Biol Res 44: 229-234, 2011

Testis-mediated gene transfer in mice: comparison of transfection reagents regarding transgene transmission and testicular damage

Marta G. Amaral, Vinicius F. Campos, Fabiana K. Seixas, Paulo V. Cavalcanti, Lisiane P. R. Selau, João C. Deschamps, Tiago Collares*

Núcleo de Biotecnologia, Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, Campus Universitário, CEP 96010-900 - Pelotas, RS, Brazil.

ABSTRACT

Testis-mediated gene transfer (TMGT) has been used as *in vivo* gene transfer technology to introduce foreign DNA directly into testes, allowing mass gene transfer to offspring via mating. In this study, we used plasmid DNA (pEGFP-N1) mixed with dimethylsulfoxide (DMSO), N,N-dimethylacetamide (DMA) or liposome (Lipofectin) in an attempt to improve TMGT. Males receiving consecutive DNA complex injections were mated to normal females to obtain F0 progeny. *In vivo* evaluation of EGFP expression, RT-PCR and PCR were used to detect the expression and the presence of exogenous DNA in the progeny. We also evaluated possible testicular damage by histological procedures. PCR and RT-PCR analyses revealed that liposome and DMSO increased the rate of TMGT. Histological analyses demonstrated that repeated (4 times) injections of DNA complexes can affect spermatogenesis. DMSO was the most deleterious among the reagents tested. In this study, we detected the presence of transgene in the progeny, and its expression in blood cells. Consecutive injections of DNA complexes were associated with impaired spermatogenesis, suggesting requirement of optimal conditions for DNA delivery through TMGT.

Key-words: TMGT, DMA (N,N-dimethylacetamide), Liposome, mice, transgenesis, histological damage.

INTRODUCTION

The use of spermatozoa has been studied in recent years for gene transfer in transgenic animal technology and several distinct approaches have been used. The first report that exogenous DNA could be introduced into sperm was made by Brackett et al. (1971). Several studies in distinct species have reported the generation of transgenic animals using spermatozoa as vectors to carrier foreign DNA to the ova (Lu et al., 2002; Webster et al., 2005; Shen et al, 2006; Hoelker et al., 2007, Collares et al., 2010, Campos et al., 2011a, Campos et al., 2011b, Campos et al., 2011c). One approach of sperm-mediated gene transfer (SMGT) is the direct introduction of foreign DNA into testes, so-called testis-mediated gene transfer (TMGT), which allows for natural mating and mass gene transfer. This technique exempts the use of other procedures such as *in vitro* fertilization (IVF) and embryo transfer (ET). Sato et al. (2002) demonstrated this method by way of direct, but surgical injection of DNA solution into testes with subsequently "*in vivo*" electroporation to improve the uptake of foreign DNA by epididymal epithelial cells. Shen et al. (2006) demonstrated efficient generation of rabbits and mice through TMGT using surgical injection in testes with a DMSO/DNA complex to improve uptake of foreign DNA by sperm cells. In addition, Dhup and Majumdar (2008) demonstrated transgenesis via permanent integration of genes in repopulating mice spermatogonial cells *in vivo*. Further advancement on TMGT technique might offer an easy way to generate transgenic animals or an important route for germ line therapy in humans, since gene transfer into testicular somatic cells in order to rescue failing spermatogenesis may one day become a reality (Coward, 2007).

On the other hand, intracellular cryoprotectants such as DMSO can improve DNA uptake by sperm cells as previously demonstrated in rabbits, mice and chicken (Li et al., 2006; Shen et al., 2006, Collares et a., 2011). Therefore other intracellular cryoprotectants such as N, N-dimetylacetamide (DMA) that have been used recently in boar sperm cryopresenvation (Bianchi et al., 2008) could also be used to increase the uptake of exogenous DNA by spermatozoa as demonstrated for chickens (Collares et al., 2011).

Here we demonstrate the efficient EGFP transgene transmission to mice offspring by TMGT with a non-surgical injection of DNA solution and without electroporation of epididymis, reducing the injury to the male and laborious handling. As transfectants we tested DNA complexes containing DMSO (dimethylsulfoxide), liposomes (Lipofectin® Transfection Reagent, Invitrogen®, USA) and for the first time, to our knowledge, DMA (N,N-dimethylacetamide), to improve the uptake of foreign DNA by sperm cells, substituting the in vivo electroporation. In addition, we evaluated injuries due to continuous injections of DNA complexes on testes by histological procedures.

