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The negative effects of exogenous DNA binding on porcine spermatozoa are caused by removal of seminal fluid

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Abstract

Sperm-mediated gene transfer (SMGT) might become the most efficient and cost effective technique to generate transgenic animals, which will significantly increase their application in biomedical research and in commercial production. Despite some successes, the technique has remained controversial for almost 20 years and despite number of studies the reasons for poor reproducibility of this promising technology has not been understood. We suggest that the reason for poor reproducibility is the presence of natural defences against exogenous DNA invasion acting in spermatozoa or in embryo. Based on previous reports we have investigated the effect of foreign DNA binding on spermatozoa by monitoring motility, viability and genomic DNA damage. Evaluation of DNA binding in sperm collected from 16 boars demonstrated that 28–45% of the added pEGFP plasmid was bound to spermatozoa with 9–32% being internalized in sperm nucleus. In agreement with previous reports, our results demonstrated that the pEGFP-treated sperm show an average a 2-fold decrease in motility ($p < 0.05$), 5-fold decrease in progressive motility ($p < 0.05$), and 1.4-fold increase in number of sperm with highly damaged DNA ($p < 0.05$) as detected by Comet assay. In contrast with previous reports, we demonstrate that all such changes were associated with the removal of seminal plasma during the washing step and not with foreign DNA binding *per se*. We suggest that poor reproducibility of SMGT most likely result from selection against DNA-loaded sperm at later stages of fertilization.

Keywords: Sperm-mediated gene transfer; Comet assay; Spermatozoa; DNA damage; Seminal plasma

1. Introduction

Sperm-mediated gene transfer (SMGT) uses sperm as a natural vector to transfer transgenes [1]. SMGT would provide the cheapest and most efficient method to produce large transgenic animals [2–5]. Despite the apparent simplicity of the method, many previous attempts to confirm SMGT efficiency have not achieved the desired result. Several groups tried to replicate the initial mouse results but unsuccessfully. Most notably Brinster et al. [6] tested 1300 offspring produced by SMGT but none of them were transgenic. Similarly, high efficiency of linker-based SMGT [4] or restriction enzyme-mediated SMGT [2] have not been reproduced to our knowledge in other laboratories. Since initial demonstration of DNA binding to rabbit sperm [7], many researchers have shown that DNA binding is a natural ability of sperm in many species [8]. Such ability would have major evolutionary implications and begs a question of how organisms protect their germline

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from invasion by exogenous DNA under natural conditions. It has been reported that incubation with exogenous DNA led to the activation of sperm nucleases [9–11] and a significant decrease in sperm motility [12,13]. Both sperm immobilization and nuclease activation might indicate the presence of natural defences activated in the sperm after the binding of exogenous DNA. It is reasonable to suggest that poor reproducibility of SMGT might also be related with activation of such defences in sperm.

The objective of our project was to investigate the effect of foreign DNA binding on spermatozoa by monitoring DNA binding and internalization, motility, viability and genomic DNA damage. Better understanding of foreign DNA effects on sperm will allow researchers to significantly improve SMGT reproducibility and better understand natural defences against foreign DNA invasion.

2. Materials and methods

2.1. Animals and handling

Eighteen sexually mature Yorkshire boars were kept at the Ponsonby Research Station, University of Guelph. Each boar was housed individually according to the guidelines of Canadian Council of Animal Care. All animal manipulations were approved by the University of Guelph Animal Care Committee.

2.2. Semen collection and preparation

Once per week, collection of sperm was performed by the gloved hand methods. Washed and DNA-treated semen was prepared as described previously [3,21]. Swine fertilization medium [SFM; 1 L contains 11.25 g glucose, 10 g sodium citrate ($2H_2O$), 4.7 g EDTA ($2H_2O$), 3.25 g citric acid (H_2O), 6.5 g Trizma adjusted to pH 7.4] supplemented with 6 mg/mL BSA (Fisher) was used as an extender [3]. To prepare seminal plasma, the supernatant was saved and centrifuged again. This saved seminal plasma was added back to the washed or DNA-treated sperm from the same boar to achieve a final concentration similar to the negative control.

