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Original article

Effect of the acrosome reaction on the efficiency of sperm-mediated DNA transfer

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Abstract

Sperm-mediated gene transfer (SMGT) is based on the ability of spermatozoa to bind exogenous DNA and transfer it into oocytes by fertilization. However, SMGT is still undergoing optimization to improve its efficiency to produce transgenic animals. The acrosome reaction is necessary for spermatozoa to carry the exogenous DNA into oocytes. In this study, the effect of the acrosome reaction on the efficiency of spermatozoa carrying exogenous DNA was evaluated. The results showed that the efficiency of the acrosome reaction was significantly higher ($p<0.05$) after incubation with 50 μmol/L progesterone compared to incubation without progesterone. It was significantly higher ($p<0.05$) in the 20, 40, and 60 min of progesterone treatment groups than in the 0 min treatment group. The spermatozoa were further incubated with cyanine dye Cy5 labeled DNA (Cy5-DNA) for 30 min at 37°C, and positive fluorescence signals were detected after the acrosome reaction was induced by progesterone at concentrations of 0 and 50 μmol/L for 40 min. The percentage of positive Cy5-DNA signals in spermatozoa was 96.61±2.06% and 97.51±2.03% following exposure to 0 and 50 μmol/L progesterone, respectively. The percentage of partial spermatozoa heads observed following combination with Cy5-DNA was 39.73±3.03% and 56.88±3.12% following exposure to 0 and 50 μmol/L progesterone, respectively. The ratio of positively stained spermatozoa combined with exogenous DNA showed no reduction after the acrosome reaction. These results suggest that the acrosome reaction might not be the key factor affecting the efficiency of SMGT.

Key words: spermatozoa, SMGT, acrosome reaction, exogenous DNA

Introduction

In comparison to other techniques, such as DNA microinjection, nuclear transfer, and retroviral infection, sperm-mediated gene transfer (SMGT) is a simpler technique by which transgenic animals can be produced (Kues and Niemann 2004, Smith and Spadafora 2005, Bacci et al. 2009, Petersen 2017). The ability of spermatozoa to accept exogenous DNA and transfer it to the oocyte during fertilization was first reported in rabbits in 1971 (Brackett et al. 1971). Since then, this technique has been used by several laboratories. A variety of transgenic animal species have been successfully produced by SMGT, including fish (Patil and Khoo 1996), chickens (Nakanishi and Iritani 1993), mice (Maione et al. 1998), and swine (Lavitrano et al. 1997).

Despite the use of SMGT to produce transgenic animals, the efficiency of the technology is still relatively

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poor, mainly because of the low uptake of exogenous DNA by spermatozoa, which thereby reduces the chances of fertilization of the oocytes with transfected spermatozoa (Anzar and Buhr 2006). In addition, the success of inter- and intra-species variability remains an unsolved challenge associated with the technology. Several factors determine the success of SMGT, including the spermatozoa donor, incubation media, exogenous DNA size and type, and the assisted reproductive technique used (Lavitrano et al. 2003, Smith and Spadafora 2005). To improve DNA-sperm binding, some authors have developed specific methodologies that use DNA-liposome complexes (Lai et al. 2001), electroporation (Horan et al. 1992), or monoclonal antibodies to link foreign DNA to spermatozoa (Chang et al. 2002). On the other hand, some groups have used dimethyl-sulfoxide (DMSO), which facilitates the transfer of foreign DNA to spermatozoa. This approach allows the efficient production of transgenic mice and rabbits (Kuznetsov et al. 2000, Shen et al. 2006).

For SMGT to be successful, the spermatozoa should incorporate or become attached to the exogenous DNA as one critical step. However, it is also necessary for the transfected spermatozoa to maintain their functionality and carry the exogenous DNA into the oocyte during fertilization. The acrosome reaction is of fundamental importance for the spermatozoa to fertilize an oocyte (Aitken et al. 1998). Mammalian spermatozoa must be acrosome-reacted before penetrating the zona pellucida. In some species, the spermatozoa undergo the acrosome reaction before binding to the zona pellucida, whereas in other species, only acrosome of intact spermatozoa can initiate binding to the zona pellucida (Harper et al. 2006). The acrosome reaction involves the fusion of the outer acrosomal membrane with the plasma membrane. The rupture of the head of the spermatozoon might lead to the loss of internalized exogenous DNA and thereby affect the amount of DNA that the spermatozoon can carry into the oocyte.

The main objective of this study was to evaluate the effect of the acrosome reaction on the efficiency of spermatozoa carrying exogenous DNA. First, the acrosome reaction of the spermatozoa was induced by progesterone *in vitro*. The spermatozoa were then incubated with Cy-5-DNA, and the effect of the acrosome reaction on spermatozoa carrying exogenous DNA was evaluated.

Materials and Methods

Animals

Institute of Cancer Research (ICR) mice (8–12 weeks old) with normal reproductive capacity were purchased from Vital River Experimental Animal Company of Beijing. All animal experiments performed in this study were approved by the Institutional Animal Care and Use Committee of Northeast Agriculture University (IACUC-02-005).