METHODS

Animals

Five groups of five male BALB/c mice, 3-6 months old, were used. After treatments each male mated with two female BALB/c. The animals were kept according to the guidelines of the Ethics Committee in Animal Experimentation of UFPel.

* Corresponding author: Tiago Collares, Centro de Biotecnologia – Universidade Federal de Pelotas, Caixa Postal 354 • CEP 96010-900, Pelotas – RS / Brazil, Phone: +55 53 32757588, collares.t@gmail.com

Received: February 2, 2010. In revised form: December 2, 2010. Accepted: December 14, 2010.

Transfection Solutions

Twenty micrograms of circular eukaryotic expression vector pEGFP-N1® (Clontech®, USA) complexed with three different transfectants: DMSO 3%, DMA 3%, and Lipofectin 3%, all diluted in phosphate-buffered saline (PBS), pH 7.2, were used. As well, 20 μg of pEGFP diluted in PBS represents the fourth group. The control group received only PBS. All treatments received 0. 1% of trypan blue (Invitrogen®, USA).

Non-surgical testis injection

Before testis injection, animals were sedated with 2 mg/kg of acepromazine (Vetnil®, Brazil) intraperitoneally. The testes were exposed in scrotal sack by a digital pressure in the abdomen and were fixed with the tip of the fingers to avoid retraction during the injection. Asepsis of the scrotal sack was carried out with 70% ethanol. Briefly, 30 μl of each solution described previously was slowly injected into each testis with 30- G needle (BD Biosciences®, USA) attached to 1-ml plastic disposable syringe at a depth of 3-4 mm through the scrotal sack. After injection the needle was removed very slowly to avoid leakage of the injected solution. Both testes were injected. Twenty-four hours after injection, each male mated for one week with two BALB/c female without superovulation. This procedure was repeated three times once a week, with the same males but mated with new females.

pEGFP vector detection

Sixty days after birth, blood was collected for DNA extraction with PureLink™ Genomic DNA Purification Kit (Invitrogen®, USA). To detect the presence of vector DNA, polymerase chain reaction was performed using EGFP-specific oligonucleotides (5'- CGGGACTTTCCAAAATGTCG -3' and 5'-GAAGATGGTGCGCTCCTGGA -3') to amplify a 500 bp fragment. PCR reactions were conducted with the following parameters: initial denaturation at 94 °C for 2 min followed by 30 cycles at 94 °C for 1min, 50 °C for 1 min and 72 °C for 1 min, plus a final extension at 72 °C for 7 min. All PCR products were sequenced in automatic DNA sequencer MegaBACE 1000 (GE Healthcare, USA).

Detection of EGFP expression

After birth, *in vivo* EGFP fluorescence was assessed using GFsP-5 miner lamp and goggles (BLS®, Hungary), which is a goggle system containing a filter set to detect EGFP fluorescence and a light to excite protein fluorescence (excitation maximum = 488 nm; emission maximum = 507 nm). EGFP expression was also evaluated by RT-PCR. Blood samples collected for PCR analysis were also used for RNA extraction. Blood was frozen and stored in liquid nitrogen until analysis. Total RNA extraction and cDNA synthesis was as described previously (Campos et al., 2010). Briefly, RNA samples were isolated using TRIzol® Reagent (Invitrogen™, Carlsbad, USA) and samples were DNase-treated with a DNA-free® kit (Ambion™, USA) following the manufacturer's protocol. First-strand cDNA synthesis was performed with 200 ng of RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, UK) according to the manufacturer's protocol. RT-PCR reactions were conducted using EGFP (5' CACGTCATTTTCCTCCTGCAT 3' and 5' GCATAGCGGCTCGTAGAGGTA 3' – product with 209 bp) and β-actin (5' TCGCTGCGCTGGTCGTCG 3' and 5' GCCAGATCTTCTCCATGTCGTCCCA 3' – product with 246 bp) primers. PCR conditions for both genes were: 35 cycles of 94°C for 15 sec, 50 °C for EGFP and 60ºC for β-actin for 30 sec and 72 °C for 30 sec, with an additional initial 1 min denaturation at 94°C and a 5 min final extension at 72°C. PCR products were electrophoresed on a 1% agarose gel containing 0.5 μg ml^{-1} ethidium bromide.

