Research in Veterinary Science xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Factors affecting porcine sperm mediated gene transfer

Francisco Alberto García-Vázquez *, Salvador Ruiz, Luis Alberto Grullón, Aitor de Ondiz, Alfonso Gutiérrez-Adán [1], Joaquín Gadea

Departamento de Fisiología, Facultad de Veterinaria, Universidad de Murcia, 30100 Murcia, Spain

ARTICLE INFO

Article history:
Received 3 June 2009
Accepted 20 September 2010
Available online xxxx

Keywords:
Reproductive technology
SMGT
AI
Piglets
Transgenic

ABSTRACT

"Sperm mediated gene transfer" (SMGT) is based on the ability of sperm cells to bind exogenous DNA. The main objective of this study was to improve the production of transgenic pigs by SMGT. Taking into account that there is a lack of repeatability in studies of SMGT and that the mechanism of binding and internalization of exogenous DNA is a question that has not been solved, different factors involved in the production of transgenic animals by SMGT method were evaluated. Here we set out to: (1) evaluate the sperm capacity to bind exogenous DNA after DMSO treatment; (2) determine the location of the transgene–spermatozoa interaction; and (3) evaluate the efficiency of production of transgenic piglets by deep intrauterine artificial insemination (AI) with sperm incubated with DNA. The percentage of DNA binding was higher than 30% after 2 h of co-culture, but it was not affected by sperm treatment with DMSO (0.3% or 3%). The integrity of the sperm plasma membrane plays a critical role in DNA interaction, and altered plasma membranes facilitate interactions with exogenous DNA. DNA bound mainly to spermatozoa with reduced viability. DNA molecules were found to be mainly associated to the post-acrosomal region (61.9%). After deep intrauterine AI a total of 29 piglets were obtained, but none of them integrated the transgene. In conclusion, although it has been confirmed that DNA can associate with boar spermatozoa, the efficiency of producing transgenic pigs by AI was not confirmed by the present experiments, mainly due to a reduced DNA binding to functional spermatozoa.

1. Introduction

In recent years, transgenic pigs have become an important tool in biomedical research, including the production of bio-molecules in the mammary gland, the development of transgenic animals to improve productivity, in *xeno-transplantation* research and as models for human diseases (Petters and Sommer, 2000; Prather et al., 2003; Lunney, 2007; Gadea and García-Vázquez, 2010a; Aigner et al., 2010). DNA pronuclear microinjection has long been the most popular system to generate transgenic animals. However, besides being expensive, this technique is still inefficient when used to generate transgenic farm animals (0.5–4%) (Niemann and Kues, 2000; Wall, 2002; Nagashima et al., 2003; Gadea and García-Vázquez, 2010b; Parrington et al., in press).

Sperm mediated gene transfer (SMGT) technology is based on the ability of sperm cells to bind, internalize and transport exogenous DNA into an oocyte during fertilization (Lavitrano et al., 1989). The first report showing that exogenous DNA could be introduced into sperm was provided by Bracketts group in rabbits (Brackett et al., 1971). Since then, SMGT has been used more or less successfully in the production of transgenic embryos and animals in a large number of species (reviewed by Smith and Spadafora (2005)). Although transgenic animals have been obtained using SMGT, the efficiency of the technique is still low, mainly due to the spermatozoon's low uptake of exogenous DNA, thereby reducing the chances of fertilizing oocytes with transfected spermatozoa (Anzar and Buhr, 2006). In addition, inter- and intra-species success variability is still an unsolved problem associated with this technology. Several factors determine the success of SMGT including the donors of spermatozoa, incubation media, exogenous DNA size and type and the assisted reproductive technique used (Lavitrano et al., 2003; Smith and Spadafora, 2005).

To be successful in the production of transgenic animals by SMGT, it is critical that the spermatozoa should incorporate or attach the exogenous DNA into the head, but it is also necessary that the "transfected" spermatozoa keep their functionality to fertilize the oocyte.

It has been previously reported that the sperm plasma membrane plays a critical role in DNA interaction (Perry et al., 1999; Anzar and Buhr, 2006; Kurome et al., 2007), so membrane alteration facilitates direct DNA interaction with the sperm chromatin thus increasing the probability of integration. To improve DNA-sperm binding, some authors have developed specific methodologies by using DNA–liposome complexes (Lai et al., 2001), electroporation

* Corresponding author. Tel.: +34 868 888009; fax: +34 868 884147.
E-mail address: fagarcia@um.es (F.A. García-Vázquez).
[1] Departamento de Reproducción Animal, INIA, 28040 Madrid, Spain.

0034-5288/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.rvsc.2010.09.015

(Horan et al., 1992) or monoclonal antibodies to link foreign DNA to sperm (Chang et al., 2002). On the other hand, some groups have used dimethyl-sulfoxide (DMSO) which helps to transfer foreign DNA to spermatozoa; an approach which allowed efficient production of transgenic mice and rabbits (Kuznetsov et al., 2000; Shen et al., 2006; Li et al., 2006). Sperm treatment with DMSO has not been used before in pig SMGT.

The production of transgenic piglets by SMGT is possible by artificial insemination (AI) (Lavitrano et al., 2002, 2003) or embryo transfer of transgenic embryos produced by intracytoplasmic sperm injection (ICSI) (Lai et al., 2001; Kurome et al., 2007; García-Vázquez et al., 2009; García-Vázquez et al., 2010). Classical AI has been used in most SMGT pig studies (Lavitrano et al., 1997, 1999, 2002, 2003; Chang et al., 2002) to place the DNA-carrying sperm in the female reproductive system to fertilize the oocytes. However, recently new alternatives have been developed using a lower number of spermatozoa, such as laparoscopic insemination (Fantinati et al., 2005) or deep intrauterine AI (Wu et al., 2008). The use of deep intrauterine AI allows the use of a reduced number of spermatozoa with subsequent reproductive performances at the same level as achieved with classical pig artificial insemination (Martinez et al., 2001).

The main objective of this study was to improve the production of transgenic pigs using deep AI by SMGT and different factors in relation with SMGT technique were evaluated. Firstly, we tried to improve the DNA-binding ability of spermatozoa by DMSO treatment and DNA-binding ability and the viability of sperm were evaluated by flow cytometry after co-incubation with exogenous DNA. Secondly, to confirm that the binding of exogenous DNA to the sperm is in a localization that allows it to enter the oocyte during fertilization, we evaluated by inmunocytochemistry the location of exogenous DNA-spermatozoa interaction by labelling DNA with digoxigenin (DIG) and later revealing using anti-DIG conjugated to Horsedish Peroxidase (HRP). Finally, we explore the production of transgenic pig by SMGT with a reduce number of spermatozoa by deep intrauterine AI, allowing spermatozoa to be deposited close to the site of fertilization, thus bypassing any possible detrimental effect of the DNA on spermatozoa transport.

2. Material and methods

2.1. Media and chemicals

All chemicals and reagents were purchased from Sigma–Aldrich Química S.A. (Madrid, Spain). Swine Fertilization Medium (SFM) (Lavitrano et al., 2003) was used for processing the spermatozoa (1L contains 11.25 g glucose, 10 g sodium citrate $2H_20$, 4.7 g EDTA-$2H_2O$, 3.25 g citric acid-H_2O, 6.5 g Trizma) supplemented with 6 mg/ml BSA (SFM/BSA).