2.3. Sperm motility and viability

Semen was prepared at a concentration of 10^7 spermatozoa/mL for motility and viability evaluation. Motility was measured by using computer-assisted semen analysis (CASA) (Spermvision, Minitube). Prepared samples were pre-warmed for 20 min before evaluation and 2 μL of sample was loaded into 4-chamber slide (Leja, Nieuw-Venner, Netherlands). In this study, sperm was considered motile if both velocity average path (VAP) was $>20\ \mu m\ s^{-1}$ and velocity curvilinear (VCL) $>5\ \mu m\ s^{-1}$ and progressively motile as those cells with VAP $>45\ \mu m\ s^{-1}$ or straightness >45% [14].

Dual fluorescent PI/SYBR 14 staining (LIVE/DEAD® Sperm Viability Kit, Invitrogen) was used to evaluate viability of sperm according to manufacturer's protocol. The number of red (dead) and green cells (live) in a total of 100 sperm was counted in duplicate for each sample with a fluorescent microscope (LEICA DMR, Germany) with the filter (450–490 nm excitation wavelengths) at 400× magnification. Viability was expressed as the average percentage of viable sperm cells from 100 counted sperm cells.

2.4. Assessment of DNA binding

A pEGFP-N1 vector containing Green Fluorescent protein reporter gene under control of CMV promoter was used for this study (BD Biosciences, Mississauga, Canada). Plasmids were extracted using Plasmid Mega Kit (Qiagen). The StuI restriction enzyme (Invitrogen, Burlington, Canada) was used to prepare linearized plasmid and digestion efficiency was checked by 1% agarose gel electrophoresis. Scintillation counting was used to assess DNA binding of sperm. The digested pEGFP-N1 was labelled with [α-^{32}P] dCTP using Nick Translation Kit (Invitrogen, Burlington, Canada). Spermatozoa (10^6 cells) were incubated with 0.4 μg of pEGFP-N1 and washed twice before quantification. Radioactivity in supernatant and pellet was analyzed in a liquid scintillation counter (Model LS6000SC, Beckman, Fullerton, CA, USA).

2.5. DNA extraction from whole sperm and sperm nuclei

Sperm genomic DNA extraction was performed using phenol:chloroform. Briefly, a sperm pellet was suspended with 100 μL of PBS and 400 μL of sperm extraction solution (2% β-mercaptoethanol, 10 mM Tris pH 8.0, 100 mM NaCl, 0.5% SDS and 10 mM EDTA) was added and incubated for 30 min at 55 °C. After that 20 μL of proteinase K (20 mg/mL, Sigma) was added and incubated at 55 °C overnight. For extraction, 500 μL of phenol:chloroform (Sigma) was added, briefly vortexed and centrifuged at 13,500 × *g* for 5 min. DNA precipitation was performed with 2

volumes of 100% ethanol and the pellet was washed with 70% ethanol, dried and dissolved in 200 μL of water.

For nuclei extraction, the sperm pellet was resuspended in 100 μL of PBS and 100 μL of DTT buffer (0.1 M DTT, 0.05 M Tris, pH 7.5) was added and incubated for 30 min on ice. Then, 800 μL of CTAB buffer (10% CTAB, 0.01 M DTT) was added to the sample and incubated again for 60 min on ice. The recovered sperm nuclei were washed twice in 50 mM Tris, pH 8.0 and centrifuged at 10,000 × *g* for 5 min. The sperm genomic DNA extraction was performed as mentioned above using phenol:chloroform. The concentration of DNA samples was measured with Picogreen dsDNA Quantification Kit (Molecular Probes, Inc., Eugene, OR) and used as a template for Real-Time PCR.

2.6. Real-Time PCR quantification

Primer sets were designed using Primer 3 (http://www.frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). In order to accurately quantify different samples, one has to ensure that the same amount of sperm DNA was used in each Real-Time PCR. The amount of DNA used in PCR was normalized for technical variability using SINE repetitive sequences [14].

Following primers were used: SINE (GGATCCGGCATTGCCGTTAG, GTCTTTTTTTGCCATTTCTTGG) and GFP (TATATCATGGCCGACAAGCA, GAACTCCAGCAGGACCATGT). Platinum® SYBR® Green qPCR SuperMix-UDG with ROX (Invitrogen) was used for Real-Time PCR quantification reaction using ABI 7700 Real Time machine. The amplification of the correct product was monitored by the melting curve during PCR, running products on agarose gel after PCR and sequencing. The standard curve method was used for quantification of pEGFP plasmid [15,16].