Histological damage analyses of injected testis

Seven days after the last injection, the males were sacrificed and the testes were dissected, fixed in Bouin's fixative for 24 h at 4°C and then subjected to standard histological procedure. Sections of 5-6 μm thickness from each testis were stained by hematoxylin-eosin (HE). Three testis regions and three slides per testis region were evaluated. To compare treatments, testicular damages were ranked according to the scores described in table I. A score was attributed to each slide. Comparison among treatments was conducted using the mean score from all slides of each treatment.

TABLE 1
Score description used for histological analysis

Score	Testicular damage description
0	Without histological damage
1	Testes with a small number of ST that show low GE
2	Testes with a small number of ST that show low GE
3	Testes with a high number of ST that show low EG and some without EG.
4	Testes with a predominance of ST that show low EG and some without EG and small area of fibrosis in the stroma
5	Testes in absence of GE in the ST and with largest area of fibrosis in the stroma and presence of lymphocytic inflammation.

ST – seminiferous tubules GE – germ epithelium

Data analyses

Data from PCR, RT-PCR and histological analyses were compared using one-way ANOVA followed by Tukey's test for multiple comparisons. Significance was considered at $p<0.05$.

RESULTS

Transgene transmission to F0 offspring by non-surgical testis injection

PCR analysis indicated that several mice born after mating with TMGT-treated males showed the presence of pEGFP-N1 vector in all treatments. The sequencing analysis showed that all PCR products belonged to the pEGFP-N1 vector (data not shown). The transgene transmission was compared among treatments only in the second injection procedure, due to the presence of mice born in all treatments (table II). Liposome and DMSO provided higher degree of gene transmission than DNA alone and DMA. No mice born in the control group showed the presence of pEGFP-N1 vector in the PCR analysis. *In vivo* EGFP fluorescence was not detected in any mice born, however, RT-PCR analysis of PCR positive animals showed EGFP expression in blood samples in several animals from all treated groups. The lipofectin group had a higher ratio of animals expressing EGFP in comparison to other groups. (Figure 1 and Table II).

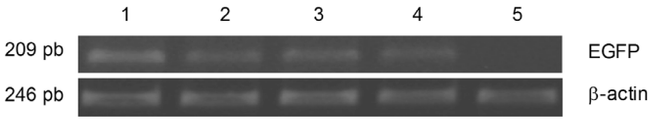

Figure 1. Gene expression analyses by RT-PCR of F0 transgenic mice obtained after mating with the injected mice males. Upper panel shows EGFP amplification and lower panel shows β-actin amplification. Lane 1 – lipofectin, Lane 2 – DMSO, Lane 3 – DMA, Lane 4 - DNA alone, Lane 5 – control (PBS alone).

TABLE II
Comparison of transfection reagents with respect to transgene transmission evaluated by PCR and transgene expression in blood cells evaluated by RT-PCR in F0 offspring obtained after TMGT

	Tranfections reagentes used														
	Lipofectin			DMSO			DMA			DNA alone			Control (PBS alone)		
	N° mice	PCR + (%)	EGFP mRNA (%)	N° mice	PCR + (%)	EGFP mRNA (%)	N° mice	PCR + (%)	EGFP mRNA (%)	N° mice	PCR + (%)	EGFP mRNA (%)	N° mice	PCR + (%)	EGFP mRNA (%)
1ª Injection	36	1 (2.7)	0	24	0 (0)	0	14	0 (0)	0	0	0 (0)	0	12	0 (0)	0
2ª Injection*	10	8(80.0)^{a}	5 (50)^{a}	9	5 (55.5)^{ab}	2 (22.3)^{ab}	18	5 (27.8)^{bc}	3 (16.6)^{ab}	26	3 (11.5)^{c}	1 (3.84)^{c}	5	0 (0)	0
3ª Injection	8	2 (25.0)	0	0	0 (0)	0	29	3 (10.3)	1	15	0 (0)	0	13	0 (0)	0
4ªInjection	11	3 (27.3)	1	1	0 (0)	0	0	0 (0)	0	9	0 (0)	0	7	0 (0)	0

*Different letters indicate statistical differences among treatments.