Test reagents

All chemicals and reagents were supplied by Sigma-Aldrich, unless otherwise indicated. Spermatozoa and embryo manipulations were carried out at 37.0°C.

Preparation of spermatozoa

Male ICR mice (8–12 weeks old) were sacrificed by cervical dislocation. In each experiment, three mice were used and the spermatozoa were mixed to exclude individual differences. Spermatozoa were collected from the epididymis, diluted with 1 mL human tubal fluid (HTF) (Millipore, China), and incubated at 37°C for 40 min to induce capacitation.

Induction of the acrosome reaction

After capacitation, the concentration of spermatozoa was adjusted to 10^7/mL in HTF. Progesterone was then added to the HTF at final concentrations of 0 μmol/L, 10 μmol/L, 20 μmol/L, 50 μmol/L, and 100 μmol/L, to induce the acrosome reaction of spermatozoa.

Detection of the acrosome reaction in spermatozoa

After the acrosome reaction was induced, the spermatozoa were washed twice in Dulbecco's phosphate-buffered saline (DPBS) and fixed in 4% (w/v) paraformaldehyde/4% (w/v) sucrose in DPBS for 20 min at room temperature. The spermatozoa were again washed twice in DPBS, stained with 100 μg/mL fluorescein isothiocyanate-labelled peanut agglutinin (FITC-PNA) for 30 min, and then washed in ddH_2O for 15 min. The spermatozoa were then stained with 5 mg/mL Hoechst 33342 for 8 min and washed twice in DPBS. They were then placed on a glass slide, after which antifade mounting medium was added, and the slide sealed with transparent nail polish. Samples were assessed using fluorescence microscopy at an excitation wavelength of 530 nm and 480 nm (Olympus BX51, Japan). At least 500 spermatozoa were counted at a time to detect the acrosome reaction. The percentage of sperm that have passed the acrosome reaction= (the number of spermatozoa that have passed the acrosome reaction /total number of spermatozoa)×100%.

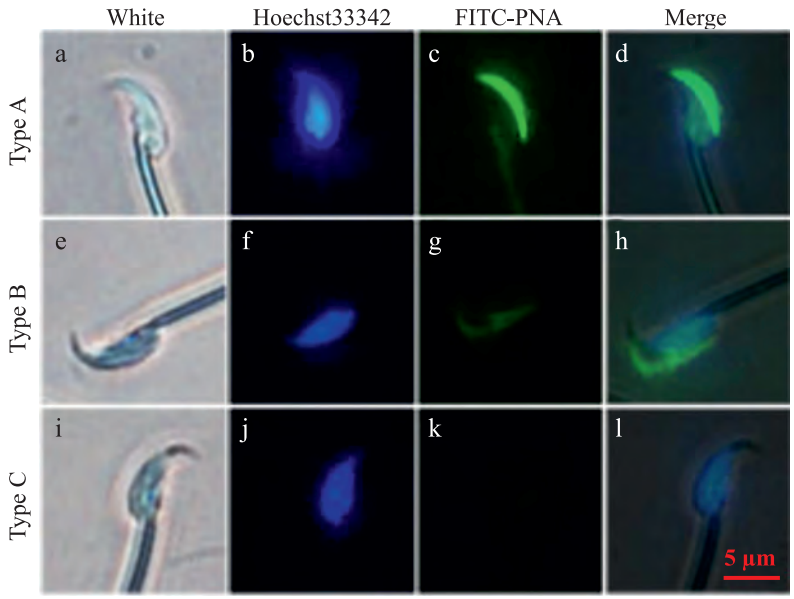

Fig. 1. Presentation of acrosome integrity after acrosome reaction of spermatozoa. Type A shows complete acrosome. Type B shows partial loss of acrosome. Type C shows complete loss of acrosome. Panels (a, e, and i) show the respective corresponding bright fields; (b, f, and j) the fluorescence in spermatozoa after being stained with Hoechst 33342; (c, g, and k) the fluorescence in spermatozoa after being stained with fluorescein isothiocyanate-labelled peanut agglutinin (FITC-PNA); and (d, h, and l) merged images.

Preparation of Cy-5 labeled DNA

The Cy-5-DNA primer sequences used were as follows: upstream 5′-TGG GCG TGG ATA GCG GTT TGA CT-3′; and downstream 5′-CCG TCG TCC TTG AAG AAG ATG GT-3′. The Cy-5-DNA primers were used to amplify a 536 bp DNA fragment from a template of the plasmid pEGFP-C1 (Clontech, China). The PCR conditions were as follows: denaturation at 94°C for 5 min; 33 cycles of denaturation at 94°C for 30 s, annealing at 63°C for 30 s, and extension at 72°C for 30s; followed by final extension at 72°C for 10 min. The Cy-5-labeled primers were synthesized by Invitrogen (Shanghai, China).

