Uptake of Mitochondrial DNA Fragment into Boar Spermatoza for Sperm-Mediated Gene Transfer

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ABSTRACT

Sperm-mediated gene transfer (SMGT) can be used to transfer exogenous DNA into the oocyte at fertilization. The main objective of this study was to assess efficiency of transferring mitochondrial DNA (mtDNA) fragment into boar spermatozoa in either presence or absence of liposome and quality of transfected spermatozoa. The mtDNA of chicken liver was isolated and purified by phenol and alkaline lysis extraction, and it was inserted to plasmid. The genome of transfected spermatozoa treated with DNase I was purified by alkaline lysis, and then amplified by the PCR analysis. After electrophoresis, DNA quantitation of each well was calculated by comparison of the band intensity with standard. As a result, exogenous DNA was composed of mtDNA fragment (1.2 kb) and plasmid (2.7 kb). On the other hand, efficiency of transfection by liposome (9.0±0.34 ng/μl) in SMGT was higher than that by DNA solution (6.9±0.53 ng/μl). However, there was no significant difference. Transfering exogenous DNA into spermatozoa was completed within 90 min of incubation. In another experiment, there were significant ($p<0.05$) differences between transfected spermatozoa using both DNA solution and DNA/liposome complexes with untreated spermatozoa for viability (70.8±1.80 and 68.0±2.16% vs. 83.3±1.69%, respectively) and motility (78.7±1.59 and 79.3±2.14% vs. 86.7±1.59%, respectively). This study indicates that exogenous mtDNA can be efficiently transferred into boar spermatozoa regardless of the presence of liposome, and transfected spermatozoa can also use insemination and *in vitro* fertilization to generate transgenic pig.

(Key words : DNA uptake, SMGT, Liposome, Transfection, Boar spermatozoa)

INTRODUCTION

The genetically modified animals are recognized as a powerful tool to investigate the function of specific genes and the mechanism regulating their expression. Transgenic animals have been routinely produced by various methods such as nuclear transfer (NT), microinjection of exogenous DNA solution and viral infection of the exogenous DNA. However, producing transgenic animals has been limited due to a low efficiency, lack of skilled researchers and expensive equipment. To resolve these problems, the new method using sperm mediated gene transfer (SMGT) has been developed and is based on the ability of spermatozoa to bind and internalize exogenous DNA and to transfer it into the oocyte at fertilization (Lavitrano *et al.*, 1992). In many studies, exogenous DNA incubated with spermatozoa localizes in the post-acrosome region of sperm head in most species, and then some exogenous DNA internalize sperm genome (Lavitrano *et al.*, 1992). SMGT appear to be a simple, efficient and applicable to all species that uses spermatozoa for reproduction. SMGT was first applied in the rabbit spermatozoa (Brackett *et al.*, 1971), as well as in other species, such as chicken (Rottmann *et al.*, 1992), sea urchins (Arezzo, 1989), zebra fish (Khoo *et al.*, 1992) and bovine (Perez *et al.*, 1991). A molecule, called inhibitory factor I (IF-I), has been isolated from the seminal fluid of mammals and blocks the interaction of DNA with sperm (Zoraqi *et al.*, 1997; Carballada *et al.*, 2001; Lavitrano *et al.*, 2003). The technology of SMGT has been limited due to species specificity and a variety of experimental conditions.

Cationic liposomes have proven to be useful tools for introducing exogenous DNA into cultured cells, gene therapy (Felgner *et al.*, 1995; Liu *et al.*, 1995) and mice spermatozoa (Bachiller *et al.*, 1991). Cationic lipo-

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somes interact with the negatively charged nucleic acid molecules and form complexes, in which the nucleic acid is coated by the lipids. The positive outer surface of the complex can then be associated with the negatively charged cell membrane, allowing the internalization of the nucleic acid.

A single mammalian cell may have hundreds of mitochondria, each containing mitochondrial DNA (mtDNA) in the form of closed circular molecules of approximately 16.5 kb in size. This mtDNA is present at about 2 to 10 copies per mitochondria, and differs from nuclear DNA in its genetic code and by its almost exclusive maternal inheritance. Indeed, modifications of mtDNA such as point mutations, fragmentation, deletions and duplications have been implicated etiologically in human diseases (Johns *et al.*, 1995) and aging (Wallace, 1992).