2.2. Transgene construction and labelling

The enhanced green fluorescent protein (EGFP) plasmid construction (5.4 kb, pEGFP-N1, Clontech Laboratories, Inc., Palo Alto, CA, USA) used in our experiments, contained the human CMV immediate early promoter and the enhanced GFP gene. This construction was linearized with Afl II prior to use. The transgene was purified using an Elu-Quit DNA Purification Kit (Schleicher & Schuell, Dassel, Germany) following the manufacturer's instructions. DNA was resuspended in TE (10 mM Tris, 0.1 mM EDTA, pH 8). Linear plasmid was labelled with fluorescein-12-dUTP (Roche®, Germany). DNA labelling was carried out by means of random primer. Briefly, mould DNA (linearized plasmid) was denatured by application of 95 °C for 5 min; nucleotides (1 mM of dATP, dCTP, and dCTP; 0.65 mM of dTTP, and 0.35 mM of the marked nucleotide) were added later as well as 5× buffer and 1 U/μl Klenow. Subsequently, the mixture was incubated for an hour at 37 °C and reaction was stopped by adding 2 μl of 0.2 M EDTA (pH 8). Marked DNA was precipitated out with cold ethanol and was finally resuspended in TE microinjection buffer. Verification of plasmid marking was determined in an agarose gel with ethidium bromide. The incorporation of the labelled nucleotide to synthesized DNA diminishes its electrophoretic mobility in relation to non-marked DNA (Gutiérrez-Adán and Pintado, 2000).

2.3. Semen collection and sperm preparation for DNA uptake

Fresh semen was routinely collected from seven mature fertile boars ("Lo Navarro" S.A. Murcia, Spain) using the manual method and a dummy. The sperm-rich fraction was collected in a pre-warmed thermos flask, and the gel fraction was held on a gauze tissue covering the thermos opening. A selection of the boars was done according to motility ($\geqslant$70%), progressive motility ($\geqslant$3; arbitrary scale 0–5) and abnormal morphology ($\leqslant$25%) (Gadea et al., 1998).

Semen was prepared according to the previously described methods (Lavitrano et al., 2003). Briefly, after collection of the sperm-rich fraction of ejaculate, the semen was diluted 1:1 in SFM medium without BSA, and transported at 37 °C. Once in the lab, it was diluted again in SFM medium (37 °C) in a 1:10 (v/v) proportion, and centrifuged at 800g for 10 min at 25 °C. Supernatant was removed by aspiration and the pellet resuspended in SFM/BSA at 25 °C and centrifuged again at 800g for 10 min at 25 °C. Supernatant was discarded and the pellet was resuspended in 1 ml of SFM/BSA at 25 °C. Motility was evaluated once spermatozoa processing had finished (to be used, it should reach a motility over 65% and a progressive motility not lower than 2.5) (Gadea et al., 1998) and final concentration was calculated using a calibrated photometer (Spermacue, Minitüb, Germany). Finally, concentration of sperm suspension was adjusted to 10^8 sperm cells/ml. Sperm cell suspension was supplemented with DMSO diluted in SFM medium at a concentration of 0.3 and 3% (v/v). The mixture was kept at 16 °C for 15 min. Then, samples (control, DMSO 0.3% and 3%) were simultaneously incubated with linearized EGFP transgene (10^8 cells/ml and 5 μg DNA/ml) labelled with fluorescein (FITC-DNA) and with propidium iodide (PI) (Gamer et al., 1994) (final concentration 5 μg/ml) in a final volume of 25 μl of SFM/BSA medium at 16 °C (García-Vázquez et al., 2009).

2.4. Analysis of seminal parameters by flow cytometry

Flow cytometric analyses were performed on a Coulter Epics XL cytometer (Beckman Coulter Inc., Miami, Florida, USA). A 15 mW argon ion laser operating at 488 nm excited the fluorophores. Data from 10,000 events per sample were collected in list mode, and four measures per sample were recorded. Flow cytometric data were analyzed using the program Expo32ADC (Beckman Coulter Inc., Miami, Florida, USA) with a gate in forward and side scatter to exclude eventual remaining debris and aggregates from the analysis.

DNA binding capacities and cellular viability were simultaneously measured by flow cytometry and the use of two fluorochromes. Spermatozoa were simultaneously incubated with transgene labelled with fluorescein (FITC-DNA) and with PI for evaluating DNA binding capacities and cellular viability during the incubation time (0, 15, 30, 60, 90 and 120 min) at 16 °C (García-Vázquez et al., 2009). Fluorescence was measured using a FL-1 sensor, at 525 nm band-pass filter to detect FITC-DNA, and a FL-2 sensor at 575 nm band-pass filter to detect PI. Cells were classified into four categories: (1) Live spermatozoa without bound DNA% (no sign of fluorescence), (2) Live spermatozoa with bound

Please cite this article in press as: García-Vázquez, F.A., et al. Factors affecting porcine sperm mediated gene transfer. Res. Vet. Sci. (2010), doi:10.1016/j.rvsc.2010.09.015

DNA% (only green fluorescence), (3) Dead spermatozoa with bound DNA% (red and green fluorescence) and (4) Dead spermatozoa without bound DNA% (red fluorescence).

This methodology has been validated with fluorescence microscopy observation and the use of a Multispectral Imaging Flow Cytometry (ImageStream®, Amnis, Seattle, WA, USA), which is a combination of quantitative image analysis and flow cytometry (Gadea et al., unpublished data).

2.5. Localization of DNA binding

The localization of the DNA bound to the sperm was detected by inmunocytochemistry techniques. DNA labelled by digoxigenin (DIG) was detected by the use of an antibody labelled with the enzyme HRP (antidigoxigenin-POD, Roche, Germany) and revealed with hydrogen peroxide (H2O2). At first, spermatozoa were incubated with plasmid labelled with DIG for 24 h. Then, spermatozoa were centrifuged at 300*g* 5 min for washing and elimination of non-bound plasmid. For blockade of cellular endogenous peroxidase activity, extensions were washed for 30 min in H_2O_2 solution. Prior to incubation with antibody, extensions were immersed in PBS supplemented with 1% BSA for 15 min to avoid nonspecific antibody binding. Then, samples were incubated in a humidity cabin at room temperature with antibody anti-DIG-HRP previously diluted 1:100 (v/v) in PBS-1% BSA for 60 min. Finally, revealing solution (Diaminobenzidine 90 μl, 100 ml of PBS, 100 μl of H_2O_2) was used for 30 min. Samples were air dried and mounted in DPX mounting medium for sperm evaluation by optical microscope observation under immersion objective (100×). (Leica®, DMLS, Wetzlar, Germany).

The localization of DNA binding was evaluated in 400 cells per sample. Two observers independently examined the samples. Spermatozoa were classified according to DNA localization in the spermatozoa: (1) DNA bound to acrosomal region, (2) DNA bound to post-acrosomal region and (3) DNA bound to acrosomal and post-acrosomal regions.

2.6. Oestrus cycle synchronization in sows

For synchronizing oestrus cycle in the five multiparous sows, prostaglandins (Dinolityc, Pfizer, Madrid, Spain) were intramuscularly injected 15 days after last cycle, in order to produce lyses of possible corpora lutea. For induction of oestrus, 48 h after PGF2α injection, 1250 IU of equine chorionic gonadotropin (eCG) (Folligon, Intervet, Barcelona, Spain) were intramuscularly administered, followed 72 h after by 750 IU of human chorionic gonadotropin (hCG) (Veterin Corion®, Divisa Farmavic S.A., Barcelona, Spain). Oestrus detection was carried out every 6 h, beginning 32 h after the hCG injection, by determination of immobility reflex, aspect of vulva, and by ultrasound evaluation following follicular growth (anechoic areas in ovary with 10–14 mm size) and moment of ovulation.