Incubation of spermatozoa with exogenous DNA

After capacitation, the concentration of spermatozoa was adjusted to 10^7/mL in HTF. They were then incubated with 100 μg/mL of Cy-5-DNA for 30 min at 37°C. After incubation with DNA, spermatozoa were washed three times with DPBS and centrifuged at 500 *g* for 5 min.

Detection of the acrosome reaction in spermatozoa incubated with exogenous DNA

The spermatozoa were incubated with different concentrations of DNA at 37°C for 30 min. After incubation with exogenous DNA, the spermatozoa were induced by 50 μmol/L progesterone for 40 min and then washed twice in DPBS. The spermatozoa were placed on a glass slide, after which antifade mounting medium was added, and the slide sealed with transparent nail polish. Samples were assessed using fluorescence microscopy at an excitation wavelength of 530 nm and 480 nm (Olympus BX51, Japan).

Statistical analysis

Experimental data were expressed as mean±SEM. Statistical analysis was performed using the SPSS 19 software (Somers, NY). Statistical significance was determined using the one-way ANOVA regression. $p<0.05$ was considered statistically significant.

Results

Detection of spermatozoa acrosome integrity after acrosome reaction

The integrity of the spermatozoa membrane was detected by FITC-PNA after the acrosome reaction. Three types of acrosomes were observed including type A, type B, and type C. Type A acrosomes were complete in structure (Fig. 1 Type A). The membranes of the acrosome were ruptured in type B and the contents of the acrosome were partially lost (Fig. 1 Type B). The acrosome in type C was completely lost (Fig. 1 Type C).