Based on this finding, this study analyzes mtDNA of chicken liver, the efficiency of cationic liposome to introduce exogenous DNA into the boar spermatozoa and quality of transfected spermatozoa.

MATERIALS AND METHODS

Semen Collection

Semen-rich fractions were collected from boars used insemination by the gloved-hand technique and filtered through cotton gauze into a pre-warm cup to remove the gel particles. Semen was extended with twice volumes of Byu-Ri extender (Sperm Gene Co., Korea). After 20 min at the room temperature, the extended semen samples were transported to the laboratory at 17℃ within 3 hr of collection. Motility of spermatozoa used this experiment was more than 85% at 39℃ by markler counting chamber, and then they were stored at 17℃.

Isolation and Purification of Mitochondrial DNA

The mtDNA was obtained from chicken liver by the alkaline lysis method (Sambrook *et al.*, 1989) which is originally developed for the isolation of plasmid DNA from bacteria (Palva *et al.*, 1985). The liver was minced with scissors and then homogenized with a glass homogenizer in homogenization buffer (250 mM sucrose, 30 mM Tris-HCl, 9 mM EDTA and 2.5 mM $CaCl_2 \cdot 2H_2O$, pH 8.0) and centrifuged at 700 ×g for 20 min at 4℃. The supernatant collected in Eppendorf centrifuge tube was centrifuged at 30,000 ×g for 20 min at 4℃, and then the pellet was vigorously mixed in 150 μl of chilled STE buffer (0.1 M NaCl, 10 mM Tris-HCl and 1 mM EDTA, pH 8.0) by vortexing. To lyse the mitochondria and denature the contaminating nuclear DNA, 300 μl of freshly prepared alkaline SDS solution (0.2 M NaOH, 1% SDS) was added and gently mixed by inverting the tubes. After the suspension followed by the incubation for 20 min, 225 μl of chilled 3 M potassium acetate buffer (60 ml of 5 M potassium acetate, 11.5 ml of glacial acetate, 28.5 ml of distilled H_2O) was added. The suspension was mixed by inverting the tube, incubated for 20 min and centrifuged at 12,000 ×g for 10 min at 4℃. The supernatant fluid was transferred to a fresh Eppendorf tube and an equal volume of equilibrated phenol (pH 8.0) was added. After the suspension followed by the incubation for 10 min, the supernatant fluid was collected by centrifugation at 14,000 ×g for 10 min at 4℃. The supernatant, transferred a fresh Eppendorf tube, was added twice volume of cold absolute ethanol and stored for 20 min at −20℃. After the supernatant fluid was removed following centrifugation at 18,000 ×g for 10 min at 4℃, the tube was dried with air at room temperature. The pellet was dissolved in 100 μl of TE buffer (5 mM Tris-HCl, pH 8.0, 1 mM EDTA), added with an equal volume of equilibrated phenol (pH 8.0). The suspension incubated for 10 min at room temperature was orderly added an equal volume of phenol/chloroform (1:1) solution, phenol : chloroform : isomayl alcohol mixture (25:24:1) and chloroform : isomayl alcohol mixture (24:1), respectively. After adding each solutions, the suspension was incubated for 10 min and centrifuged at 14,000 ×g for 10 min at 4℃. The last supernatant, transfered a fresh Eppendorf tube, was added in twice volume of cold absolute ethanol and stored for 20 min at −20℃. The suspension was centrifuged at 15,000 ×g for 10 min at 4℃, and the tube was dried with air at room temperature. This mtDNA pellet was then resuspended in 100 μl of TE buffer, treated with RNase, and phenol extracted twice. The pellet was dried in air at room temperature, dissolved in 100 μl of TE buffer and stored at −20℃.

Construction of Exogenous DNA

After purified mtDNA was digested with *Eco*R I and *Hin*dIII, they were fractionated on a 1% low-melting agarose preparative gel, agarose containing DNA ranging from 1.2 kb was sliced with a blade under UV transillumination. The DNA fragment was purified by phenol extraction and then inserted into the multiple cloning site (MCS) of the pUC19 digested with *Eco*R I and *Hin*dIII. The recombined plasmids for transformation were introduced into competent cells in ECOS™ (Real Biotech Corp) and cloned according to the manufacture's protocol. Plasmid cultured for overnight was isolated and purified by alkaline lysis method, and then stored at −20℃ until used.

