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Sperm-mediated gene transfer: effect on bovine *in vitro* embryo production

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Summary

The technique of sperm-mediated gene transfer (SMGT) can be used to delivery exogenous DNA into the oocyte. However, it has low repeatability and produces inconsistent results. In order to optimize this technique, it is necessary to study the mechanism by which DNA enters the sperm cell and integrates in the sperm genome. Furthermore, studies must focus in the maintenance of sperm cell viability and function. The aim of this study was to evaluate different SMGT protocols of sperm electroporation or capacitation (CaI) aiming to maintain sperm viability in the production of bovine embryos *in vitro.* Frozen–thawed semen was divided in two experimental groups (electroporation or CaI) and one control group (non-treated cells). For the electroporation method, five different voltages (100, 500, 750, 1000 or 1500 V) with 25 μF capacitance were used. For CaI treatment, combinations of two CaI concentrations (250 nM or 500 nM), two incubation periods of sperm cells with CaI (1 or 5 min) and two incubation periods that mimicked time of sperm cell interaction with exogenous DNA molecules (1 or 2 h) were evaluated. According to our data, electroporation and CaI treatments do not prevent sperm penetration and oocyte fertilization and can be an alternative method to achieve satisfactory DNA delivery in SMGT protocols.

Keywords: Bovine, Calcium ionophore, Electroporation, SMGT, IVP embryo

Introduction

Recent progress in basic and applied studies on embryo technologies has stimulated the development of reproductive techniques used for commercial purposes. Used alone or in combination, artificial insemination (AI), embryo transfer (ET) and *in vitro* embryo production (IVP) have been used successfully to increase the number of offspring from valuable animals.

Advances in techniques such as the use of frozen spermatozoa at AI, use of sexed semen as well as sperm-mediated gene transfer (SMGT) have offered new tools to produce domestic animals of greater genetic merit (Galli *et al.*, 2003; Carvalho *et al.*, 2010; Humblot *et al.*, 2010). SMGT is not yet used routinely, although the mechanisms of binding and internalization of DNA by sperm cells are becoming clearer. Several studies have demonstrated that a part of the high variability in the outcome of IVP of bovine embryos may be due to the different ability of sperm from different bulls to fertilize oocytes. Thus, SMGT protocols associated with IVP must be well

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established in regard to sperm quality because viable spermatozoa have better chances to fertilize the oocyte (Iranpour *et al.*, 2000). In order to raise SMGT efficiency, electroporation and induced membrane alteration protocols can be used to increase exogenous DNA uptake by sperm cells (Gagné *et al.*, 1991; Perry *et al.*, 1999). However, it is not known if such modifications could influence sperm fertilizing ability and embryo development in cattle.

Electroporation causes a transient increase in the permeability of cell membranes after exposure to a high electric field. This effect happens because, when the transmembrane voltage induced by an external electric field exceeds a certain threshold, a rearrangement of the molecular structure of the membrane occurs, leading to pore formation and a considerable increase in permeability to molecules of sizes ranging from ions to macromolecules (Chen *et al.*, 2006). Because of this characteristic, electroporation can be used to improve the number of DNA or other molecules taken up by sperm cells. According to Rieth *et al.* (2000), the electric field formed during electroporation should increase the binding between exogenous DNA molecules and sperm cells. As a consequence, the electroporated cells retain DNA more efficiently than non-electroporated ones, but that does not yield a higher percentage of transfected cells (Müller *et al.* 1992). Moreover, high voltage protocols could reduce sperm motility after electroporation (Gagné *et al.*, 1991).

Similar to electroporation, there are chemical agents that may induce alterations in the sperm membrane that could facilitate exogenous DNA entrance. The lipid disorganization that occurs during the sperm capacitation process is a physiological example of these membranes alterations. Canovas *et al.* (2010) induced sperm capacitation with heparin followed by incubation with exogenous DNA. In the past, some authors have suggested that there was a potentially negative effect of DNA binding to sperm cells in the presence of glycosaminoglycans (Camaioni *et al.*, 1992; Jonák *et al.*, 2000; Kuznetsov *et al.*, 2000); thus semen treated with calcium ionophore (CaI), another capacitation agent, could be an alternative (Pereira *et al.*, 2000).