2.7. Comet assay

Comet assay was performed as described previously with some modifications [17,18]. Collected and washed semen was diluted to a concentration of 5×10^6 spermatozoa/mL. The pellet was resuspended in 98 μL of PBS, mixed with 2 μL of 2% β-mercaptoethanol and incubated for 1 h at 4 °C. Cells were further mixed with 200 μL of lysis buffer (1.25 M NaCl, 0.01% sodium *N*-lauroyl sarcosine, 50 mM sodium salt of EDTA, 100 mM Tris–HCl, pH 10) and incubated for 2 h at 4 °C. Sperm were centrifuged at 1500 × *g* for 5 min to remove lysis buffer and 200 μL of incubation buffer (50 mM Tris–HCl, pH 8.0, 10 mM EDTA) was added. After 1 h incubation at 37 °C, 12.5 μL of proteinase K (50 mg/mL) were added and samples were incubated overnight at 55 °C. For the treated sperm sample, the diluted sample (65 μl) was mixed with 65 μL of 1% of low melting agarose (Sigma–Aldrich) pre-warmed at 55 °C. 50 μL of this mixture were loaded onto a microgel slide pre-coated with 1% normal agarose. Then 50 μL of 0.5% of low melting agarose gel was poured as a 3rd layer to protect the sample layer. The slides went through an unwinding step for 25 min in fresh alkaline electrophoresis buffer (300 mM sodium hydroxide, 1 mM EDTA, 0.2% DMSO, 0.1% 8-hydroxyquinoline, pH >13) in horizontal electrophoresis unit and electrophoresed in fresh alkaline electrophoresis buffer for 20 min at 20 V. After electrophoresis slides were immersed three times in 0.4 M Tris–HCl, pH 7.4 for neutralization and finally immersed into 70% of ethanol and completely dried. Slides were stained with 500 μL of 10,000 times dilution of SYBR Gold Nucleic Acid Gel Stain (Molecular Probes Company) for 10 min. To quantify the amount of sperm genomic DNA damage, five classes from zero to four were used [19,20].

2.8. Statistical analysis

All data are reported as the mean of three independent replications. Analysis of variance (ANOVA) among the groups was tested and their interactions were analyzed by GLM using Tukey test. The experimental design was a randomized completed block (RCB) design, in which the blocks were boars. A p-value less than 0.05 was considered significant and between $0.05 < p < 0.1$ as a tendency in all experiments.

3. Results

3.1. Binding and internalization of pEGFP-N1 plasmid by porcine sperm

To evaluate DNA binding in our research pig population a total of 3 ejaculates were collected from 16 boars and thoroughly washed to remove seminal plasma which contains inhibitors of DNA binding [10,22]. DNA binding was evaluated by incubation of sperm with ^{32}P-labelled linearized pEGFP-N1 and any unbound plasmid was removed by two washing steps. DNA binding rates varied from 28% to 45% of the

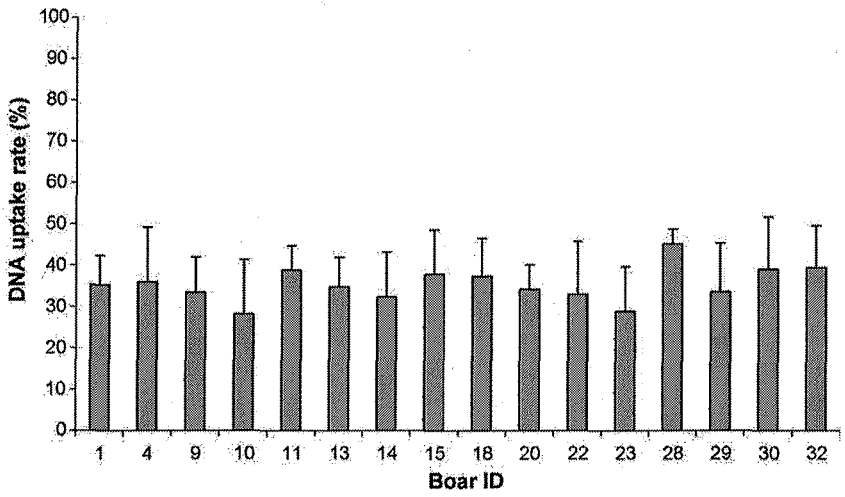

Fig. 1. DNA binding rate (%) by porcine sperm. No significant differences were detected among boars ($p = 0.86$) (mean ± S.D., $N = 3$ ejaculations/boar).

added DNA (Fig. 1). No significant differences were detected between boars in DNA binding.