Spermatoza Transfection

Ninety seven microlitter of Androhep extender (glucose 26.0 g, EDTA 2.4 g, sodium citrate 8.0 g, sodium bicarbonate 1.25 g and HEPES 9.0 g in 1.0 l of distilled water) were gently mixed with 2 μl of liposome (Sig-

ma) for 5 min, and then mixed with 5 μl exogenous DNA solution (40 ng/μl) for 20 min at room temperature. 10^6 spermatozoa were washed twice in Androhep extender. The resuspended pellet in DNA solution or DNA/liposome complexes was incubated for 180 min at 17℃.

Assessment of DNA Uptake

A transfected spermatozoa under different conditions were washed twice by centrifuging at 500 ×g for 5 min. The pellet resuspended with DNase I (15 U) was incubated for 1 hr at 37℃ to remove exogenous DNA bounded cell membrane of spermatozoa. For PCR analysis, spermatozoa washed twice by centrifuging were isolated using the alkaline lysis method. The PCR amplification was performed according to the standard protocol using forward primer 5'-GTTTTCCCAGTCACGAC-3' and reverse primer 5'-TCACACAGGAAACAGGTATGAC-3'. The PCR reaction conditions consisted of denaturation at 94℃ for 1 min, followed by 35 amplification cycles: denaturation at 94℃, for 30 sec; annealing at 55℃, for 30 sec; extension at 72℃, for 30 sec. Cycle 35 contained an additional extension at 72℃, for 5 min. Non-treated spermatozoa were used as a negative control and plasmid DNA of a 1.2 kb DNA fragment was used as a positive control. 10 μl of the reaction mixture was then analyzed on 1.0% agarose gel. The gels were stained with ethidium bromide, and amplified DNA bands were visualized by ultraviolet transillumination. To analysis transfection efficiency under different conditions, quantitation of exogenous DNA was estimated by visual comparison of the band intensity with the standards.

Quality of Spermatozoa

The viability and motility of spermatozoa incubated with either DNA solution or DNA/liposome complexes for 90 min was promptly assessed using SYBR-14/PI assay (Garner *et al.*, 1995) and Makler counting chamber (Makler, 1978) according to the standard protocol, respectively.

Statistical Analysis

Each experiment was replicated four times. Statistical analysis between treatments was carried out by analysis of variance (ANOVA). Significant differences between means were determined using Fisher's protected least significant difference (LSD) and Duncan's multiple range test using the SAS version 8.01 for Windows (SAS Institute, 1990). Data from each experiment are presented as the mean±SE and $p<0.05$ was considered to be statistically significant.

RESULTS

Exogenous DNA Containing Chicken mtDNA

To use exogenous DNA for introducing into boar spermatozoa, the DNA was extracted from mitochondria of chicken liver by both phenol and alkaline lysis method. The mtDNA separated in 1.0% agarose gel was approximately 12,000 base pair, and then 1,200 bp DNA fragment was confirmed by digesting mtDNA with *Eco*R I and *Hin*dIII (Fig. 1A). Furthermore, no contaminating chromosomal DNA was detected in this analysis, and no problems were encountered in obtaining total digests of the mtDNA with the enzymes used. The exogenous DNA composed of both mtDNA fragment (1,200 bp) and the pUC19 (2,700 bp) were approximately 3,900 bp using electrophoresis (Fig. 1B).