The aim of this study was to test the effects of two different SMGT protocols (electroporation or capacitation with calcium ionophore – CaI) on the viability of sperm used to produce bovine embryos *in vitro.*

Materials and methods

Unless otherwise indicated, all chemicals were obtained from Sigma Chemical (St. Louis, MO, USA). Tissue culture media (TCM199) (HEPES and bicarbonate) and fetal calf serum (FCS) were obtained from Gibco (Grand Island, NY, USA).

Frozen semen from a single bull was thawed in a water bath (37°C, 30 s) and prepared by centrifugation on a 45–90% discontinuous Percoll gradient for 30 min at 600 *g*. Selected sperm cells were then washed by centrifugation (200 *g* for 5 min) in Sperm-Talp medium (Parrish *et al.*, 1988) to remove Percoll residues. Sperm motility was evaluated (×200 magnification) and the cells were diluted to a final concentration of 1×10^6 cells/ml. Sperm cells were divided randomly between experimental groups (CaI and electroporation) and the control group (untreated). Each experiment was replicated 10 times.

Electroporation

Electroporation protocol was performed with 5×10^6 sperm. Cells were pre-incubated in the chamber for 10 min and then exposed to 100, 500, 750, 1000 or 1500 V and 25 μF capacitance (CellJect Pro, Hybaid, UK) for 12 ms. Electroporated cells stayed in the electroporation solution for an additional 10 min. This later incubation was meant to mimic a period in which sperm cell would interact with exogenous DNA molecules if they had been added to the solution. Cells were then washed by centrifugation on Sperm-Talp medium (200 *g*, 5 min) and evaluated for motility and concentration. A total of 626 oocytes were used for *in vitro* maturation and 171 embryos were produced using electroporated sperm cells.

Calcium ionophore (A23187)

A combination of two CaI concentrations ([CaI]; 250 nM or 500 nM) diluted in IVF medium (Parrish *et al.*, 1988), two intervals of incubation of sperm cells with CaI (tCaI; 1 or 5 min) and two total incubation periods (tINC; 1 or 2 h) were evaluated in eight treatment combinations. Semen samples (5×10^6 sperm cells/ml per group) were incubated with one of the two [CaI] at room temperature (22–25°C) for 1 or 5 min (tCaI). Next, sperm cells were incubated for 1 or 2 h (tINC) at 38.5°C in 5% (v/v) CO_2 in moisturized air. This later incubation was meant to mimic a period in which sperm cell would interact with exogenous DNA molecules if they had been added to the solution. After tINC, spermatozoa were washed by centrifugation in Sperm-Talp medium (200 *g*, 5 min) and sperm motility and concentration were assessed. A total of 1210 oocytes were used for *in vitro* maturation and 291 embryos were produced using sperm cells capacitated with CaI.

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Oocyte *in vitro* maturation

Embryos were produced according to Yamada *et al.* (2007). Briefly, cumulus–oocyte complexes (COCs) were obtained by aspirating follicles from ovaries collected at a slaughterhouse. Groups of 15–30 COCs with homogeneous ooplasm and multilayer compact cumulus cells were placed in a 90 μl maturation medium droplet, covered with mineral oil and cultured for 24 h at 38.5°C under 5% CO_2 in air and high humidity conditions. The maturation medium was TCM 199 bicarbonate supplemented with 10% (v/v) FCS, 22 μg/ml pyruvate, 50 μg/ml gentamycin, 0.5 μg/ml follicle-stimulating hormone (FSH) (Follitropin-V; Vetrepharm Inc., Ontario, Canada), 50 μg/ml hCG (Chorulon, Intervet Schering-Plough, The Netherlands) and 1 μg/ml 17β-estradiol).

In vitro fertilization

For *in vitro* fertilization (IVF), COCs were washed in fertilization medium before being transferred in groups of 15–30 into plates that contained 90 μl droplets of IVF-Talp medium, that consisted of 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% fatty acid free–BSA under mineral oil. For each experimental group, droplets were inseminated at a final concentration of 1×10^6 sperm/ml. *In vitro* fertilization was carried out at 38.5°C under an atmosphere of 5% CO_2 in air and high humidity.