2.7. Deep intrauterine insemination

Spermatozoa were incubated with linearized EGFP transgene (10^8 cells and 5 μg DNA) in a total volume of 10 ml of SFM/BSA medium. This suspension was incubated for 2 h at 16 °C. Sample was heated at 37 °C for 10 min before artificial insemination. Two inseminations (separated by 12 h) with semen from the same ejaculate preserved at 16 °C were carried out for each sow.

Insemination was carried out 36–40 h after hCG administration before ovulation in five multiparous sows. Two catheters were used; a conventional cervical insemination catheter and a flexible catheter 1.80 m long, external diameter 4 mm and internal diameter 1.8 mm (Fireflex, Magapor S.L., Zaragoza, Spain).

2.8. Pregnancy evaluation

Early pregnancy diagnosis was developed with a 100 FALCO-VET scan (Esaote España S.A., Barcelona, Spain) equipped with a 6–8 MHz endorectal transducer and a 5–7.5 MHz microconvex transducer for trans-abdominal application.

2.9. Analysis of DNA integration and EGFP expression

The piglets born were evaluated for transgene integration (PCR) and EGFP expression. Direct evidence of gene integration was carried out by the extraction of genomic DNA from tissues obtained from piglets for posterior analysis by PCR. Once DNA was purified, its concentration was quantified in a spectrophotometer. Fifty-one hundred nanogram of purified DNA was used for the PCR of EGFP. Primers used were GFP-1 (5′-TGA ACC CGA TCG AGC TGA AGG G-3′) and GFP-2 (5′-TCC AGC AGG ACC ATG TGA TCG C-3′), which specifically amplify a section of 340 base pairs of the DNA of EGFP. Amplification was carried out in a total volume of 25 μl, using 1U of Taq polymerase (Promega Biotech Ibérica, Madrid, Spain), 2.5 μl of 10× buffer, 100 μM dNTP, 0.10 M of each primer and 1.5 mM $MgCl_2$. PCR cycles consisted of a first denaturing cycle at 92 °C (2 min), followed by 30 cycles at 92 °C (30 s), 59 °C (30 s), and 72 °C (30 s); and a final extension cycle of 5 min. The PCR product was run on an agarose gel at 1.5% in TBE buffer and subsequently stained by ethidium bromide to evaluate the presence of the gene product by visualization under UV light. Positive (DNA of EGFP-expressing transgenic mice) and negative (DNA of non-transgenic pig) controls were used in each PCR. Furthermore, each sample was amplified with primers for ZFX/ZFY to confirm its quality (Gutiérrez-Adán et al., 1996).

EGFP expression was evaluated by direct by direct observation in different piglet tissues (blood, tail and ear skin, liver, kidney, lung, heart, spleen and muscle) through blue light with a maximum excitation of 488 nm by a UV emitting lamp.

2.10. Statistical analysis

Data are presented as means ± SEM and analyzed by ANOVA considering the specific sperm treatment as the main variable. When ANOVA revealed a significant effect, values were compared by the least significant difference pairwise multiple comparison post hoc test (Tukey). Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Experiment 1. Effect of the DMSO treatment on DNA-spermatozoa interaction

Seven replicates were conducted for this experiment. Percentage of total DNA-bound spermatozoa, dead and live DNA-bound spermatozoa, and viability were recorded and analyzed. The capacity to bind to the exogenous DNA and sperm viability were not affected by the treatment with DMSO (Table 1). Exogenous DNA bound mainly to spermatozoa with reduced viability in all the groups of spermatozoa evaluated (Control: 32.42 ± 0.75%; DMSO 0.3%: 31.35 ± 0.95%; DMSO 3%: 30.11 ± 0.97%, Table 1). Consequently, only a low percentage of live spermatozoa was bound to DNA (mean value lower than 2% in any treatment). A higher percentage of live cells bound to DNA was detected when 3% DMSO-treated spermatozoa were used than in the other treatments ($p < 0.01$).

Incubation time in each treatment did not significantly affect sperm viability, although viability tended to decrease during each

Please cite this article in press as: García-Vázquez, F.A., et al. Factors affecting porcine sperm mediated gene transfer. Res. Vet. Sci. (2010), doi:10.1016/j.rvsc.2010.09.015

Table 1
Mean values (mean ± SEM) for sperm viability and DNA-binding to the spermatozoa during incubation (120 min) measured by flow cytometry. Control and DMSO (0.3% and 3%) treated spermatozoa.

Spermatozoa treatment	% Dead spermatozoa	% Bound spermatozoa		
		Total	Dead	Live
Control	33.04 ± 0.74	33.80 ± 0.76	32.42 ± 0.75	1.39 ± 0.04[a]
DMSO 0.3%	31.95 ± 0.93	32.97 ± 0.94	31.35 ± 0.95	1.62 ± 0.06[b]
DMSO 3%	31.34 ± 0.98	31.95 ± 0.97	30.11 ± 0.97	1.84 ± 0.07[c]

[a,b,c] in the same column indicate significant differences ($p < 0.01$).

time evaluated ($p = 0.13$) (Fig 1a). Similarly, the percentage of total DNA-bound spermatozoa (Fig 1b) and viable spermatozoa bound to foreign DNA (Fig 1c) was not affected by the time of incubation in the three experimental groups.

Considering the three treatments data together in each incubation time (independently of the treatment used), the percentage of viable spermatozoa bound to foreign DNA significantly decreased with the time of incubation ($p < 0.01$, Fig 1d).

In all the parameters studied, the three treatments showed a similar pattern during the time of study, so the *p*-values for the interaction of treatment and time in the ANOVA model is higher than 0.85.

Finally, we confirmed that DNA-binding capacity was inversely related to sperm viability (by study of the linear regression (Fig 2, $r = 0.99$, $p < 0.01$).

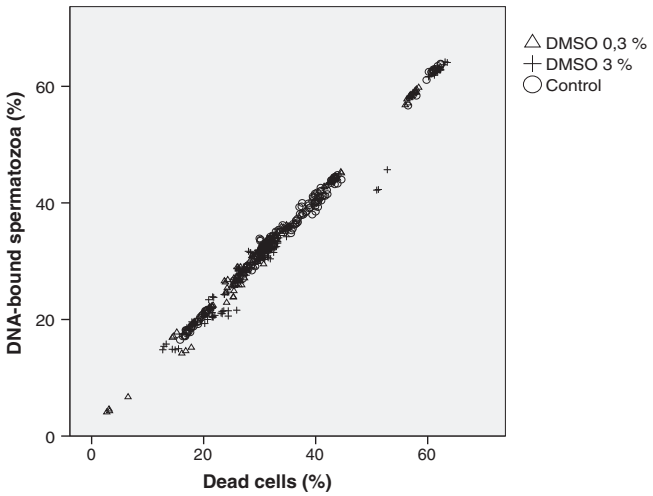

Fig. 2. Regression plots for dead cells (%) and DNA-bound spermatozoa (%) in boar spermatozoa throughout 120 min of incubation evaluated by flow cytometry after different sperm treatments. Pearson correlation coefficient (r) = 0.99, $p < 0.01$.

