS). Plasmid DNA was amplified as PCR-positive control. PCR products were submitted to 1% agarose gel electrophoresis stained with ethidium bromide.

DNA sequencing

The amplified products (272bp) were sliced from the gel and purified with *Rapid Gel Extraction System* (Marligen Biosciences Inc., USA). The purified product (5 μL) was added to 5 pMol of forward or reverse specific primer and 2 μL of distilled water and sequenced by *BigDye2 dideoxy terminators system* (Applied Biosystems, CA, USA). At least one sample from each group was sequenced.

Statistical Analysis

All data were evaluated using SAS System for Windows (SAS Institute Inc., Cary, NC, USA). Residue normality (Gaussian distribution) and variance homogeneity of each variable were evaluated using the Guided Data Analysis. Adjusted means were obtained using the Least-squares means (LSMEANS) and treatments effects (I, CaI, E and L) were analyzed using the Tukey test. Response variables considered

were cleavage, blastocyst, hatching, and DNA-positive embryo rates. A probability value of P < 0.05 was considered statistically significant. Results are reported as untransformed means +/- standard error of the mean (SEM).

Results

Results show that the sperm fertilizing ability was not affected by protocols used for exogenous DNA-uptake as no significant differences on embryo development rates (cleavage, blastocyst and hatching rates) between experimental groups were observed (Table 1). Although the rate of positive embryo was constant among all groups we observed a low reproducibility for all methods. However rates of DNA-positive blastocysts did not differ among E, L, CaI and I groups (Figure 1). The molecular analysis of blastocysts DNA revealed the presence of the plasmid sequence (Figure 2). No trace of target sequence was found at culture medium or washing medium (data not shown).

Discussion

Results presented here show that DNA-uptake by bovine sperm occurs after electroporation, lipofection, CaI and incubation protocols with the same efficiency. Protocols used for exogenous DNA incorporation did not affect the fertilizing capacity of sperm as there was no significant difference in embryo

development among experimental and control groups. Additionally, although the rate of positive embryo was constant among all groups we observed a low reproducibility for all methods. Therefore, other factors such as exogenous DNA sequence, size and concentration should be considered to improve SMGT (SCIAMANNA et al., 2000).

SMGT is not yet fully established as a reliable genetic manipulation. Furthermore, is known that stable integration is rarely observed with SMGT protocols (SMITH; SPADAFORA, 2005; SPADAFORA, 2008). One reason for the low efficiency may be related to endonucleases, which are activated according to the DNA concentration. This activity leads to exogenous sequences degradation or an apoptosis-like process in the sperm, significant decrease in spermatozoa motility eventually causing cell death (SPADAFORA, 1998; SMITH; SPADAFORA, 2005). On the other hand, our group showed that in bovine species the exogenous DNA concentration had no apparent effect on endogenous DNA integrity and it could be due to the chromatin structure of sperm DNA (FEITOSA et al., 2010). Since bovine spermatozoa lack protamine

Table 1 – Embryo development rates (cleavage, blastocyst and blastocyst hatching) for in vitro fertilization using different protocols for exogenous DNA-uptake by sperm cells (E, L, CaI or I). Data presented as LSMEANS ± SEM - São Paulo - 2006

