

Testing Sperm-Mediated Gene Transfer (SMGT) in Pigs

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Introduction

A number of transgenic animals have been created for agricultural and biomedical purposes during last two decades. Several methods to create genetically modified animals have been developed such as: pronuclei microinjection, viral vectors, sperm-mediated gene transfer method, and gene targeting followed by nuclear transfer. However, there are still problems with application of these methods to livestock due to high cost and low efficiency. Sperm-mediated gene transfer (SMGT) has been proposed as cheap and efficient method which uses sperm as a natural vector to transfer transgene to the egg (Lavitrano et al., 1989; Lavitrano et al., 2002). Recent successes with production of transgenic pigs is very promising, as SMGT was used to produce transgenic pig with human decay accelerating factor construct (Lavitrano et al., 2002) and multi-transgenic pigs carrying up to three fluorescent proteins (Webster et al., 2005). Despite apparent simplicity of the method, many attempts to apply SMGT did not achieve the desired result, most notably Brinster et al. (1989) tested 1300 mouse offspring produced by SMGT but none of them was transgenic. Similarly, high efficiency of linker based SMGT (Chang et al., 2002) or restriction enzyme-mediated SMGT (Shemesh et al., 2000) have not been replicated to our knowledge in other laboratories. These data indicate that some important variables affecting SMGT have not been identified yet. The goal of current project was to provide independent confirmation for applicability of SMGT in pigs and better understand factors responsible for conflicting results. There is also fundamental question of how organisms protect their germline from invasion by DNA present in the environment. Such problem is especially acute in species with external fertilization, such as fish, but a similar problem also arise in mammals where sperm, oocyte, or embryo, might be exposed to microbial, viral, and endogenous cellular DNA in reproductive tract. It is possible that difficulty in reproducibility of SMGT is an indication of natural defences against exogenous DNA invasion functioning in sperm or embryo.

Results and Discussion

Despite indication of reliable protocol for SMGT in pigs finally established, we have not been able to reproduce it. No GFP positive transgenic embryos were detected after testing 105 fertilized (≥ 2 cells) embryos produced by 9 sows inseminated with DNA treated sperm from 3 different boars and even a 20% higher fertilization rate in DNA treated sperm was elucidated (Figure 1). Based on previously published results around 50% of transgenic embryos were expected (Lavitrano et al., 2002). GFP is very sensitive reporter and injection of just 1-3 pEGFP plasmids directly into nucleus can produce GFP positive cells. Absence of pEGFP plasmid was also confirmed by WGA-PCR. That seems to indicate that no pEGFP plasmids were transferred to embryos by sperm in our experiments. It was important to confirm first that in our experiment porcine sperm bound and internalized sufficient amount of pEGFP plasmid. To compare our results with Lavitrano et al. (2003), actual copy number of plasmids bound per sperm was calculated. For SMGT, Lavitrano et al. (2003) used 400 mkg of 6.8 kb construct incubated with 10^9

spermatozoa. Boars were used with 58% and 67% rate of DNA uptake with around 20% of plasmid internalized in the sperm nucleus. According to our calculations this should lead to $1.2 - 1.4 \times 10^4$ copies of plasmid per spermatozoa nucleus. In our experiment, similar estimation for boars used for SMGT resulted in 1.4×10^4 (boar ID 10) and 0.6×10^4 (boar ID 28) plasmids internalized in the nucleus. This is similar to Lavitrano et al. (2003) and 100 fold more than was found previously for pigs (3.8×10^2) (Horan et al., 1991).

Even if sperm internalized sufficient number of plasmids in the nucleus, but if it is unable to reach and fertilize oocyte, no transgenic animals will be produced. Our hypothesis was that DNA binding to sperm results in inability of DNA loaded sperm to participate in fertilization process. We did not detect any major negative effect from DNA treatment on sperm motility or viability. Significant decrease in motility seems to be associated with removal of seminal plasma and not the DNA treatment. It does not explain why DNA loaded sperm does not reach oocyte. DNA treatment did result in additional increase in DNA damage in boar spermatozoa (Figure 2). Number of researchers suggested that mature spermatozoa contain nucleases which are induced after sperm damage (Sotolongo et al., 2005). It was also shown that binding of exogenous DNA to sperm lead to activation of endogenous nuclease activity and degradation of both sperm endogenous chromosomal DNA and added transgene (Spadafora, 1998).