3.2. Experiment 2. Localization of the interaction between DNA and spermatozoa

A total of three replicates were carried out for this experiment. Evaluation of binding of digoxigenin-11-dUTP-labelled DNA

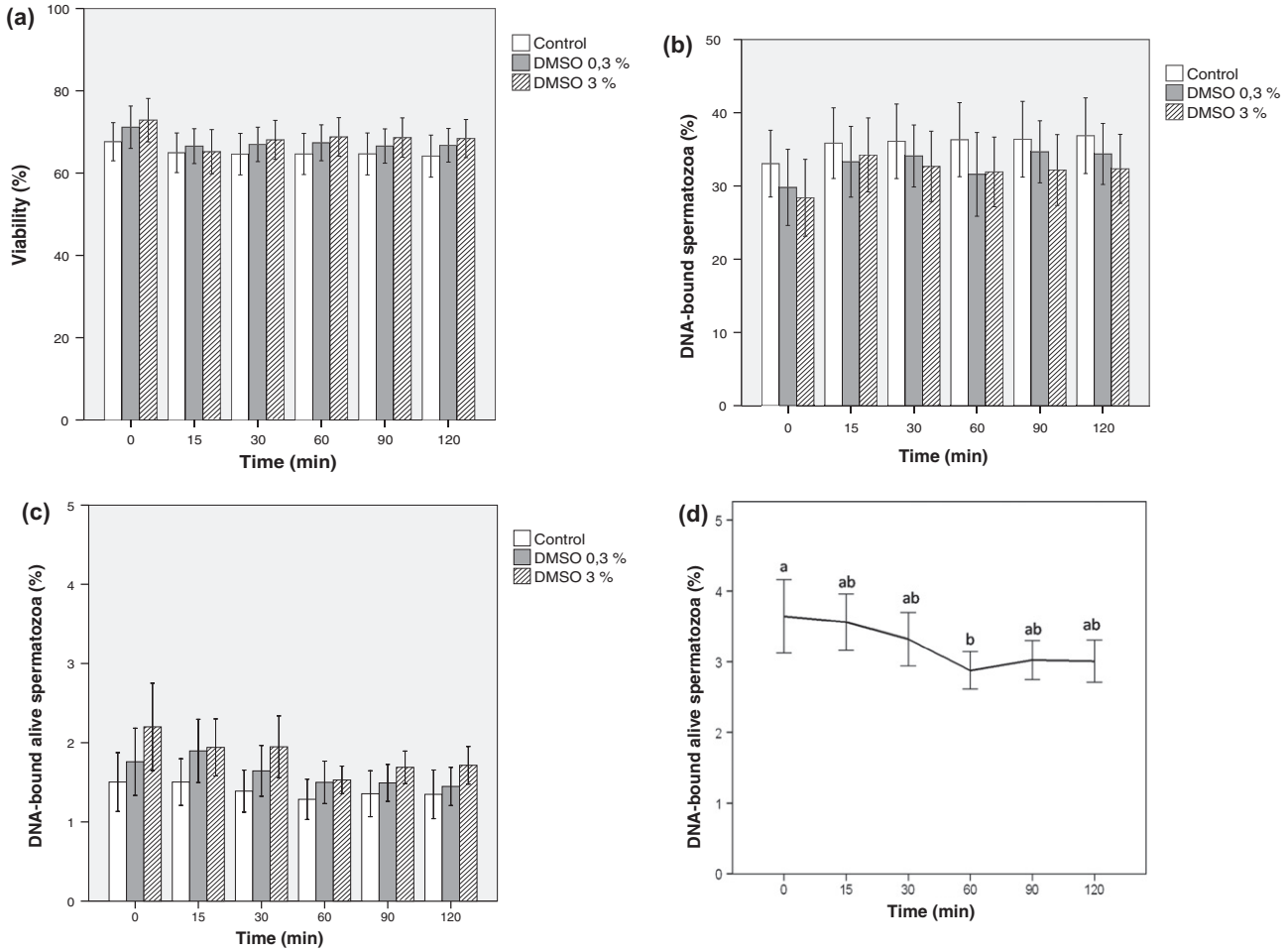

Fig. 1. Results of DNA binding kinetic (mean ± SEM) between spermatozoa and exogenous DNA throughout 120 min of incubation evaluated by flow cytometry. Control and DMSO (0.3% and 3%) treated spermatozoa. (a) Viability measured by propidium iodide staining. (b) Percentage of DNA-bound spermatozoa. (c) Percentage of DNA-bound alive spermatozoa ($p < 0.01$). (d) Percentage of DNA-bound alive spermatozoa independently of the treatment used ($p < 0.01$).

Please cite this article in press as: García-Vázquez, F.A., et al. Factors affecting porcine sperm mediated gene transfer. Res. Vet. Sci. (2010), doi:10.1016/j.rvsc.2010.09.015

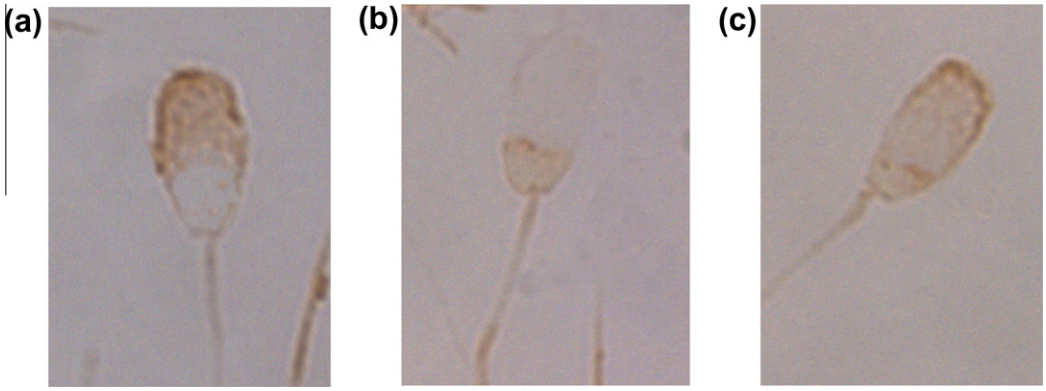

Fig. 3. Location of DNA binding to intact spermatozoa. Evaluation by digoxigenin-HRP staining technique. The binding was classified in three groups: (a) acrosomal region, (b) post-acrosomal and (c) whole head.

molecules to spermatozoa showed that 51.08 ± 6.79% of the cells were bound to DNA after 24 h of co-incubation. The DNA molecules were mainly located at the head, although with different binding patterns. Localization in the post-acrosomal region was the most frequently observed (61.93 ± 5.23%), followed by 27.03 ± 5.31% of sperm showing the transgene in the whole head (acrosomal and post-acrosomal region) and only 11.03 ± 3.06% of the sperm localized to the binding site in the acrosomal region (Fig. 3). No significant differences in the percentages were found between the two observers ($p = 0.77$).

3.3. Experiment 3. Use of deep intrauterine artificial insemination with spermatozoa incubated with exogenous DNA

Four of the five inseminated sows were diagnosed as pregnant by ultrasound evaluation at 25–28 days post-insemination (fecundation rate 80%). After 113–117 pregnancy days, sows farrowed a total of 29 piglets (14 females and 15 males). Expression of EGFP in the newborn piglets was not observed by direct epifluorescent examination under UV light. Samples from blood, tail and ear tissue from 2 days old piglets were evaluated by PCR amplification with negative results for EGFP transgene integration. At 30 days after birth, the animals were euthanized and different tissues (liver, kidney, lung, heart, spleen, muscle) were analyzed by PCR and fluorescence microscopy with negative results.