Histological damage analysis of injected testis

Each testis was histologically inspected 7 days after 4 consecutive injections. In the control group (injection with PBS alone) no testicular damage (scored 0) was found (Table III; Fig. 2/A). For treatment with DNA alone, DNA/liposome, or DNA/DMA, testicular damages were scored between 1.6 and 3.0 (Table III; C-E of Fig. 2/C-E), but no significant differences were found among them. On the other hand, injections of DNA/DMSO complex resulted in high degree of damages (scored 3.4; Table III). These testes had atrophic seminiferous tubules lacking spermatogonia and Sertoli cells together with stromal fibrosis, absence of Leydig cells and lymphocytic infiltration (B of Fig. 2/B).

DISCUSSION

The present study reports on the non-surgical testis injection coupled to DNA complexes as an innovative DMA (N, N-dimethylacetamide) to improve transgene transmission. Although we did not obtain the best results with DMA, this compound demonstrated a good potential for TMGT procedures. Further studies should be conducted focused on the optimal concentration, exogenous DNA concentration, incubation temperature and sperm damage to improve DMA utilization to generate transgenic TMGT.

TABLE 3
Histopatolological analyses of testis damage after four injections

Treatment	Histological damage*
Control (PBS alone)	0 ^{a}
DNA alone	1.6 ± 0.26 ^{b}
DNA/Lipofectin complex	2.7 ± 0.25 ^{b}
DNA/DMA complex	3.0 ± 0.21 ^{b}
DNA/DMSO complex	3.4 ± 0.22 ^{c}

*Data are expressed as means ± SEM (n=10) and represent the mean of scores (Table I) in each treatment. Different letters indicate statistical differences among means.

Non-surgical TMGT was able to transmit the pEGFP-N1 vector to the offspring. The use of DNA complexes to enhance uptake of exogenous DNA by sperm was previously demonstrated by Sato et al. (2002) and Shen et al. (2006). Shen et al. (2006) carried out experiments with DNA/DMSO complex and subsequent injection into to mouse and rabbit testes and demonstrated that respectively 61 and 55% of offspring born was genetically transformed. In our study, we obtained similar results using DMSO transfection and when the exogenous DNA was injected without transfectant.

Liposome and DMSO were found to increase the rate of TMGT. Kim et al. (1997) carried out the experiments with commercial liposome transfection agent. They observed a low transfection efficiency of the seminiferous tubule cells in mice at 1 to 12 weeks after injection. Therefore, the transgene was transferred by sperm to F0 progeny, but it was lost from most tissues during growth. Using a lipid-based method of transfection, Celebi et al. (2002) reported the transient transmission of a transgene in the progeny of male mice undergoing *in vivo* germ cell transfection. Yonezawa et al., (2001) produced transgenic rats by means of TMGT using liposomes and showed that one month after birth only 4% of the progeny were foreign-DNA-positive. Here, we demonstrated that two months after birth we still detect the EGFP gene in 80% of offspring using TMGT associated with liposome.

In this work, the transgene was successfully transmitted to offspring but *in vivo* EGFP fluorescence in the body was not detected. Yonezawa et al., (2001) demonstrated that more than 80% of morula-stage embryos expressed EGFP. Then they detected introduced DNA in the progeny by PCR and found that the ratio of animals carrying the foreign DNA decreased