Based on our results and analysis of available data from other experiments it seems unlikely that selection against DNA loaded sperm happens at DNA/sperm interaction stage. Sperm seems to be able to internalize relatively large number of plasmids without any significant effects on sperm motility and viability *in vitro*. Similarly selection against exogenous DNA seems unlikely at the embryo level, both pronuclear microinjection and ICSI were used successfully to produce transgenic offspring. Yet, absence of transgenic progeny after SMGT seems to indicate that *in vivo* certain deficiencies of DNA loaded sperm become apparent and selected against *in vivo*. We suggest that either DNA degradation in DNA loaded sperm or even DNA binding to sperm might be recognized as fragmented/abnormal DNA and selected against *in vivo*. It has been shown that human cervical mucus can act as a selective sieve preventing progress of spermatozoa with fragmented DNA and chromatin structural abnormalities, and that through binding in tubal reservoirs, sperm with low DNA damage is selected. Attached exogenous DNA might also physically interfere with sperm interaction with oviductal cells, oocyte or with sperm movement through reproductive tract. In sperm autoimmunity, spermatozoa bound with antisperm antibodies are unable to penetrate human cervical mucus (Yanagimachi, 2003). It is possible that DNA bound to sperm membrane receptor might also create a physical drag on sperm progress in oviduct. This hypothesis is attractive, as it would explain selective elimination of DNA loaded sperm from fertilization process.

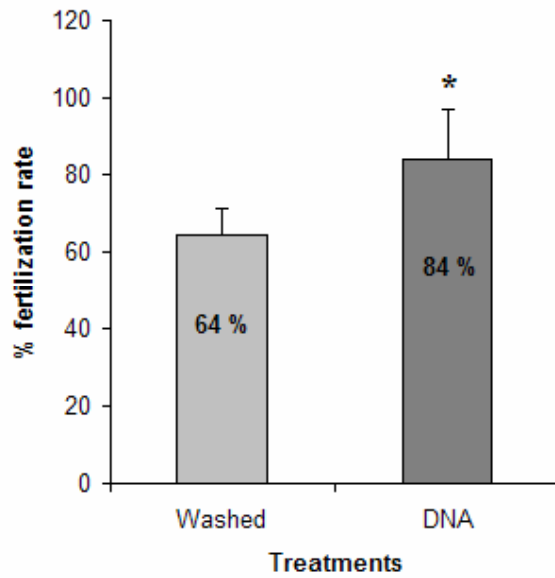


Figure 1. Fertilization rate.

Fertilization rate. Fertilization rate was calculated as the sum of embryos recovered vs total number of oocytes and embryos recovered (100%). Superscripts (*) indicate significant difference with negative control (ANOVA, proc glimmix model; $p < 0.05$).

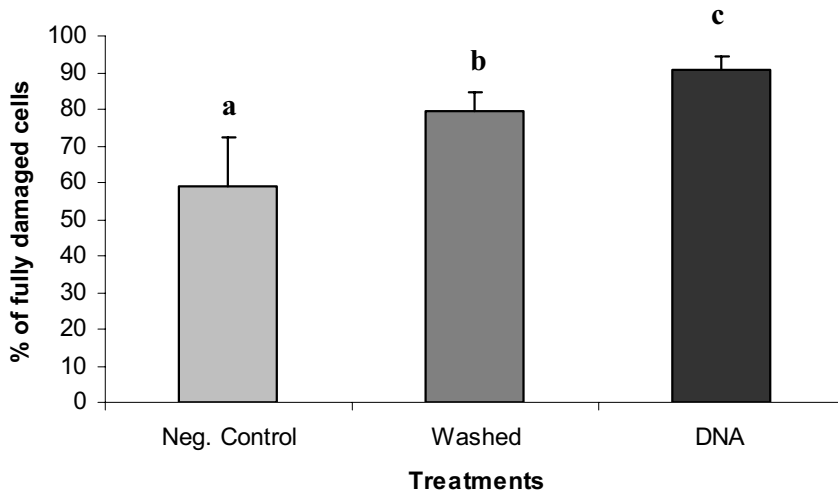


Figure 2. Effect of DNA treatments on sperm DNA damage (mean \pm SD).

Neg. Control: diluted semen; washed: washed semen; and DNA: washed and DNA (pEGFP-N1) treated semen. All incubations were performed at 17°C for 24 hr; Different letters (a, b) indicate significant difference among treatments (tukey-test; $p < 0.05$).

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