4. Discussion

SMGT has proved to be a viable method for generating transgenic embryos and animals in a considerable number of species (Smith and Spadafora, 2005). However issues still remain to be addressed regarding its repeatability, efficiency in different species, and our understanding of the underlying molecular basis of its action. Such issues will be important to resolve if this approach is to be employed for generation of transgenic animals on a routine basis (Parrington et al., in press).

Several factors could determine the success of results using this technique including boar donors, construct used, incubation media, incubation time (Lavitrano et al., 2003) and sperm treatments (Perry et al., 1999; Kang et al., 2008; García-Vázquez et al., 2009). Herein, we evaluated several factors related to the efficiency of porcine SMGT: the effect of different sperm treatments on the DNA-binding process, the localization of the bound DNA and the efficiency of deep intrauterine AI for producing transgenic pigs.

To be successful in the production of transgenic animals by SMGT, one critical step is for the spermatozoa to incorporate or attach the exogenous DNA into the head, but it is also necessary that the "transfected" spermatozoa keep their functionality to fertilize the oocyte. Several groups have developed treatments to improve DNA binding to the spermatozoa such as electroporation (Tsai et al., 1997), liposome/DNA complexes (Lai et al., 2001; Celebi et al., 2003) or monoclonal antibodies to link foreign DNA to sperm (Chang et al., 2002). Our first objective in this study was to increase DNA-binding capacity by treatment with DMSO which has permeabilizing effects on cell membranes (Liu et al., 1999) and has been successfully used for in vitro fertilization in rabbit and mouse (Kuznetsov et al., 2000; Li et al., 2006; Shen et al., 2006). Under our experimental conditions, DMSO treatment did not increase boar sperm DNA-binding capacity and did not negatively affect viability (Table 1 and Fig 1). A significant but only very limited DMSO effect on the percentage of DNA-bound live cells, which increased in a dose–effect way, was detected. However, this statistical increase, which is based on a precise cytometry measurement, has in our opinion a very low biological value. These results are in accordance with other reports where a decrease in sperm fertility when DMSO treatment was used to improve exogenous DNA-spermatozoa interaction was observed (Liu et al., 1999). However, treatment with DMSO has been successfully used for in vitro fertilization in rabbit and mouse (Kuznetsov et al., 2000; Li et al., 2006; Shen et al., 2006). These experiments using mice and rabbit spermatozoa included a cooling 10–20 min period at 4 °C for the spermatozoa, in some cases followed by heat shock (42 °C for 20 min) (Kuznetsov et al., 2000) and this DMSO treatment significantly increased the percentage of sperm bound to DNA. However, in our experimental conditions the cold shock period is not applicable for boar spermatozoa because at this temperature (4 °C) the boar spermatozoa suffer a severe cold shock (Pursel et al., 1973) that damages their functionality. However, the factors leading to this high success rate were not known and further studies must be carried out to evaluate different DMSO concentrations and the relation between DNA concentration and proportion circular/linear DNA as previously have been reported in rabbit sperm (Li et al., 2006).

The determination of DNA-binding to the sperm cells is a key point in this technique. Radiolabeled measurements, fluorescent microscopy and inmunohistochemistry techniques have been used to measure DNA binding. Flow cytometry looks like a very valuable tool for evaluating DNA-binding and viability (García-Vázquez et al., 2009; Cánovas et al., 2010), as it is possible to evaluate the kinetics of the binding process. It allows us to know the number of live spermatozoa carrying exogenous DNA which could fertilize in conventional IVF or AI systems. The percentage of DNA binding detected after 2 h of incubation is in the range of 30–40%, but less than 2% of cells are viable and with DNA bound, significantly reduced compared to the percentage showed by radiolabeled

Please cite this article in press as: García-Vázquez, F.A., et al. Factors affecting porcine sperm mediated gene transfer. Res. Vet. Sci. (2010), doi:10.1016/j.rvsc.2010.09.015

measurements (Camaioni et al., 1992; Lavitrano et al., 2002,2003) and in the same range as others using a non-radioactive method (Horan et al., 1992; Gandolfi et al., 1996; De Cecco et al., 2010). Although these methods localize labelled DNA in situ, new studies must be developed with real-time PCR to quantify the amount of exogenous DNA associated with spermatozoa (Feitosa et al., 2010).

In our study, most of the exogenous DNA is bound to dead cells or to cells with severe membrane alterations (PI stained) (Table 1, Fig 2). Some reports have proposed that a direct relationship exists between the binding of exogenous DNA to spermatozoa and the integrity of the plasma membrane (Perry et al., 1999; Anzar and Buhr, 2006) or capacitation status (Wang et al., 2003). These results confirmed previous studies in boar (García-Vázquez et al., 2009) and bull spermatozoa (Cánovas et al., 2010). Although the cause of this process is not known, several hypotheses could be explored. It has been previously reported that there is a window of opportunity in which the exogenous DNA binds to spermatozoa, which coincides with the early stage of capacitation. DNA is ideally added within 30 min after washing the sperm and not later than 60 min (Lavitrano et al., 2003). Firstly, it is possible that under our experimental conditions, sperm capacitation occurs very quickly, so DNA binding can occur but the acrosome reaction takes place too early and thus the spermatozoa die. In this case, changes in the sperm membrane functionality might modulate the time of DNA binding. Secondly, 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 (Maione et al., 1998; Spadafora, 1998; Szczygiel et al., 2003) and it is possible that DNA-binding induces the alteration and death of the cell by endonuclease activation in an apoptotic-like process (Maione et al., 1997; Cánovas et al., 2010). Finally, the medium used in this experiment (SFM) contained EDTA and was free of bivalent ions such as calcium allowing proper uptake without activation of the nucleases that are present in spermatozoa and that are known to be a cause of DNA rearrangements (Lavitrano et al., 2003). A very early event in sperm capacitation is the activation of sperm motility. The vigorous movement of the flagellum starts immediately after sperm are released from the epididymis and come into contact with high bicarbonate and calcium concentrations present in the seminal fluid (Visconti, 2009), but under our conditions the seminal plasma was discarded. In contrast to the fast activation of sperm motility, other capacitation-associated processes require longer incubation periods. These slower processes can be accomplished in vitro by using sperm incubated in defined media. In all cases, in vitro capacitation media contain a protein source, usually BSA, and an assortment of ions including bicarbonate and calcium (Visconti, 2009). The media composed of BSA but without bicarbonate and calcium (such as SFM), probably only modulate the elimination of sperm membrane cholesterol, but is not a total capacitation media. So, further investigations concerning modulation of sperm capacitation sperm as associated with SMGT must be carried out.