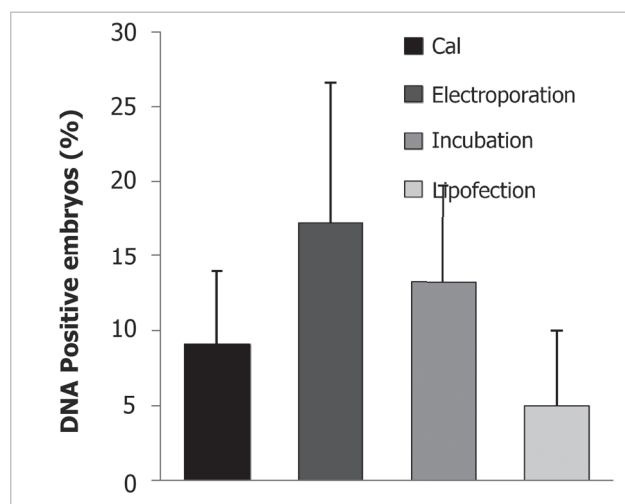| GROUPS	CLEAVAGE (LSmeans ± SEM)	BLASTOCYST (LSmeans ± SEM)	HATCHING (LSmeans ± SEM)
CaI	63.84 ± 3.89	28.07 ± 4.43	32.32 ± 9.72
Electroporation	65.53 ± 3.59	26.61 ± 4.98	41.72 ± 9.38
Incubation	65.02 ± 5.24	23.95 ± 3.00	38.30 ± 9.88
Lipofection	57.22 ± 3.45	27.41 ± 5.20	28.82 ± 8.67
Control group	67.55 ± 5.55	21.76 ± 2.92	31.57 ± 8.44

Figure 1 – Percentage of DNA-positive embryos produced by in vitro fertilization using different protocols for exogenous DNA-uptake by sperm cells (electroporation, lipofection, CaI, or incubation groups). Data presented as LSMEANS ± SEM
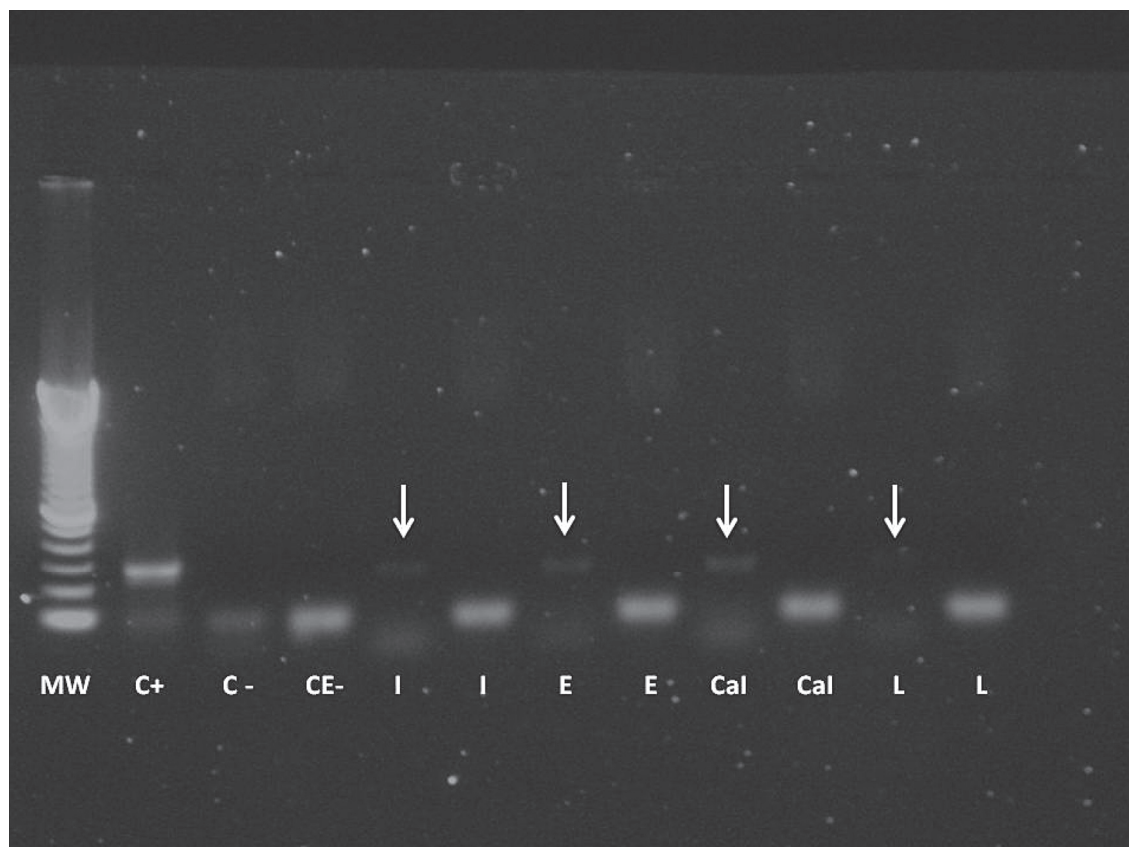
Source: (SIMÕES, 2013)