To determine what percentage of bound pEGFP was internalized into the sperm nuclei, quantification by Real-Time PCR was developed. To quantify the amount of pEGFP all DNA (membrane bound and internalized in the nucleus) was extracted from sperm incubated with the plasmid. To quantify pEGFP internalized in the nucleus, DNA was extracted from purified sperm nuclei. Internalization rate in the sperm nucleus varied from 8.6% to 31.5% of total bound pEGFP, but no significant differences were detected between the boars (Fig. 2).

3.2. *Effect of DNA binding on sperm motility*

To evaluate the effect of exogenous DNA binding on sperm motility, the spermatozoa were evaluated before and after incubation with pEGFP. It was found that while untreated sperm showed normal motility, the pEGFP-treated sperm showed on average a 2-fold decrease in motility and a 5-fold decrease in progressive motility ($p < 0.05$) (Fig. 3). To identify the cause for decreased motility the effect of different sperm treatments was further tested with three boars. It was found that motility and progressive motility was again drastically decreased after incubation with DNA

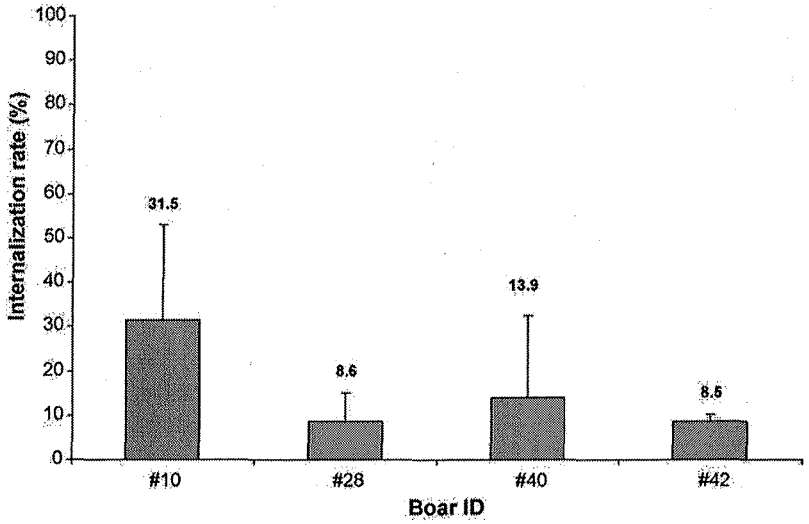

Fig. 2. Internalization rate of sperm-bound pEGFP-N1 into nucleus as detected by Real-Time PCR. No significant differences among boars were detected ($p = 0.2$) (mean ± S.D.; $N = 3$ ejaculations/boar).