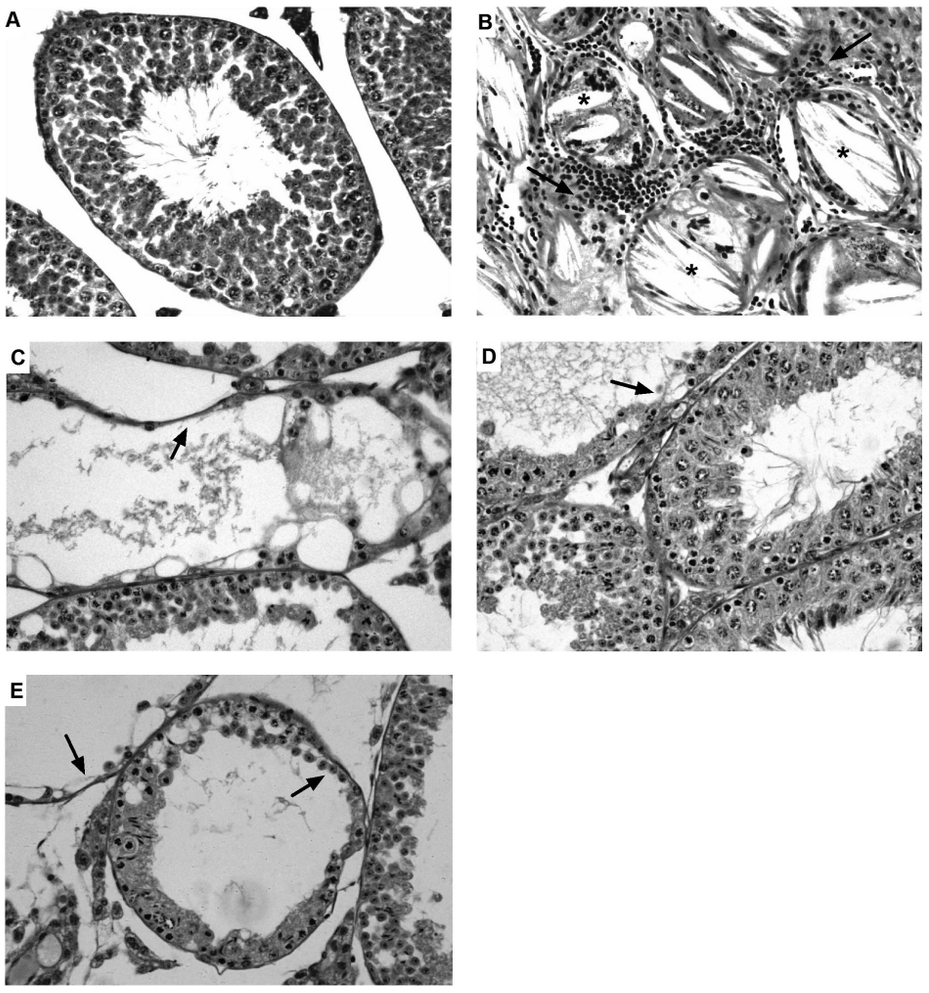

Figure 2. Hematoxylin and eosin staining of testes injected with different DNA complexes. (A) Control (injections of PBS alone), seminiferous tubules without any histological damage are seen. (B) Injections of DNA/DMSO complex, atrophic seminiferous tubules lacking germ cells and Sertoli cells are seen (asterisks). Stromal fibrosis and absence of Leydig cells are indicated by arrowhead and lymphocytic infiltration (arrows) are also evident in the interstitial space. (C) Injections of DNA/DMA complex, seminiferous tubules with reduced number of spermatogonial cells are seen (arrow). (D) Injections of DNA alone, some seminiferous tubules exhibit a reduced number of spermatogonial cells (arrow). (E) Injections of DNA/liposome complex, some seminiferous tubules exhibit a reduced number of spermatogonial cells (arrows). The magnification is x 400 in all panels.

as they developed, and that only a part of postpartum progeny were foreign-DNA-positive with high incidence of mosaicism. Here, we believe that absence of EGFP expression in the body could by caused by the mosaicism, since EGFP expression was detected by RT-PCR in blood cells of some animals. This result coincides with previous reports that CMV promoter could drive EGFP expression for leucocytes of transgenic chickens, produced by sperm-mediated gene transfer (Harel-Markowitz et al., 2009). Using mouse model, Kato et al. (1999) reported that 40% of morula-stage embryos that had been subjected to pronuclear microinjection of the same gene construct used in the present study were EGFP-positive, among which 62% showed mosaic fluorescence. Thus, the efficiency in transferring foreign DNA into the egg is much higher by the TMGT method than by the pronuclear microinjection method. The reason for this difference may be that foreign DNA is introduced into the egg under much more physiological condition by the TMGT method, using the sperm as a vector, than with the microinjection method. In addition, the difference in the timing of the introduction of foreign DNA into the egg between the TMGT method (at the time of fertilization) and the microinjection method (after formation of the pronucleus) could account for the difference in the ratio of mosaic embryos.