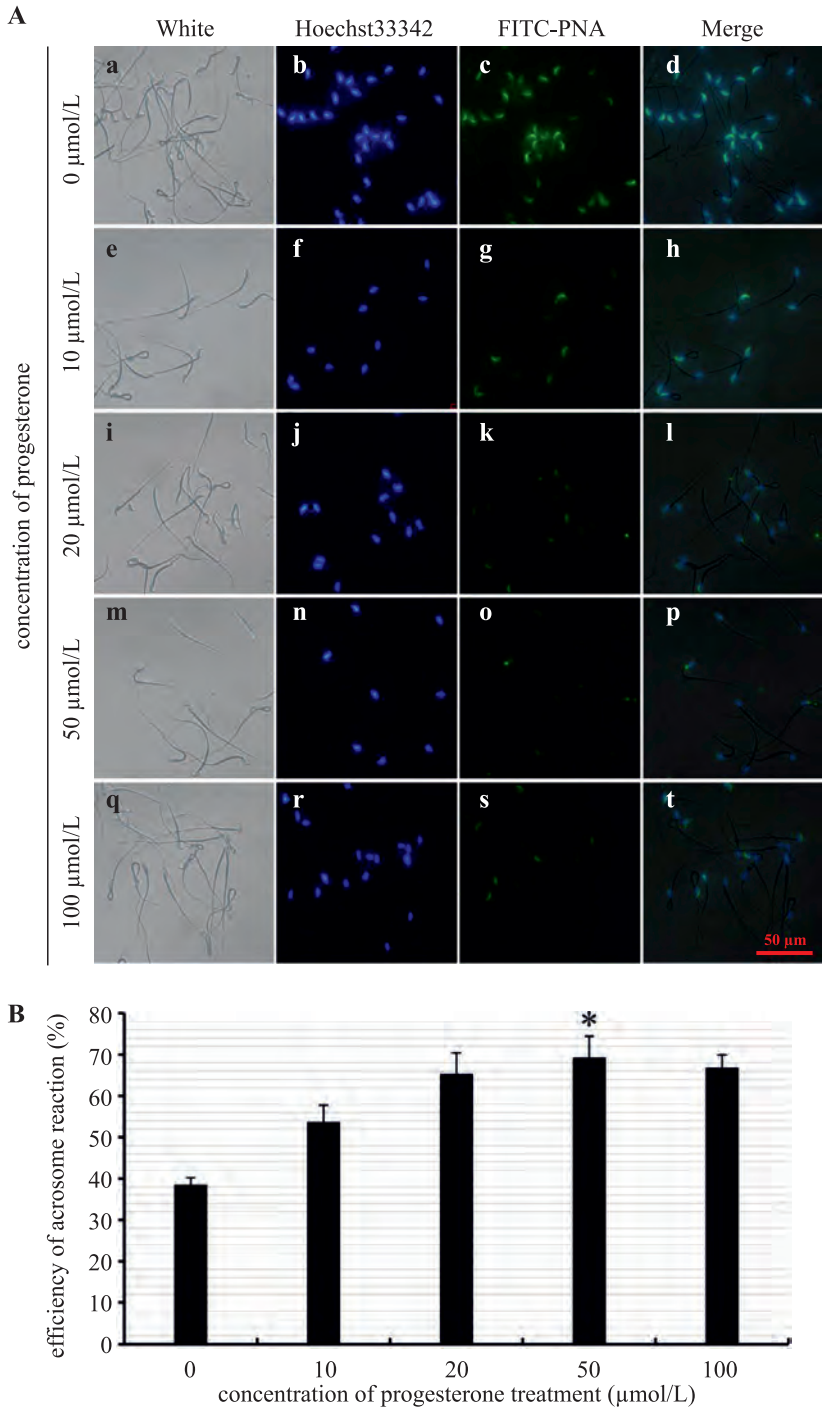

Fig. 2. Effect of progesterone concentration on efficiency of the acrosome reaction. (A) Spermatozoa acrosomal reactions were induced by progesterone at concentrations of 0, 10, 20, 50, and 100 μmol/L. Panels (a, e, i, m, and q) show the corresponding bright fields; (b, f, j, n, and r) fluorescence in spermatozoa after being stained with Hoechst 33342; (c, g, k, o, and s) fluorescence in spermatozoa after being stained with fluorescein isothiocyanate-labelled peanut agglutinin (FITC-PNA); and (d, h, l, p, and t) merged images. (B) Efficiency of acrosomal reaction induced by progesterone at concentrations of 0, 10, 20, 50, 100 μmol/L. *$p<0.05$ indicates significant differences in relation to incubation with 0 μmol/L of progesterone.

Effect of progesterone concentration on efficiency of the acrosome reaction

The integrity of the spermatozoa membrane was detected by FITC-PNA after the acrosome reaction was induced by progesterone at concentrations of 0, 10, 20, 50, and 100 μmol/L for 20 min (Fig. 2A). The results showed that the efficiency of the acrosome reaction was 38.54±1.67%, 53.75±4.12%, 65.42±5.01%, 69.38±5.10%, and 66.87±3.01% after induction by progesterone