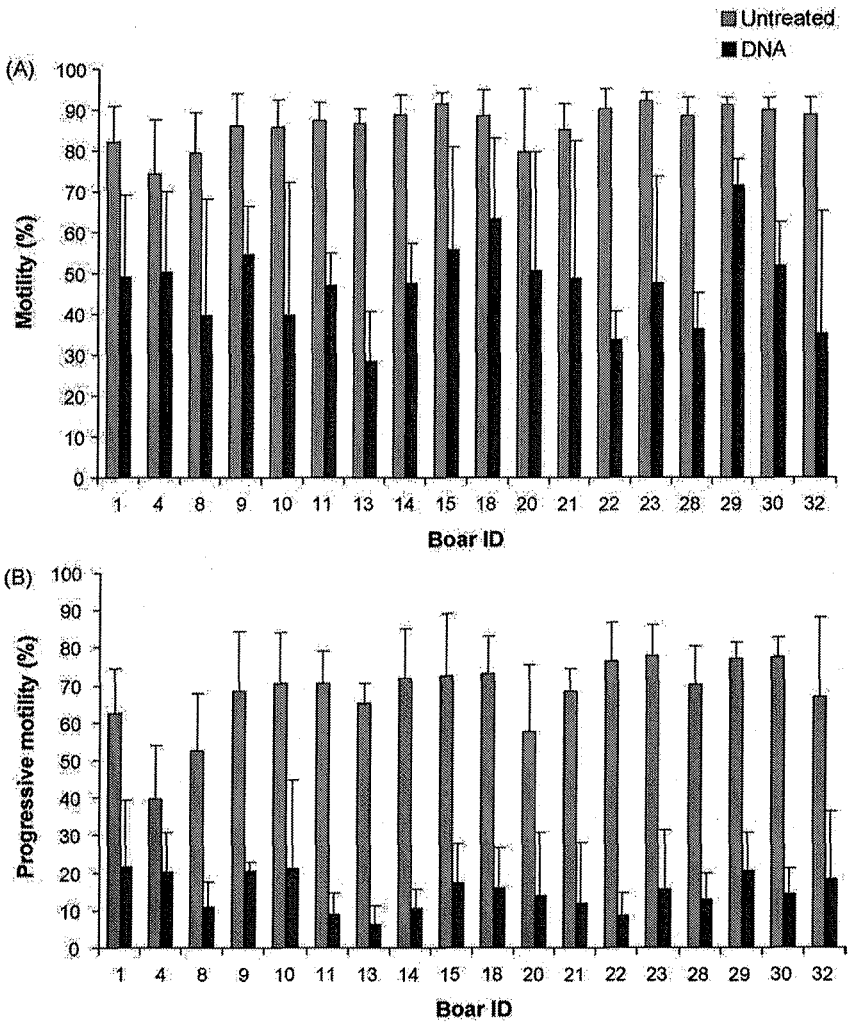

Fig. 3. Effect of DNA incubation on (A) sperm motility and (B) progressive motility. Untreated: no treatment; DNA: sperm was washed and incubated with pEGFP. Significant difference between control and treatment was found for all boars in (A) and (B) (Tukey test; $p < 0.05$).

($p < 0.05$). At the same time there was a similar decrease in motility after the washing step (Fig. 4). There was no additional decrease of sperm motility after pEGFP incubation ($p > 0.05$). Thus, the washing step alone seems to cause a decrease in sperm motility and not the incubation with pEGFP. Addition of seminal plasma after washing returned sperm motility to normal in both washed and pEGFP-treated samples (Fig. 4). Therefore, the removal of seminal plasma led to the decrease in sperm motility.

3.3. Damage of sperm DNA

Alkaline Comet assay is a sensitive technique for detection of DNA damage at a single cell level [17], and was used to detect damage in sperm incubated with pEGFP. Data from three boars indicates that both sperm washing (125%, $p < 0.001$) and DNA treatment (135%, $p < 0.0001$) resulted in an increase in the number of sperm with highly damaged DNA compared to control (100%) (not shown). At the same time, there were no differences in the DNA damage between washed and DNA-treated groups ($p = 0.14$). Therefore, it might be concluded that the washing step was primarily responsible for increased DNA damage detected in sperm genomic DNA.

Sperm viability was determined for all boars by measuring plasma membrane integrity using PI/SYBR 14 staining [23]. Viability after 24 h incubation with pEGFP varied from 63.3% to 78.5% with no differences between boars ($p > 0.05$) (not shown). In addition, the effect of each treatment including washing and