The highest ration of transgene transmission was obtained in the second injection/mating. Shen et al. (2006) performed weekly consecutive injections, but did not evaluate transgene transmission after each injection. After the fourth injection, a severe reduction was observed, and in some cases the absence of progeny. We observed the presence of vaginal plugs, indicating the occurrence of mating in the females after injections in all treatments. In addition, no behavioral changes were observed in males, leading to the conclusion that this reproductive deficiency was caused by the reduced spermatogenesis observed after multiple injections.

Injections into testes could produce testicular damage as demonstrated by our histological analysis. In the control group no damage was observed. In contrast, testes injected with DNA alone, DNA/DMA or DNA/liposome complex were significantly damaged (see Table 3; A vs. C-E of Fig. 1). Remarkably, the testes injected with DNA/DMSO complex showed a high degree of damage (see table 3; Fig. 2/B). All of these testes examined exhibited fibrosis and lymphocytic infiltration. We believe that consecutive injections of DMSO-containing solution can induce testicular degeneration and reduce vascularization around seminiferous tubules. As a result, spermatogenesis is impaired, as evidenced by a reduced number of spermatogonial cells.

In summary, we reported transgene transmission in mice by non-surgical TMGT using different transfectants. As well, we demonstrated reduction of germ epithelium of testes after DNA complex injections, fact that must be elucidated to further applications of TMGT for *in vivo* gene transfer technology.

AKNOWLEDGEMENTS

This work was supported by FAPERGS/Edital PROADE III (# 05/2328.6). V. F. Campos is a student of the Graduate Program in Biotechnology at Universidade Federal de Pelotas and is supported by CAPES. J.C. Deschamps is a research fellow of CNPq.

REFERENCES

BIANCHI I, CALDERAM K, MASCHIO EF, MADEIRA EM, DA ROSA ULGUIM R, CORCINI CD, BONGALHARDO DC, CORRÊA EK, LUCIA T JR, DESCHAMPS JC, CORRÊA MN (2008) Evaluation of amides and centrifugation temperature in boar semen cryopreservation. Theriogenology 69:632-638.

BRACKETT BG, BORANSKA W, SAWICKI W, KOPROWSKI H (1971) Uptake of heterologous genome by mammalian spermatozoa and its transfer to ova through fertilization. Proc Natl Acad Sci USA 68:353-357.

CAMPOS VF, AMARAL MG, SEIXAS FK, POUEY JLF, SELAU LPR, DELLGOSTIN OA, DESCHAMPS JC, COLLARES T (2011b) Exogenous DNA uptake by South American catfish (*Rhamdia quelen*) spermatozoa after seminal plasma removal. Anim Reprod Sci (in press).

CAMPOS VF, COLLARES T, DESCHAMPS JC, SEIXAS FK, DELLAGOSTIN OA, LANES CFC, SANDRINI J, MARINS, LF, OKAMOTO M, SAMPAIO LA, ROBALDO RB (2010) Identification, tissue distribution and evaluation of brain neuropeptide Y gene expression in the Brazilian flounder *Paralichthys orbignyanus*, J Biosc 35:405-413.

CAMPOS VF, KOMNINOU ER, URTIAGA G, LEON PM, SEIXAS FK, DELLAGOSTIN OA, DESCHAMPS JC, COLLARES T (2011a) NanoSMGT: transfection of exogenous DNA on sex-sorted bovine sperm using nanopolymer. Theriogenology 75:1476-1481.

CAMPOS VF, LEON PM, KOMNINOU ER, DELLAGOSTIN OA, DESCHAMPS JC, SEIXAS FK, COLLARES T (2011c) NanoSMGT: transgene transmission into bovine embryos using halloysite clay nanotubes or nanopolymer to improve transfection efficiency. Theriogenology (in press).

CELEBI C, AUVRAY P, BENVEGNU T, PLUSQUELLEC D, JEGOU B, GUILLAUDEUX 209 T (2002) Transient transmission of a transgene in mouse offspring following *in vivo* transfection of male germ cells. Mol Reprod Dev 62:477-482.