In vitro embryo culture (IVC)

At approximately 18 h post insemination (hpi), presumptive zygotes were partially denuded by gentle pipetting and washed three times in SOFaa medium (Tervit *et al.*, 1972) supplemented with 5% (v/v) FCS, MEM non-essential amino acids and MEM essential amino acids. The embryos were co-cultured with granulosa cells in 90 μl droplets of SOFaa under mineral oil, at 38.5 °C under an atmosphere of 5% CO_2 in air and high humidity. Embryo culture was carried out for 12 days starting at IVF (day 0). Cleavage rate was recorded at 72 hpi and blastocyst and hatching rates were respectively recorded at days 9 and 12 post insemination.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) and means were compared by orthogonal contrast, when appropriated, with a 5% level of significance.

Table 1 Blastocyst (day 9) and hatched blastocyst (day 12) rates of *in vitro* produced embryos using electroporated spermatozoa at different voltages

Voltage (KV)	Oocytes (*n*)	Blastocyst LS means ± SE (*n*)	Hatched blastocyst LS means ± SE (*n*)
0	92	59.79 ± 9.87^{a} (51)	$100.87 \pm 19.03^{a,d}$ (50)
100	108	$37.14 \pm 7.91^{a,c}$ (43)	$107.27 \pm 15.26^{a,c}$ (43)
500	115	$20.90 \pm 6.59^{b,c}$ (24)	$84.58 \pm 12.7^{a,c}$ (20)
750	107	$22.50 \pm 6.59^{b,c}$ (24)	$50.0 \pm 12.7^{b,e}$ (12)
1000	102	$18.48 \pm 6.59^{b,c}$ (18)	$58.33 \pm 12.7^{d,b,e}$ (10)
1500	102	8.10 ± 7.91^{b} (11)	40.6 ± 15.26^{e} (4)

$^{a–e}$Values within columns differ, $P < 0.05$. SE, standard error. LS mean, least squares mean.

Results

Electroporation

Blastocyst production using electroporated spermatozoa decreased as the voltage increased. Blastocyst rate from group 100 V did not differ from groups 500, 750 and 1000 V, but was different than that of group 1500 V. Hatching blastocyst rates from groups 100 V and 500 V were higher than those of 750, 1000 and 1500 V groups (Table 1).

Calcium ionophore

There was an interaction among calcium concentration ([CaI]), period of spermatozoa exposure to calcium (tCa) and period mimicking sperm exposure to exogenous DNA molecules (tINC), on blastocyst rate ($P < 0.02$). Groups of cells treated with 250 nm of CaI (1, 2, 3 and 4) showed lower blastocyst rates than the control group ($P < 0.05$). Group 3 had a better blastocyst rate than the other experimental groups ($P < 0.05$). In contrast, blastocyst rates from groups of cells treated with 500 nm (5, 6, 7 and 8) were similar to that of the control group ($P < 0.05$).

Moreover, blastocyst rates from groups 3 and 5 were higher when compared with other experimental groups ($P < 0.05$), however they were not different from that of the control group.

Hatching rates from groups 2 and 5 did not differ from other groups. Except from groups 6 and 8, all experimental groups showed hatching rates similar to the control group ($P > 0.05$) (Table 2).

Discussion

Our study demonstrated that electroporation and sperm membrane alteration by CaI influence sperm fertilizing capacity. As this process is essential to

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Table 2 Blastocyst (day 9) and hatched blastocyst (day 12) rates of *in vitro* produced embryos after sperm was treated with different calcium ionophore (CaI) concentration, exposure period and incubation period prior to CaI treatment

Groups	[CaI] (mM)	tCa (min)	tINC (h)	Oocytes(*n*)	Blastocyst LS means ± SE (n)	Hatched blastocyst LS means ± SE (*n*)
Control	0	0	0	151	30.50 ± 4.73^{a} (46)	58.6 ± 11.75^{a} (26)
1	250	1	1	150	26.48 ± 4.73^{b} (39)	66.17 ± 11.75^{a} (26)
2	250	1	2	144	25.63 ± 4.73^{b} (37)	34.0 ± 11.75^{ab} (13)
3	250	5	1	127	36.01 ± 4.73^{a} (46)	57.88 ± 11.75^{a} (26)
4	250	5	2	200	15.30 ± 4.73^{b} (31)	52.29 ± 11.75^{a} (16)
5	500	1	1	112	37.40 ± 4.73^{a} (42)	41.3 ± 11.75^{ab} (17)
6	500	1	2	185	8.07 ± 4.73^{b} (15)	15.0 ± 11.75^{b} (2)
7	500	5	1	141	24.80 ± 4.73^{b} (35)	64.29 ± 11.75^{a} (22)
8	500	5	2	180	8.31 ± 4.73^{b} (15)	15.0 ± 11.75^{b} (2)

^{a,b}Values within columns differ, $P < 0.05$.