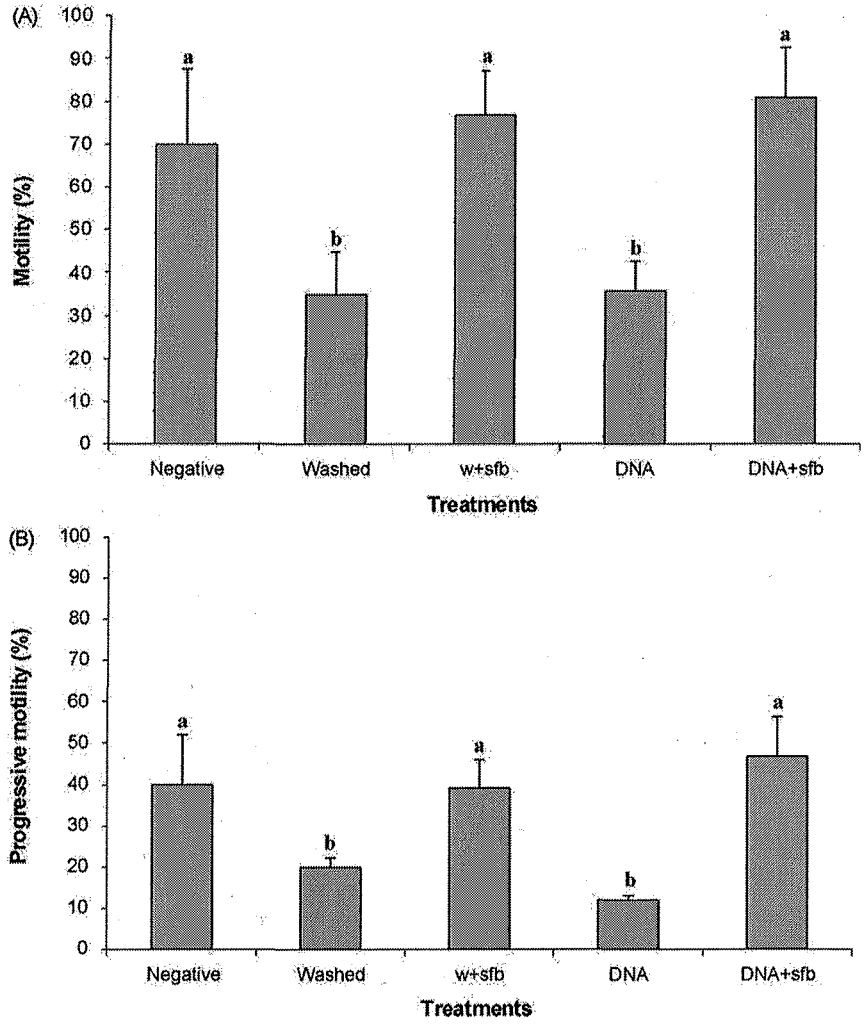

Fig. 4. Evaluation of effect of DNA treatment on (A) sperm motility and (B) progressive motility. Negative: no treatment; washed: sperm was washed; w + sfb: sperm was washed and seminal plasma added back; DNA: sperm was washed and incubated with pEGFP; and DNA + sfb: sperm was washed, seminal plasma was added back and sperm was incubated with pEGFP (mean ± S.D., $N = 3$ boars, 3 ejaculations/boar). Different letters (a, b) indicate significant difference (Tukey test, $p < 0.05$).

incubation with pEGFP was evaluated with sperm from three boars. The results show no differences in sperm viability among the untreated, washed, and pEGFP-treated sperm ($p > 0.05$) (not shown).

4. Discussion

In our previous testing of SMGT 105 fertilized embryos (≥2 cells) were produced by 18 sows inseminated with pEGFP-treated sperm from 3 different boars. Neither GFP expression nor presence of pEGFP plasmid by PCR was detected in tested embryos (not shown). It was important to confirm that in our experiment porcine sperm could bind and internalize plasmid DNA. Evaluation of DNA binding in sperm collected from 16 boars demonstrated that 28–45% of the added pEGFP plasmid was bound to spermatozoa with 9–32% being internalized in sperm nucleus with no significant differences between boars. In contrast, considerable differences were reported in the ability of porcine sperm to bind exogenous DNA between different experiments and even between different boars [21,24]. To compare our results, we calculated an actual copy number of plasmids internalized per spermatozoa. In our experiment, we estimate that 0.6×10^4 (boar # 28) and 1.4×10^4 (boar # 10) plasmids were internalized per spermatozoa. This is similar to 1.3×10^4 internalized plasmids achieved by Lavitrano et al. [21] and 16–37-fold more than what was found previously for pigs (3.8×10^2) [24]. While it might be

possible to increase DNA binding and internalization by using electroporation or lipofection [25], based on our results, it seems unlikely that failures of SMGT can be explained by problems at the DNA/sperm interaction stage. It is known that pronuclear microinjection of around 500 copies of plasmid per egg produces optimal transgenesis results and even injection of just 5 copies is sufficient to produce transgenic mice [26].