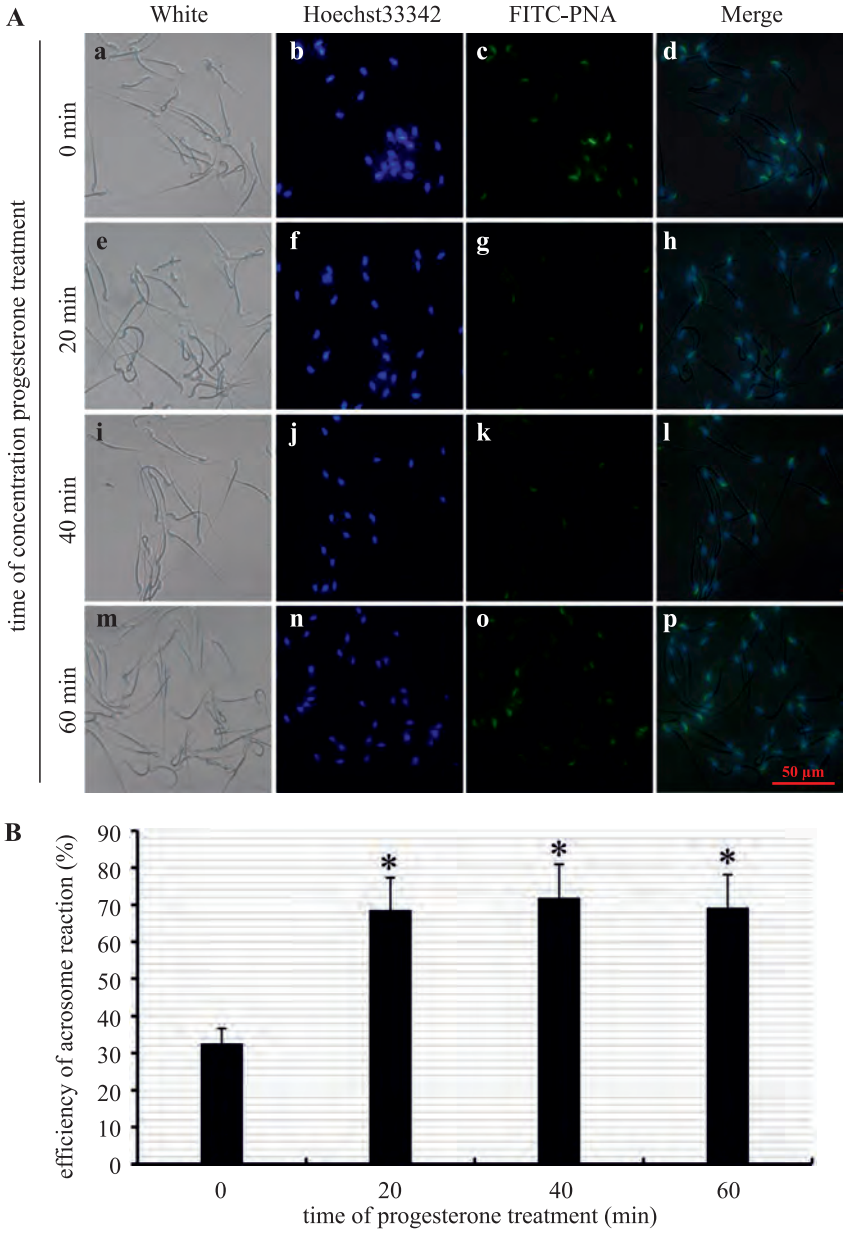

Fig. 3. Effect of progesterone induction time on efficiency of the acrosome reaction. (A) Spermatozoa acrosomal reactions were induced by 50 μmol/L progesterone for 0, 20, 40, and 60 min. Panels (a, e, i, and m) show the corresponding bright fields; (b, f, j, and n) fluorescence in spermatozoa after being stained with Hoechst 33342; (c, g, k, and o) fluorescence in spermatozoa after being stained with fluorescein isothiocyanate-labelled peanut agglutinin (FITC-PNA); and (d, h, l, and p) merged images. (B) Efficiency of acrosomal reaction induced by 50 μmol/L progesterone for 0, 20, 40, and 60 min. *$p<0.05$ indicates significant differences in relation to incubation with progesterone for 0 min.

at concentrations of 0, 10, 20, 50, and 100 μmol/L, respectively (Fig. 2B). The efficiency of the acrosome reaction was significantly higher in the 10, 20, 50, and 100 μmol/L progesterone groups than in the 0 μmol/L group. In addition, the efficiency of the acrosome reaction was slightly higher in the 50 μmol/L progesterone group than in the 10, 20, and 100 μmol/mL groups. However, this efficiency was not significantly different between the 10, 20, 50, and 100 μmol/L progesterone groups.

Effect of progesterone induction time on efficiency of the acrosome reaction

The integrity of the spermatozoa membrane was detected by FITC-PNA after the acrosome reaction was induced by progesterone at a concentration of 50 μmol/L for 0, 20, 40, and 60 min (Fig. 3A). The results showed that the efficiency of the acrosome reaction was 32.69±4.02%, 68.64±8.72%, 71.91±9.02%, and 69.29± 8.85% after induction by progesterone for 0, 20, 40, and 60 min, respectively (Fig. 3B). In addition,