DNA Uptake of Spermatozoa

After spermatozoa genome transfected by either DNA

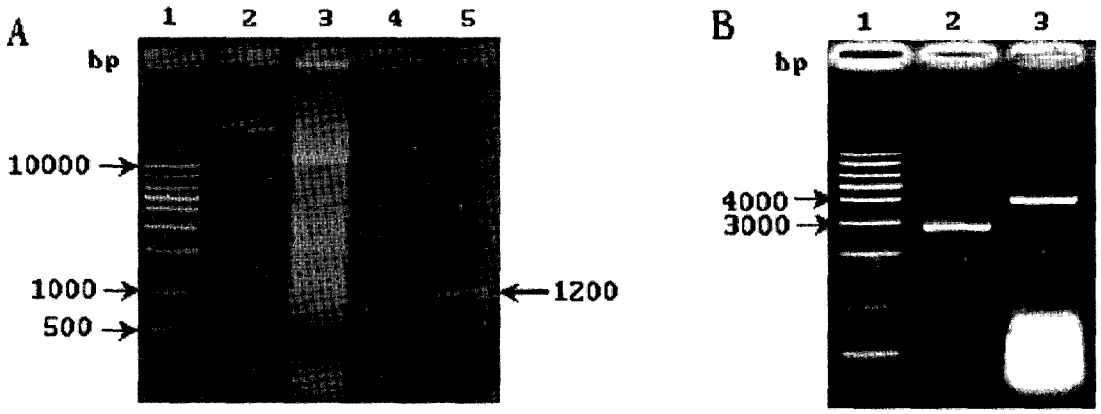

Fig. 1. The isolation of DNA from mitochondria of chicken liver (A) and construction of exogenous DNA (B). In panel A, lane 1, 10 kb DNA ladder; lane 2, chicken liver mtDNA; lane 3, mtDNA digested with *Eco*R I and *Hin*dIII; lane 4 and 5, isolated mtDNA fragment. In panel B, lane 1, 10 kb DNA ladder; lane 2, pUC19; lane 3, recombinant plasmid.

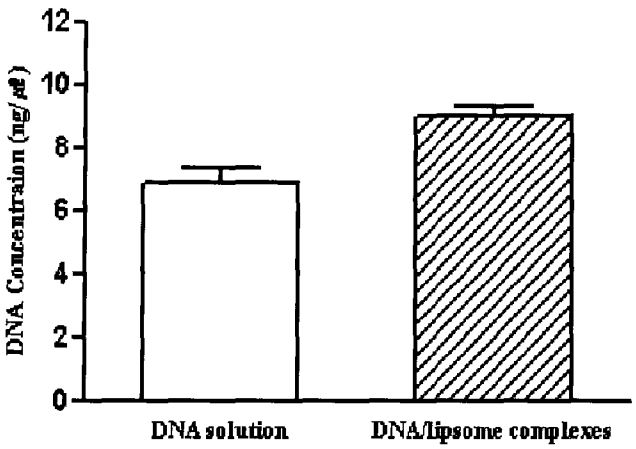

Fig. 2. Efficiency of DNA uptake in boar spermatoza by liposome.

solution or DNA/liposome complexes was extracted using alkaline extraction method, we performed polymerase chain reaction and electrophoresis. DNA quantitations were estimated by band intensity comparing with the standard. As shown in Fig. 2, transfection efficiency of by DNA/liposome complexes (9.0±0.34 ng/μl) in spermatozoa was higher than that of DNA solution alone (6.9±0.53 ng/μl), but there was no significant difference.

Additionally, transfection efficiency of spermatozoa by increasing periods of incubation was shown in Fig. 3. The transfection efficiency of spermatozoa incubated with DNA/liposome complexes for 120 min (9.0±0.14 ng/μl) was significantly ($p<0.05$) higher compared with 10, 30 and 60 min (6.0±0.27, 7.1±0.62 and 7.4±0.20 ng/μl, respectively). Transfection efficiency was gradually increased in both treatments for 90 min of incubation.

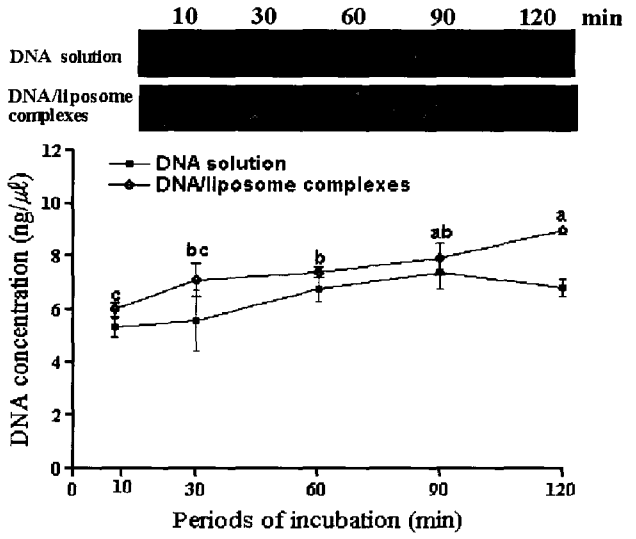

Fig. 3. Analysis of DNA uptake in boar spermatozoa during periods of incubation. [a~c] Different letters above the bar in the liposome/DNA complex indicated significant differences.