COLLARES T, CAMPOS VF, LEON PMM, CAVALCANTI PV, AMARAL MG, DELLAGOSTIN OA, SEIXAS FK, DESCHAMPS JC (2011) Transgene transmission in chicken by sperm-mediated gene transfer after seminal plasma removal using exogenous DNA complexed with dimethylsulfoxide or N,N-dimethylacetamide. J Biosci (in press).

COLLARES T, CAMPOS VF, SEIXAS FK, CAVALCANTI PV, DELLAGOSTIN OA, MOREIRA HL, DESCHAMPS JC (2010) Transgene transmission in South American catfish (*Rhamdia quelen*) larvae by sperm-mediated gene transfer. J Biosci 35:39-47.

COWARD K, KUBOTA H, PARRINGTON J (2007) In vivo gene transfer into testis and sperm: developments and future application. Arch Androl 53:187-197.

DHUP S, MAJUMDAR SS (2008) Transgenesis via permanent integration of genes in repopulating spermatogonial cells in vivo. Nat Methods 5:601-603.

HAREL-MARKOWITZ E, GUREVICH M, SHORE LS, KATZ A, STRAM Y, SHEMESH M (2009) Use of sperm plasmid DNA lipofection combined with REMI (Restriction Enzyme- Mediated Insertion) for production of transgenic chickens expressing EGFP (Enhanced Green Fluorescent Protein) or Human follicle-stimulating hormone. Biol Reprod 80:1046-1052.

HOELKER M, MEKCHAY S, SCHNEIDER H, BRACKET BG, TESFAYE D, JENNEN D, THOLEN E, GILLES M, RINGS F, GRIESE J, SCHELLANDER K. 2007. Quantification of DNA binding, uptake, transmission and expression in bovine sperm mediated gene transfer by RT-PCR: Effect of transfection reagent and DNA architecture. Theriogenology 67:1067-1107.

KATO M, YAMANOUCHI K, IKAWA M, OKABA M, NAITO K, TOJO H (1999) Efficient selection of transgenic mouse embryos using EGFP as a marker gene. Mol Reprod Dev 54:43-48.

KIM JH, JUNG-HA HS, LEE HT, CHUNG KS. 1997. Development of a positive method for male stem cell-mediated gene transfer in mouse and pig. Mol Reprod Dev 46:515-526.

LAVITRANO M, CAMAIONI A, FAZIO VM, DOLCI S, FARACE MG, SPADAFORA C (1989) Sperm cells as vectors for introducing foreign DNA into eggs: Genetic transformation of mice. Cell 57:717-723.

LI L, SHEN W, MIN L, DONG H, SUN Y, PAN Q (2006) Human lactoferrin transgenic rabbits produced efficiently using dimethylsulfoxide-sperm-mediated gene transfer. Reprod Fertil Dev 18:689-695.

LU JK, FU BH, WU JL, CHEN TT (2002) Production of transgenic silver sea bream (Sparus sarba) by different gene transfer methods. Mar Biotech 4:328-337.

OGAWA S, HAYASHI K, TADA N, SATO M, KURIHARA T, IWAYA M (1995) Gene expression in blastocysts following direct injection of DNA into testis. J Reprod Dev 41:379-238 382.

SATO M, ISHIKAWA A, KIMURA M (2002) Direct injection of foreign DNA into mouse testis as a possible in vivo gene transfer system via epididymal spermatozoa. Mol Reprod Dev 61:49-56.

SHEN W, LI L, PAN QJ, MIN LJ, DONG HS, DENG JX (2006) Efficient and simple production of transgenic mice and rabbits using the new DMSO-sperm mediated exogenous DNA transfer method. Mol Reprod Dev 73:589-594.

WEBSTER NL, FORNI M, BACCI ML, GIOVANNONI R, RAZZINI R, FANTINATI P, ZANNONI A, FUSETTI L, DALPRA L, BIANCO MR, PAPA M, SEREN E, SANDRIN MS, MCKENZIE I, LAVITRANO M (2005) Multi-transgenic pigs expressing three fluorescent proteins produced with high efficiency by sperm mediated gene transfer. Mol Reprod Dev 72:68-76.

YONEZAWA T, FURUHATA Y, HIRABAYASHI K, SUZUKI M, TAKAHASHI M, NISHIHARA M (2001) Detection of transgene in progeny at different developmental stages following testis-mediated gene transfer. Mol Reprod Dev 60:196-201.