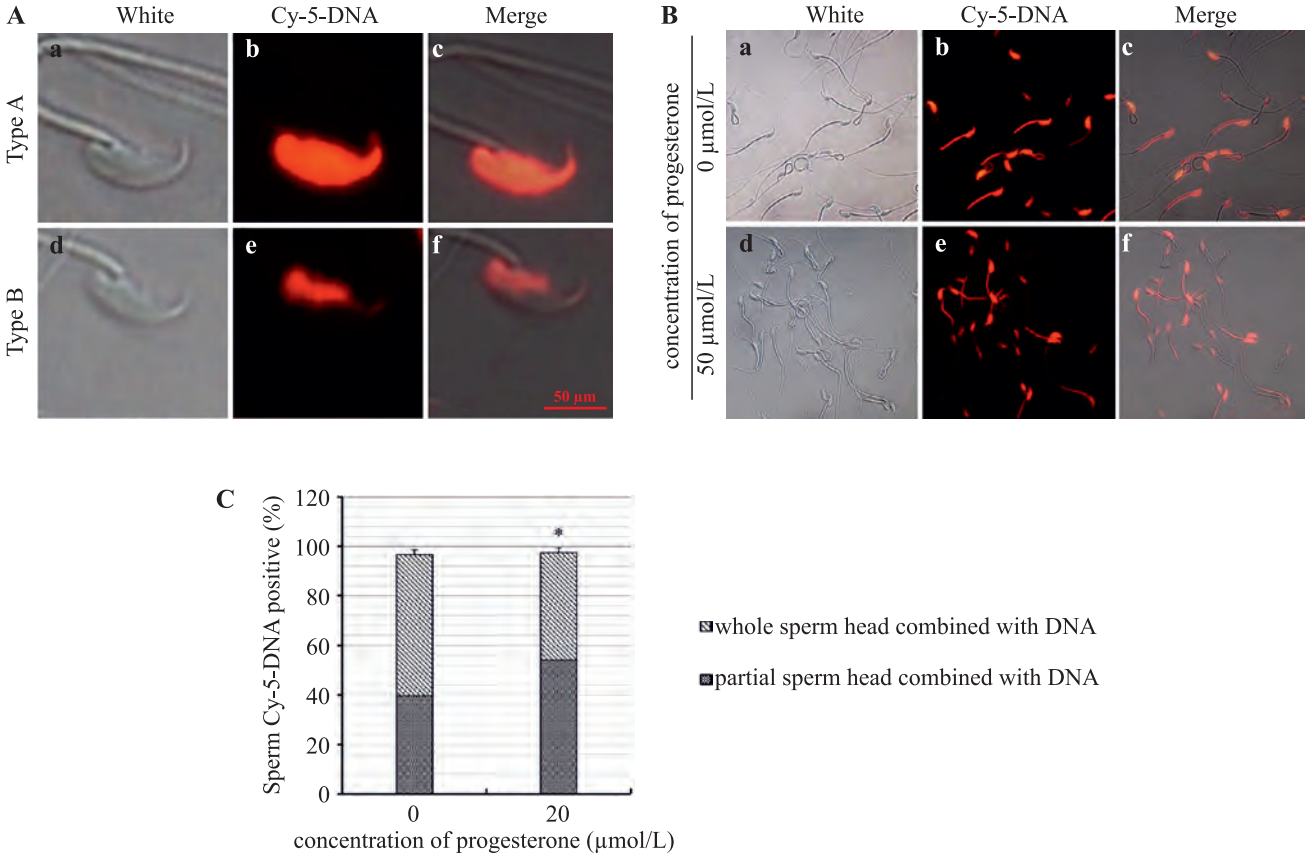

Fig. 4. Detection and efficiency of the acrosome reaction on spermatozoa carrying exogenous DNA. (A) The position of Cy5-DNA on the head of spermatozoon. Type A shows the whole sperm head combined with DNA. Type B shows a partial sperm head combined with DNA. Panels (a and d) show corresponding bright fields; (b and e) fluorescence in spermatozoa after co-incubation with DNA; and (c and f) merged images. (B) Spermatozoa acrosomal reactions were induced by 0 and 50 μmol/L progesterone for 40 min. Panels (a and d) show corresponding bright fields; (b and e) fluorescence in spermatozoa after co-incubation with DNA; and (c and f) merged images. (C) Percentage of positive signals in spermatozoa after the acrosome reaction. *$p<0.05$ indicates significant differences.

the efficiency of the acrosome reaction was significantly higher in the 20, 40, and 60 min progesterone treatment groups than in the 0 min treatment group. Moreover, the efficiency of the reaction was slightly higher in the 40 min progesterone treatment group than in the 20 and 60 min treatment groups. However, this efficiency was not significantly different between the 20, 50, and 60 min progesterone treatment groups.

Detection and efficiency of the acrosome reaction on spermatozoa carrying exogenous DNA

Positive signals in spermatozoa were detected by fluorescence microscopy after incubation with Cy5 labeled DNA. Two types of spermatozoa were observed to be bound to the DNA. In type A spermatozoa, the whole spermatozoon head was combined with the DNA (Fig. 4A). In type B spermatozoa, the spermatozoon head was partially combined with the DNA (Fig. 4A). Positive signals in spermatozoa were also detected by fluorescence microscopy after the acrosome reaction was induced by progesterone at concentrations of 0 and 50 μmol/L for 40 min (Fig. 4B). The results showed that the positive signals detected in spermatozoa were 96.61±2.06% and 97.51±2.03% in the 0 and 50 μmol/L progesterone groups, respectively (Fig. 4C). The percentage of partial spermatozoa heads combined with DNA was 39.73±3.03% and 56.88±3.12% in the 0 and 50 μmol/L progesterone groups, respectively (Fig. 4C). Furthermore, the percentage of partial spermatozoa heads combined with DNA was significantly higher in the 50 μmol/L progesterone treatment group than in the 0 μmol/L group.

Discussion

The SMGT technique has proved to be a viable method to generate transgenic embryos and animals in a considerable number of species (Smith and Spadafora 2005). However, some issues remain to be addressed regarding its repeatability, efficiency in different spe-

cies, and our understanding of the underlying molecular basis of its action. The resolution of such issues would be important if this approach is to be employed to produce transgenic animals on a routine basis (Coward et al. 2007, Parrington et al. 2011).

During SMGT, in order to fuse to and fertilize an oocyte, spermatozoa undergo a complex set of molecular events, including the induction of capacitation *in vitro*, co-incubation with DNA *in vitro*, and the acrosome reaction. The acrosome reaction is a prerequisite, before capacitated spermatozoa can interact with an oocyte in a species-specific manner, undergo acrosomal exocytosis, and fertilize the oocyte (Abou-Haila and Tulsiani 2000). During the acrosome reaction, the apical outer membrane and the spermatozoon plasma membrane fuse at multiple sites. The release of various enzymes in the apical body and exposure of the acrosome intima eventually lead to fusion of the spermatozoon plasma membrane and the plasma membrane of the oocyte cells (Tulsiani et al. 1998). In the present study, two outcomes were noted after the acrosome reaction: partial loss and complete loss of the acrosome.

Progesterone, a hormone produced during ovulation, reportedly induces the acrosome reaction by interacting with the spermatozoon plasma membrane in a receptor-mediated manner (Roldan and Fraser 1998). When spermatozoa are treated with progesterone, there is a transient elevation of intrasperm Ca^{2+} (Kirkman-Brown et al. 2002), and the involvement of progesterone receptors on the spermatozoon plasma membrane (Liu et al. 2009) induce a progesterone-activated influx of Ca^{2+}. The dynamics in Ca^{2+} signaling during the progesterone-activated acrosome reaction have demonstrated the involvement of the tyrosine kinase pathway (Garcia et al. 1999) and gamma aminobutyric acid (GABA) A like receptor (Kuroda et al. 1999) as mediators of the Ca^{2+} signal that trigger the reaction.