Figure 2 – Electrophoresis on 1% agarose gel stained with ethidium bromide for detection of plasmid DNA. MW: DNA ladder; C+: plasmid DNA positive control, C-: PCR negative control (no sample); CE-: embryo from control group; I: embryos from incubation group; E: embryos from electroporation group; CaI: embryos from calcium ionophore group; L: embryos from lipofection group. White arrows indicate DNA-positive blastocyst

Source: (SIMÕES, 2013)

2 (only protamine 1 has been detected) and DNA fragmentation is more related to protamine 2, this could offer a better resistance to DNA damage to endonuclease activity compared to other species like murine and human. In the present study we used the same concentration of exogenous DNA as (FEITOSA et al., 2010). We can speculate that exogenous DNA concentration was not able to trigger endonuclease activity; hence, embryo development rates from all experimental groups were not different (p > 0.05) from control group which suggests that endonuclease activity is not a barrier for SMGT in this species.

Feitosa et al. (2009), reported that incubation time could reduce sperm viability causing acrosome membrane disruption during increased incubation periods. Our data is in accordance with Canovas,

Gutierrez-Adan and Gadea (2010) that showed that after 5 min of incubation, more than 20% of bovine spermatozoa bind to exogenous DNA. García-Vázquez et al. (2009) described that exogenous DNA mainly bound to spermatozoa with reduced viability and as a consequence, only a low percentage of living cells were bound to DNA. According to these authors, frozen-thawed spermatozoa present a lower viability but a greater DNA-binding capacity when compared to fresh sperm cells, probably because the freezing process induces plasma membrane changes that facilitate the DNA-binding. Moreover, it is known that frozen semen have plasma membrane alterations similar to modifications occurring during sperm capacitation (HARRISON, 1997; LEAHY; GADELLA, 2011). Sperm DNA-binding may occur during this

capacitation-like process, but since acrosome reaction may also take place prematurely, the sperm cell could die. This is a possible explanation for our results since the majority of oocytes could be fertilized by sperm without exogenous DNA, resulting in a low percentage of positive embryos with exogenous DNA.

It was showed that all protocols for exogenous DNA-uptake by bovine spermatozoa can be used to produce transgenic livestock. Other methods could be used in order to produce transgenic farm animals such as direct microinjection of the egg pronuclei and viral-based constructs as vectors to introduce the foreign DNA into embryos. However, these methods show some disadvantages as (I) low efficiency since the zygote of most farm animals are opaque due to the lipid content in the cytoplasm, which makes the pronuclei visualization very difficult; (II) elevated costs and skills; (III) embryo manipulation at early stages of development; (IV) gene silencing by DNA methylation due to the presence of viral sequences and a high frequency of mosaicism in founder animals. Moreover, the use of retroviral vectors is affected by safety issues (YANG et al., 2000; HOFMANN et al., 2006; LAVITRANO et al., 2006). The ability of transferring genes into animal genome is a prerequisite for further progress in animal transgenesis. However, the improvement of methods for genetic modification is needed. Key aspects to be improved include the DNA-uptake efficiency, the localization of the gene in the nucleus and gene targeting of the transgene in the genome.

Conclusion

The results showed that all protocols for exogenous DNA-uptake are equally effective. All methods had a constant embryo production, despite the low reproducibility of exogenous DNA positive embryo rates. Therefore, other factors such as exogenous DNA sequence, size and concentration should be considered to improve SMGT.

Acknowledgements

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