In our second experiment, the localization of DNA binding was evaluated. DNA molecules showed a preferential affinity for the post-acrosomal region of the sperm head confirming the information reported for different species, including pig (Brackett et al., 1971; Atkinson et al., 1991; Camaioni et al., 1992; Lavitrano et al., 1992; Gandolfi et al., 1996; Kuznetsov et al., 2000). In accordance with another report (Camaioni et al., 1992) in a minority of labelled cells (11.03%) bound DNA was present in the acrosomal region. The presence of regional domains, with different composition and functions is a well-known characteristic of spermatozoa (Holt, 1984). Plasmatic sperm membrane in the post-acrosomal and mid piece regions are implicated in gamete fusion in the final process of fertilization (Bedford et al., 1979; Shalgi and Phillips, 1980; Yanagimachi, 1981; Francolini et al., 1993). It is known that at fertilization the apical sperm membrane and the equatorial segment of the acrosome are not incorporated into the egg plasma membrane, but are internalized by the egg as a composite vesicle, made up of spermatozoa and egg membranes (revised by Camaioni et al. (1992)). The fate of such vesicles is unknown, but they might act as DNA carriers into the egg, not only of DNA bound to the sperm membrane, but also by entrapping DNA in solution in the culture medium (Camaioni et al., 1992).

Finally, we explored the use of deep intrauterine AI as a simple and low-cost methodology to produce transgenic pigs. The interaction, internalization and integration of exogenous DNA within sperm cells is not merely the consequence of a passive and uncontrolled process, it is regulated by factor-specific mechanisms that metabolically activate the cells (Smith and Spadafora, 2005). Thus, SMGT might involve energy consumption (Feitosa et al., 2009). The use of deep intrauterine AI allows the use of a reduced number of spermatozoa and for sperm to be deposited close to the site of fertilization to minimize the possibility of competition between DNA bound sperm and non-bound sperm. In our study, the fertility rate was 80% and the litter size was 7.25 using only 10^8 spermatozoa per animal, 30-fold times less than classical insemination. However, none of the different tissues from 29 piglets analyzed by PCR integrated the foreign gene.

Lavitrano et al. (1997, 2002, 2003) reported and confirmed high efficiency of transgenesis after AI. Having confirmed by flow cytometry that the DNA binds to sperm we can hypothesize the causes of the absence of transgenic piglets after AI. Under our experimental conditions the percentage of sperm DNA binding is close to 30–40% and only less than 2% of cells are viable and with DNA bound; so it is logical to expect that there was no evidence of transgenesis in any of the piglets born. Our results are consistent with those obtained by Gandolfi et al. (1996), who reported absence of transgenesis in 126 piglets from 35 females inseminated. Similarly, in previous report of SMGT (Kang et al., 2008), 105 fertilized embryos ($\geqslant$2 cells) were produced by 18 sows inseminated with pEGFP-treated sperm from three different boars and neither GFP expression nor presence of EGFP plasmid by PCR was detected in embryos tested (data not shown).

Another likely possible cause is that the "transgenic spermatozoa" are less competitive compared to "unbound" cells in reaching the oocytes or in penetrating the egg coats, particularly when fertilization takes place in vivo, as previously was suggested by Gandolfi et al. (1996). In fact, Kang et al. (2008) demonstrated that the DNA-treated sperm show a significant decrease in motility and progressive motility, and there is an increase in the number of sperm with highly damaged DNA. Such changes in transgene-loaded sperm clearly might negatively affect the ability to participate in fertilization and could explain repeated failure of SMGT (Kang et al., 2008). DNA degradation might decrease the possibility that sperm carrying exogenous DNA will participate in fertilization and 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 (Ellington et al., 1990; Bianchi et al., 2004). In addition, it has been reported that a similar decrease in sperm motility and plasma membrane integrity was observed after DNA treatment in bull spermatozoa (Schellander et al., 1995; Anzar and Buhr, 2006; Cánovas et al., 2010), but no explanation for decreased motility was provided.

An alternative to producing transgenic pigs by SMGT is the use of ICSI in combination with this method. When ICSI is applied many normal selection steps of fertilization are bypassed, such as sperm interaction with the reproductive tract and oocyte, and sperm functionality is not necessary. Transgenic animals and embryos have been produced using ICSI-SMGT in several species such as pig (Lai et al., 2001; Yong et al., 2006; García-Vázquez et al.,

Please cite this article in press as: García-Vázquez, F.A., et al. Factors affecting porcine sperm mediated gene transfer. Res. Vet. Sci. (2010), doi:10.1016/j.rvsc.2010.09.015

2010), mouse (Perry et al., 1999), rhesus monkey (Chan et al., 2000) and horse (Pereyra-Bonnet et al., 2008). Another alternative is the use of sorter cytometry, to allow the separation and collection of sperm subpopulations. For example in our case, the DNA-bound live spermatozoa subpopulation could be collected and used to fertilize by surgical AI using a minimal number of sperm.

In conclusion, this study confirmed that under our experimental conditions porcine sperm could bind plasmid DNA, with binding localized mainly in the post-acrosomal area of the spermatozoa head. Under our experimental conditions, the production of transgenic pigs by deep AI is limited because the percentage of spermatozoa carrying exogenous DNA and with plenty of functionality is limited. An extensive study of the factors implicated in the process must be carried out to improve the performance of the technique.

Acknowledgments

The authors would like to thank JA. Carvajal and S. Rodriguez for their generous technical assistance. This work was supported by the Regional Government of Murcia (BIOCARM 10BIO2005/01-6463), Fundación Séneca (08752/PI/08) and Spanish Ministry of Science and Innovation (AGL-2006-03495 and AGL-2009-12512-C02-01). We also thank Ruth Tunn (University of Oxford) for help with the language in the manuscript.

References

Aigner, B., Renner, S., Kessler, B., Klymiuk, N., Kurome, M., Wünsch, A., Wolf, E., 2010. Transgenic pigs as models for translational biomedical research. Journal of Molecular Medicine 88, 653–664.

Anzar, M., Buhr, M.M., 2006. Spontaneous uptake of exogenous DNA by bull spermatozoa. Theriogenology 65, 683–690.

Atkinson, P.W., Hines, E.R., Beaton, S., Matthaei, K.I., Reed, K.C., Bradley, M.P., 1991. Association of exogenous DNA with cattle and insect spermatozoa *in vitro*. Molecular, Reproduction and Development 29, 1–5.

Bedford, J.M., Moore, H.D., Franklin, L.E., 1979. Significance of the equatorial segment of the acrosome of the spermatozoon in eutherian mammals. Experimental Cell Research 119, 119–126.

Bianchi, P.G., De Agostini, A., Fournier, J., Guidetti, C., Tarozzi, N., Bizzaro, D., Manicardi, G.C., 2004. Human cervical mucus can act in vitro as a selective barrier against spermatozoa carrying fragmented DNA and chromatin structural abnormalities. Journal of Assisted Reproduction and Genetic 21, 97–102.

Brackett, B.G., Boranska, W., Sawicki, W., Koprowiski, H., 1971. Uptake of heterologus genome by mammalian spermatozoa and its transfer to ova through fertilization. Proceedings of the National Academy of Sciences of the United States of America 68, 353–357.

Chan, A.W., Luetjens, C.M., Dominko, T., Ramalho-Santos, J., Simerly, C.R., Hewitson, L., Schatten, G., 2000. Foreign DNA transmission by ICSI: injection of spermatozoa bound with exogenous DNA results in embryonic GFP expression and live rhesus monkey births. Molecular Human Reproduction 6, 26–33.

Camaioni, A., Russo, M.A., Odorisio, T., Gandolfi, F., Fazio, V.M., Siracusa, G., 1992. Uptake of exogenous DNA by mammalian spermatozoa specific localization of DNA on sperm heads. Journals of Reproduction and Fertility 96, 203–212.