When the acrosome reaction is induced without an external stimulus (agonist) in a time-dependent manner, it is referred to as a spontaneous acrosome reaction (Abou-Haila and Tulsiani 2000). The non-physiological acrosome reaction depends on several factors, including the species and capacitation medium. In the mouse and several other species, spermatozoa that have undergone a spontaneous acrosome reaction are unable to bind to, and fertilize, a zona-intact oocyte; however, these spermatozoa are perfectly capable of fertilizing a zona-free oocyte *in vitro* and producing normal pups (Tulsiani et al. 1998). The spontaneously acrosome-reacted spermatozoa have typically lost their receptors that recognize and bind to the zona pellucida, but otherwise remain fertile. In this study, the control group spermatozoa were those that underwent a spontaneous acrosome reaction. Thus, these spermatozoa could not fertilize an oocyte with an intact zona pellucida *in vitro*.

The localization of DNA binding was evaluated using Cy5 labeled DNA. The DNA molecules showed an affinity for the post-acrosomal region of the spermatozoa head and the whole spermatozoa head, confirming previous reports on different species, including the pig (Francolini et al. 1993, Patil and Khoo 1996, Kuznetsov et al. 2000). These findings are also consistent with those of another report that demonstrated the presence of labeled cells bound to DNA in the acrosomal region (Camaioni et al. 1992). The different regions of spermatozoa show differences in composition and functions during fertilization (Medarde et al. 2013). The plasma membrane of spermatozoa in the post-acrosomal and mid piece regions are involved in gamete fusion during the final process of fertilization (Francolini et al. 1993, Patil and Khoo 1996). The apical membrane of spermatozoa and the equatorial segment of the acrosome are not incorporated into the plasma membrane of the oocyte, but are internalized by the oocyte as a composite vesicle, comprising both spermatozoa and oocyte membranes at fertilization (Camaioni et al. 1992). The fate of such vesicles is unknown. However, they might act as DNA carriers into the oocyte, both for DNA bound to the spermatozoon membrane, and DNA entrapped in solution in the culture medium (Camaioni et al. 1992). Spermatozoa are able to both bind to DNA and internalize DNA into the spermatozoon head during incubation (Eghbalsaied et al. 2013, Mu et al. 2018). After the acrosome reaction, exogenous DNA bound to the acrosomal region is lost, whereas DNA bound at the post-acrosomal and mid-piece regions of spermatozoa can still be transferred into the oocyte.

In conclusion, this study confirms that under the experimental conditions described, spermatozoa could bind to exogenous DNA, by localized binding of the whole spermatozoa head or the post-acrosomal area. After the acrosome reaction, exogenous DNA bound at the acrosomal region of the spermatozoa is lost, and DNA bound at the post-acrosomal and mid-piece regions are retained. The ratio of positively stained spermatozoa combined with exogenous DNA showed no reduction after the acrosome reaction. These results suggest that the acrosome reaction might not be the key factor affecting the efficiency of SMGT.

Acknowledgements

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References

Abou-Haila A, Tulsiani DR (**2000**) Mammalian sperm acrosome: formation, contents, and function. Arch Biochem Biophys 379: 173-182.

Aitken RJ, Harkiss D, Knox W, Paterson M, Irvine DS (**1998**) A novel signal transduction cascade in capacitating human spermatozoa characterised by a redox-regulated, cAMP-mediated induction of tyrosine phosphorylation. J Cell Sci 111 : 645-656.

Anzar M, Buhr MM (**2006**) Spontaneous uptake of exogenous DNA by bull spermatozoa. Theriogenology 65: 683-690.

Bacci ML, Zannoni A, De Cecco M, Fantinati P, Bernardini C, Galeati G, Spinaci M, Giovannoni R, Lavitrano M, Seren E, Forni M (**2009**) Sperm-mediated gene transfer--treated spermatozoa maintain good quality parameters and in vitro fertilization ability in swine. Theriogenology 72: 1163-1170.

Brackett BG, Baranska W, Sawicki W, Koprowski H (**1971**) Uptake of heterologous genome by mammalian spermatozoa and its transfer to ova through fertilization. Proc Natl Acad Sci U S A 68: 353-357.

Camaioni A, Russo MA, Odorisio T, Gandolfi F, Fazio VM, Siracusa G (**1992**) Uptake of exogenous DNA by mammalian spermatozoa: specific localization of DNA on sperm heads. J Reprod Fertil 96: 203-212.