Our results demonstrated that the pEGFP-treated sperm show an average a 2-fold significant decrease in motility, 5-fold decrease in progressive motility, and 1.4-fold increase in number of sperm with highly damaged DNA. Such changes in pEGFP-loaded sperm clearly might negatively affect its ability to participate in fertilization and explain repeated failure of SMGT. High motility of sperm has been always an important indicator for successful fertilization in swine [27]. Selection of donor boars with high percentage of motile sperm is also one of the major criteria for successful SMGT [21]. It has been previously reported that a similar decrease in sperm motility was observed after DNA treatment in porcine [28] and bull spermatozoa [12,13] but no explanation for decreased motility was provided. Before incubation with pEGFP, sperm is repeatedly centrifuged and washed to remove seminal plasma inhibitors of DNA binding. Thus either centrifugation, removal of seminal plasma, or incubation with DNA might have caused the loss of motility. Our results clearly demonstrate that a decrease in the motility of DNA-treated sperm resulted from removal of seminal plasma during sperm washing. Seminal plasma contains many factors that maintain sperm motility, so it is not surprising that its removal decreased sperm motility [29,30]. Similarly, it has been suggested that mature spermatozoa contain nucleases induced by internalization of foreign DNA that might cause degradation of both sperm chromosomal DNA and added exogenous DNA [9–11]. Such DNA degradation might decrease possibility that sperm carrying exogenous DNA will participate in fertilization. For example, it has been previously demonstrated that human cervical mucus can act as a selective sieve preventing progress of spermatozoa with fragmented DNA and chromatin structural abnormalities [31,32]. Even if such sperm reaches the oocyte, it is unlikely to produce viable embryos due to poor implantation and abortion [33]. While a significant increase in sperm genomic DNA damage was detected by the Comet assay in our experiment, once more, it seems that the washing step was primarily responsible for this increase. It is well known that spermatozoa deficiency in nuclear DNA repair systems results in their increased susceptibility to oxidative stress and DNA fragmentation [34]. Seminal plasma is a major source of protective antioxidant activity for spermatozoa and its removal likely increased the production of reactive oxygen species by seminal leukocytes and spermatozoa resulting in increased DNA damage [35].

In conclusion, poor reproducibility of SMGT and evolutionary common sense does suggest presence of natural barriers against sperm carrying exogenous DNA. Despite significant amount of plasmid binding and internalization by porcine sperm similar to reported by Lavitrano et al. [21] we did not detect any major negative effects on sperm motility, viability or DNA damage. A significant decrease in motility and an increase in DNA damage seem to be associated with the washing step alone and do not explain why only spermatozoa not carrying exogenous DNA are able to participate in fertilization. Our results seemingly indicate the absence of specific defences against foreign DNA in spermatozoa, so it is likely such barriers exist at later stages of sperm transport or fertilization. The reproductive tract is designed as an obstacle course for spermatozoa with the number of selection steps at which damaged or fertilization-deficient spermatozoa are eliminated [36,37]. This enormous selection pressure decreases the number of participating spermatozoa from 10^9 to just a few actually reaching the oocyte. It seems likely that DNA-loaded sperm are selected against *in vivo*, and as a result either does not reach the egg, cannot properly participate in fertilization or produce nonviable embryos. Another indication that DNA-loaded sperm might be selected against *in vivo* is that, unlike SMGT, direct ICSI of DNA-loaded sperm into oocyte has been consistently successful in producing transgenic animals with high efficiency [38–40]. ICSI bypasses many normal selection steps of fertilization, such as sperm interaction with the reproductive tract and oocyte. Further experiments must be performed to understand the transportation of DNA carrying sperm in the reproductive tract *in vivo*, its ability to participate in the fertilization process and the fate of foreign DNA delivered to oocytes. Better understanding of such natural defences against foreign DNA invasion will allow finally solving long standing SMGT reproducibility problems and permitting wider use of this promising technology to generate transgenic animals for biomedical research and commercial production.

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