IVF outcome, the choice of the best protocol is decisive for the success of this technique. In this study, embryo development rates were negatively influenced increasing the electroporation voltage. According to Nishikage *et al.* (2004), higher voltage increases cellular transfection efficiency, but based on our results it diminishes sperm viability. The SMGT associated with electroporation has been described in some species, leading to better results in transfection rates than sperm-DNA incubation alone (Gagné *et al.*, 1991; Sin *et al.*, 1995, 2000; Tsai, 2000). In all cases, electric pulse conditions were crucial to the technique efficiency, since higher voltages can induce damage of electroporated cells and alter embryo development rates (Rieth *et al.*, 2000; Nishikage *et al.*, 2004). Sin *et al.* (1995) reported that for black-footed abalone (*Haliotis iris*), even with decreased sperm motility, the optimal electroporation conditions for DNA uptake was 1000 V/cm with two pulses of 27.4 ms each. Therefore, sperm electroporation should be performed only in species in which an efficient protocol for DNA uptake is difficult because it does not yield a higher percentage of sperm-DNA uptake (Lavitrano *et al.*, 2006).

In our study, blastocyst rates decreased as voltage increased, but there were no difference among rates at 500, 750 and 1000 V. However, hatching rates decreased when voltages higher than 500 V were used. Our experiments confirmed that the influence of electric pulses is not only harmful to sperm function, but also affects embryo development. According to Gagné *et al.* (1991), higher voltages can interfere with late embryo development because they induce nuclear alterations or sperm-DNA damage. Hence, voltages higher than 500 V could decrease SMGT outcome.

Another alternative to optimize sperm-DNA association, CaI supplementation during sperm-DNA incubation, was evaluated in this report. Our results showed that in 250 nM CaI groups, blastocyst rates after 1 min calcium ionophore-sperm incubation (tCaI) were lower than those incubated for 5 min. This result is in accordance with findings by Pereira *et al.* (2000), which described that the percentage of acrosome-reacted spermatozoa was higher when incubation time increased. Nevertheless, 500 nM CaI for 5 min, decreased blastocyst rates and better results were obtained after 1 min incubation with the same CaI concentration. Because high extracellular calcium concentration promotes more rapid sperm capacitation, only a few minutes are necessary to achieve this condition, while a toxic effect may occur with a longer incubation period. According to Landim-Alvarenga *et al.* (2004) excessive entry of calcium inside the sperm could change calcium homeostasis in the midpiece, affecting mitochondrial function and reducing sperm motility. This effect could explain why in the present study there was lower embryo development after a higher CaI concentration treatment, associated with longer incubation periods.

Results from the incubation period mimicking sperm interaction with exogenous DNA molecules (tINC) showed that incubation for 2 h was harmful for embryo development, regardless of CaI concentration and incubation period with sperm cells. This result suggests that not only length of the incubation period, but also CaI concentration is crucial for maintenance of sperm viability. In a previous work, our group described that incubation period was a factor in decreased sperm viability, causing acrosome membrane disruption during greater incubation periods (Feitosa *et al.*, 2009). Because sperm viability is crucial for sperm–oocyte interaction during the fertilization process, prolonged incubation period and higher CaI concentration could diminish SMGT efficiency.

Overall, the present results suggest that even with lower blastocysts rates, the manipulation of sperm

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aiming to optimize DNA-sperm interaction is possible, as embryos were produced with sperm cells exposed to electrical fields or to CaI.

Conclusion

This work allowed us to conclude that sperm treatment did not prevent oocyte fertilization, in spite of the reduction in embryo production. Conditions described herein can be used as an alternative method to enhance DNA integration for species in which it is difficult to standardize a SMGT protocol.

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