Chang K, Qian J, Jiang M, Liu YH, Wu MC, Chen CD, Lai CK, Lo HL, Hsiao CT, Brown L, Bolen J, Jr., Huang HI, Ho PY, Shih PY, Yao CW, Lin WJ, Chen CH, Wu FY, Lin YJ, Xu J, Wang K (**2002**) Effective generation of transgenic pigs and mice by linker based sperm--mediated gene transfer. BMC Biotechnol 2: 5.

Coward K, Kubota H, Parrington J (**2007**) In vivo gene transfer into testis and sperm: developments and future application. Arch Androl 53: 187-197.

Eghbalsaied S, Ghaedi K, Laible G, Hosseini SM, Forouzanfar M, Hajian M, Oback F, Nasr-Esfahani MH, Oback B (**2013**) Exposure to DNA is insufficient for in vitro transgenesis of live bovine sperm and embryos. Reproduction 145: 97-108.

Francolini M, Lavitrano M, Lamia CL, French D, Frati L, Cotelli F, Spadafora C (**1993**) Evidence for nuclear internalization of exogenous DNA into mammalian sperm cells. Mol Reprod Dev 34: 133-139.

Garcia MA, Meizel S (**1999**) Progesterone-mediated calcium influx and acrosome reaction of human spermatozoa: pharmacological investigation of T-type calcium channels. Biol Reprod 60: 102-109.

Harper CV, Barratt CL, Publicover SJ, Kirkman-Brown JC (**2006**) Kinetics of the progesterone-induced acrosome reaction and its relation to intracellular calcium responses in individual human spermatozoa. Biol Reprod 75: 933-939.

Horan R, Powell R, Bird JM, Gannon F, Houghton JA (**1992**) Effects of electropermeabilization on the association of foreign DNA with pig sperm. Arch Androl 28: 105-114.

Kirkman-Brown JC, Punt EL, Barratt CL, Publicover SJ (**2002**) Zona pellucida and progesterone-induced Ca2+ signaling and acrosome reaction in human spermatozoa. J Androl 23: 306-315.

Kues WA, Niemann H (**2004**) The contribution of farm animals to human health. Trends Biotechnol 22: 286-294.

Kuroda Y, Kaneko S, Yoshimura Y, Nozawa S, Mikoshiba K (**1999**) Influence of progesterone and GABAA receptor on calcium mobilization during human sperm acrosome reaction. Arch Androl 42: 185-191.

Kuznetsov AV, Kuznetsova IV, Schit IY (**2000**) DNA interaction with rabbit sperm cells and its transfer into ova in vitro and in vivo. Mol Reprod Dev 56 (Suppl 2): 292-297.

Lai L, Sun Q, Wu G, Murphy CN, Kuhholzer B, Park KW, Bonk AJ, Day BN, Prather RS (**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, Forni M, Bacci ML, 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. Mol Reprod Dev 64: 284-291.

Lavitrano M, Forni M, Varzi V, Pucci L, Bacci ML, 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. Transplant Proc 29: 3508-3509.

Liu DY, Liu ML, Baker HW (**2009**) Enhancement of sperm-zona pellucida (ZP) binding capacity by activation of protein kinase A and C pathways in certain infertile men with defective sperm-ZP binding. Hum Reprod 24: 20-27.

Maione B, Lavitrano M, Spadafora C, Kiessling AA (**1998**) Sperm-mediated gene transfer in mice. Mol Reprod Dev 50: 406-409.

Medarde N, Munoz-Munoz F, Lopez-Fuster MJ, Ventura J (**2013**) Variational modularity at the cell level: insights from the sperm head of the house mouse. BMC Evol Biol 13: 179.

Mu Y, Jiao M, Zhao Y, Lv J, Wang J, Hao J, Zhang X, Kong Q, Liu Z (**2018**) A method for tracing exogenous DNA uptake in live spermatozoa and embryos. Pol J Vet Sci 21: 193-202.

Nakanishi A, Iritani A (**1993**) Gene transfer in the chicken by sperm-mediated methods. Mol Reprod Dev 36: 258-261.

Parrington J, Coward K, Gadea J (**2011**) Sperm and testis mediated DNA transfer as a means of gene therapy. Syst Biol Reprod Med 57: 35-42.

Patil JG, Khoo HW (**1996**) Nuclear internalization of foreign DNA by zebrafish spermatozoa and its enhancement by electroporation. J Exp Zool 274: 121-129.

Petersen B (**2017**) Basics of genome editing technology and its application in livestock species. Reprod Domest Anim 52 (Suppl 3): 4-13.

Roldan ER, Fraser LR (**1998**) Protein kinase C and exocytosis in Mammalian spermatozoa. Trends Endocrinol Metab 9: 296-297.

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. Mol Reprod Dev 73: 589-594.

Smith K, Spadafora C (**2005**) Sperm-mediated gene transfer: applications and implications. Bioessays 27: 551-562.

Tulsiani DR, Abou-Haila A, Loeser CR,Pereira BM (**1998**) The biological and functional significance of the sperm acrosome and acrosomal enzymes in mammalian fertilization. Exp Cell Res 240: 151-164.