Cánovas, S., Gutiérrez-Adán, A., Gadea, J., 2010. Effect of exogenous DNA bovine sperm functionality using sperm mediated gene transfer (SMGT) technique. Molecular, Reproduction and Development 77, 687–698.

Celebi, C., Guillaudeux, T., Auvray, P., Vallet-Erdtmann, V., Jegou, B., 2003. The making of "transgenic spermatozoa". Biology of Reproduction 68, 1477–1483.

Chang, K., Qian, J., Jiang, M., Liu, Y.H., Wu, M.C., Chen, C.D., Lai, C.K., Lo, H.L., Hsiao, C.T., Brown, L., Bolen Jr., J., Huang, H.I., Ho, P.Y., Shih, P.Y., Yao, C.W., Lin, W.J., Chen, C.H., Wu, F.Y., Lin, Y.J., Xu, J., Wang, K., 2002. Effective generation of transgenic pigs and mice by linker based sperm-mediated gene transfer. BMC Biotechnology 2, 5.

De Cecco, M., Spinaci, M., Zannoni, A., Bernardini, C., Seren, E., Forni, M., Bacci, M.L., 2010. Coupling sperm mediated gene transfer and sperm sorting techniques: a new perspective for swine transgenesis. Theriogenology 74, 856–862.

Ellington, J.E., Broemeling, L.D., Broder, S.J., Jones, A.E., Choker, D.A., Wright, R.W., 1990. Comparison of fresh and cryopreserved human sperm attachment to bovine oviduct (uterine tube) epithelial cells in vitro. Journal of Andrology 20, 492–499.

Fantinati, P., Zannoni, A., Bernardini, C., Webster, N., Lavitrano, M., Forni, M., Seren, E., Bacci, M.L., 2005. Laparoscopic insemination technique with low numbers of spermatozoa in superovulated prepuberal gilts for biotechnological application. Theriogenology 63, 806–817.

Feitosa, W.B., Milazzotto, M.P., Simões, R., Rovegno, M., Nicacio, A.C., Nascimento, A.B., Gonçalves, J.S., Visintin, J.A., Assumpção, M.E., 2009. Bovine sperm cells viability during incubation with or without exogenous DNA. Zygote 17, 315–320.

Feitosa, W.B., Mendes, C.M., Milazzotto, M.P., Rocha, A.M., Martins, L.F., Simões, R., Paula-Lopes, F.F., Visintin, J.A., Assumpção, M.E., 2010. Exogenous DNA uptake by bovine spermatozoa does not induce DNA fragmentation. Theriogenology 74, 563–568.

Francolini, M., Lavitrano, M., Lamia, C.L., French, D., Frati, L., Cotelli, F., Spadafora, C., 1993. Evidence for nuclear internalization of exogenous DNA into mammalian sperm cells. Molecular, Reproduction and Development 34, 133–139.

Gadea, J., Matás, C., Lucas, X., 1998. Prediction of porcine semen fertility by homologous in vitro penetration (hIVP) assay. Animal Reproduction Science 54, 95–108.

Gadea, J., García-Vázquez, F.A., 2010a. Methodologies for generating transgenic pigs. ITEA – Informacion Tecnica Economica Agraria 106, 15–29.

Gadea, J., García-Vázquez, F.A., 2010b. Applications of transgenic pigs in biomedicine and animal production. ITEA – Informacion Tecnica Economica Agraria 106, 30–45.

Gandolfi, F., Terqui, M., Modina, S., Brevini, T.A., Ajmone-Marsan, P., Foulon-Gauze, F., Courot, M., 1996. Failure to produce transgenic offspring by intra-tubal insemination of gilts with DNA-treated sperm. Reproduction, Fertility and Development 8, 1055–1060.

García-Vázquez, F.A., Ruiz, S., Matás, C., Izquierdo-Rico, M.J., Grullón, L.A., De Ondiz, A., Vieira, L., Avilés-López, K., Gutiérrez-Adán, A., Gadea, J., 2010. Production of transgenic piglets using ICSI-SMGT in combination with rcombinase RecA. Reproduction 140, 259–272.

García-Vázquez, F.A., García-Roselló, E., Gutiérrez-Adán, A., Gadea, J., 2009. Effect of sperm treatment on efficiency of EGFP-expressing porcine embryos produced by ICSI-SMGT. Theriogenology 72, 506–518.

Gamer, D.L., Johnson, L.A., Yue, S.T., Roth, B.L., Haugland, R.P., 1994. Dual DNA staining assessment of bovine sperm viability using SYBR-14 and propidium iodide. Journal of Andrology 16, 620–629.

Gutiérrez-Adán, A., Behboodi, E., Andersen, G.B., Medrano, J.F., Murray, J.D., 1996. Relationship between stage of development and sex of bovine IVM-IVF embryos cultured *in vitro* versus in the sheep oviduct. Theriogenology 46, 515–525.

Gutiérrez-Adán, A., Pintado, B., 2000. Effect of flanking matrix attachment regions on the expression of microinjected transgenes during preimplantation development of mouse embryos. Transgenic Research 9, 81–89.

Horan, R., Powell, R., Bird, J.M., Gannon, F., Houghton, J.A., 1992. Effects of electropermeabilization on the association of foreign DNA with pig sperm. Archives of Andrology 28, 105–114.

Holt, W.V., 1984. Membrane heterogeneity in the mammalian spermatozoon. In: Bourne, G.H., Danielli, J.F. (Eds.), International Review of Cytology. Academic Press, New York, pp. 159–194.

Kang, J.H., Hakimov, H., Ruiz, A., Friendship, R.M., Buhr, M., Golovan, S.P., 2008. The negative effects of exogenous DNA binding on porcine spermatozoa are caused by removal of seminal fluid. Theriogenology 70, 1288–1296.

Kurome, M., Saito, H., Tomii, R., Ueno, S., Hiruma, K., Nagashima, H., 2007. Effects of sperm pretreatment on efficiency of ICSI-mediated gene transfer in pigs. Journal of Reproduction and Development 53, 1217–1226.

Kuznetsov, A.V., Kuznetsova, I.V., Schit, I.Y., 2000. DNA interaction with rabbit sperm cells and its transfer into ova in vitro and in vivo. Molecular Reproduction and Development 56, 292–297.

Lai, L., Sun, Q., Wu, G., Murphy, C.N., Kuhholzer, B., Park, K.W., Bonk, A.J., Day, B.N., Prather, R.S., 2001. Development of porcine embryos and offspring after intracytoplasmic sperm injection with liposome transfected or non-transfected sperm into *in vitro* matured oocytes. Zygote 9, 339–346.

Lavitrano, M., Bacci, M.L., Forni, M., Lazzereschi, D., Di Stefano, C., Fioretti, D., Giancotti, P., Marfe, G., Pucci, L., Renzi, L., Wang, H., Stoppacciaro, A., Stassi, G., Sargiacomo, M., Sinibaldi, P., Turchi, V., Giovannoni, R., Della Casa, G., Seren, E., Rossi, G., 2002. Efficient production by sperm-mediated gene transfer of human decay accelerating factor (hDAF) transgenic pigs for xenotransplantation. Proceedings of the National Academy of Sciences of the United States of America 99, 14230–14235.

Lavitrano, M., Camaioni, A., Fazio, V.M., Dolci, S., Farace, M.G., Spadafora, C., 1989. Sperm cells as vectors for introducing foreign DNA into eggs: genetic transformation of mice. Cell 57, 717–723.

Lavitrano, M., Forni, M., Bacci, M.L., Di Stefano, C., Varzi, V., Wang, H., Seren, E., 2003. Sperm mediated gene transfer in pig: selection of donor boars and optimization of DNA uptake. Molecular Reproduction and Development 64, 284–291.

Lavitrano, M., Forni, M., Varzi, V., Pucci, L., Bacci, M.L., Di Stefano, C., Fioretti, D., Zoraqi, G., Moioli, B., Rossi, M., Lazzereschi, D., Stoppacciaro, A., Seren, E., Alfani, D., Cortesini, R., Frati, L., 1997. Sperm-mediated gene transfer: production of pigs transgenic for a human regulator of complement activation. Transplantation Proceedings 29, 3508–3509.

Lavitrano, M., French, D., Zani, M., Frati, L., Spadafora, C., 1992. The interaction between exogenous DNA and sperm cells. Molecular Reproduction and Development 31, 161–169.

Lavitrano, M., Stoppacciaro, A., Bacci, M.L., Forni, M., Fioretti, D., Pucci, L., Di Stefano, C., Lazzereschi, D., Rughetti, A., Ceretta, S., Zannoni, A., Rahimi, H., Moioli, B., Rossi, M., Nuti, M., Rossi, G., Seren, E., Alfani, D., Cortesini, R., Frati, L., 1999. Human decay accelerating factor transgenic pigs for xenotransplantation obtained by sperm-mediated gene transfer. Transplantation Proceedings 31, 972–974.

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. Reproduction Fertility and Development 18, 689–695.

Please cite this article in press as: García-Vázquez, F.A., et al. Factors affecting porcine sperm mediated gene transfer. Res. Vet. Sci. (2010), doi:10.1016/j.rvsc.2010.09.015

Liu, X.Y., Zohar, Y., Knibb, W., 1999. Association of foreign DNA with sperm of gilthead seabream, sparus aurata, after sonication, freezing, and dimethyl sulfoxide treatments. Marine Biotechnology 1, 175–183.

Lunney, J.K., 2007. Advances in swine biomedical model genomics. International Journal of Biological Science 3, 179–184.

Maione, B., Lavitrano, M., Spadafora, C., Kiessling, A.A., 1998. Sperm-mediated gene transfer in mice. Molecular, Reproduction and Development 50, 406–409.

Maione, B., Pittoggi, C., Achene, L., Lorenzini, R., Spadafora, C., 1997. Activation of endogenous nucleases in mature sperm cells upon interaction with exogenous DNA. DNA and Cell Biology 16, 1087–1097.

Martinez, E.A., Vazquez, J.M., Roca, J., Lucas, X., Gil, M.A., Parrilla, I., Vazquez, J.L., Day, B.N., 2001. Successful non-surgical insemination with small numbers of spermatozoa in sows. Reproduction 122, 289–296.

Nagashima, H., Fujimura, T., Takahagi, Y., Kurome, M., Wako, N., Ochiai, T., Esaki, R., Kano, K., Saito, S., Okabe, M., Murakami, H., 2003. Development of efficient strategies for the production of genetically modified pigs. Theriogenology 59, 95–106.

Niemann, H., Kues, W.A., 2000. Transgenic livestock: premises and promises. Animal Reproduction Science 60–61, 277–293.

Parrington, J., Coward, K., Gadea, J. Sperm and testis mediated DNA transfer as a means of gene therapy. Systems Biology in Reproductive Medicine, in press. doi:10.3109/19396368.2010.514022.

Pereyra-Bonnet, F., Fernández-Martin, R., Olivera, R., Jarazo, J., Vichera, G., Gibbons, A., Salamone, D., 2008. A unique method to produce transgenic embryos in ovine, porcine, feline, bovine and equine especies. Reproduction Fertility and Development 20, 741–749.

Perry, A.C., Wakayama, T., Kishikawa, H., Kasai, T., Okabe, M., Toyoda, Y., Yanagimachi, R., 1999. Mammalian transgenesis by intracytoplasmic sperm injection. Science 284, 1180–1183.

Petters, R.M., Sommer, J.R., 2000. Transgenic animals as models for human disease. Transgenic Research 9, 347–351.

Prather, R.S., Hawley, R.J., Carter, D.B., Lai, L., Greenstein, J.L., 2003. Transgenic swine for biomedicine and agriculture. Theriogenology 59, 115–123.

Pursel, V.G., Johnson, L.A., Schulman, L.L., 1973. Effect of dilution, seminal plasma and incubation period on cold shock susceptibility of boar spermatozoa. Journal of Animal Science 37, 528–531.

Schellander, K., Peli, J., Schmoll, F., Brem, G., 1995. Artificial insemination in cattle with DNA-treated sperm. Animal Biotechnology 41, 50.

Shalgi, R., Phillips, D.M., 1980. Mechanism of sperm entry in cycling hamsters. Journal of Ultrastructure Research 71, 154–161.

Shen, W., Li, L., Pan, Q., Min, L., Dong, H., Deng, J., 2006. Efficient and simple production of transgenic mice and rabbits using the new DMSO-sperm mediated exogenous DNA transfer method. Molecular Reproduction and Development 73, 589–594.

Smith, K., Spadafora, C., 2005. Sperm-mediated gene transfer: applications and implications. BioEssays 27, 551–562.

Spadafora, C., 1998. Sperm cells and foreign DNA: a controversial relation. BioEssays 20, 955–964.

Szczygiel, M.A., Moisyadi, S., Ward, W.S., 2003. Expression of foreign DNA is associated with paternal chromosome degradation in intracytoplasmic sperm injection-mediated transgenesis in the mouse. Biology of Reproduction 68, 1903–1910.

Tsai, H.J., Lai, C.H., Yang, H.S., 1997. Sperm as a carrier to introduce an exogenous DNA fragment into the oocyte of Japanese abalone *(Haliotis divorsicolor suportexta)*. Transgenic Research 6, 85–95.

Visconti, P.E., 2009. Understanding the molecular basis of sperm capacitation through kinase design. Proceedings of the National Academy of Sciences of the United States of America 106, 667–668.

Wall, R.J., 2002. New gene transfer methods. Theriogenology 57, 189–201.

Wang, H.J., Lin, A.X., Chen, Y.F., 2003. Association of rabbit sperm cells with exogenous DNA. Animal Biotechnology 14, 155–165.

Wu, Z., Li, Z., Yang, J., 2008. Transient transgene transmission to piglets by intrauterine insemination of spermatozoa icubated with DNA fragments. Molecular Reproduction and Development 75, 26–32.

Yanagimachi, R., 1981. Mechanisms of fertilization in mammals. In: Mastroianni, L., Biggers, J.D. (Eds.), Fertilization and Embryonic Development *In Vitro*. Plenum Press, New York, p. 182.

Yong, H.Y., Hao, Y., Lai, L., Li, R., Murphy, C.N., Rieke, A., Wax, D., Samuel, M., Prather, R.S., 2006. Production of a transgenic piglet by a sperm injection technique in which no chemical or physical treatments were used for oocytes or sperm. Molecular Reproduction and Development 73, 595–599.

Please cite this article in press as: García-Vázquez, F.A., et al. Factors affecting porcine sperm mediated gene transfer. Res. Vet. Sci. (2010), doi:10.1016/j.rvsc.2010.09.015