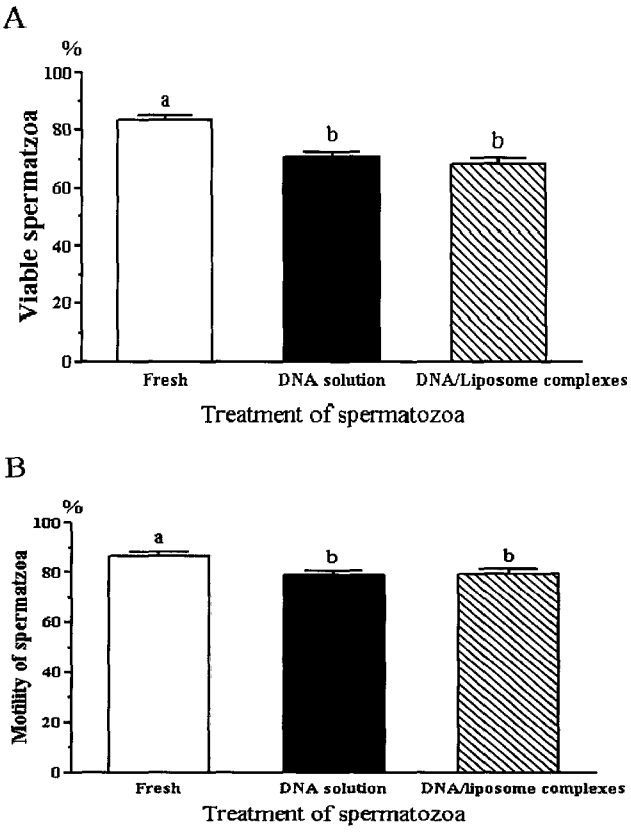

Fig. 4. Viability (A) and motility (B) of transfected spermatozoa. [a,b] Different letters above the bar donate significant differences.

Viability and Motility of Trnasfected Spermatozoa

Viability (A) and motility (B) of spermatozoa incubated with DNA solution or DNA/liposome complexes for 90 min were shown in Fig. 4. Both viability and motility of spermatozoa transfected by DNA solution alone (70.8±1.79 and 78.7±1.59%, respectively) and DNA /liposome complexes (68.0±2.16 and 79.3±2.14%, respectively) were significantly ($p<0.05$) higher than those of fresh spermatozoa (83.3±1.69 and 86.7±1.60%, respectively).

DISCUSSION

In the present study, mtDNA was isolated and purified from chicken liver by alkaline lysis to introduce exogenous DNA into boar spermatozoa. The mtDNA is associated with a generalized physiological decline that is common to all aging organisms. Deletions in mtDNA are responsible for a number of disease often affecting skeletal and cardiac muscle (Holt *et al.*, 1988). Thus transgenic animal introduced with mtDNA could be used to undertake not only expression and function of specific gene but also diseases and aging.

Furthermore, exogenous DNA containing mtDNA can be efficiently transferred into boar spermatozoa incubating with exogenous DNA. This result is consistent with previous report that post-acrosome region of spermatozoa heads could spontaneously be bounded with foreign DNA. There were specific proteins binding the DNA to spermatozoa head that specific molecular might have been structural substrate of the interaction between the exogenous DNA and spermatozoa (Zani *et al.*, 1995). Some DNA fragment binding the spermatozoa could be internalized into genome of spermatozoa, which was associated with CD4 molecules. Spermatozoa from CD4 knockout mice were capable of binding exogenous DNA, but lost the ability to further internalize it. Additionally, there were major histocompatibility complex (MHC) class II molecules as a part of the mechanism between spermatozoa and exogenous DNA. Spermatozoa from MHC class II knockout mice showed a reduced ability to bind DNA compared with spermatozoa from wild-type animals (Lavitrano *et al.*, 1997). Besides, inhibitory factor I (IF-I) blocks to bind spermatozoa with exogenous DNA. IF-I could be isolated from seminal plasmid of variety animals as 37-kDa DNA-binding glycoprotein (Zani *et al.*, 1995). Thus, the complex of spermatozoa and exogenous DNA must be controlled by a variety of conditions, ie, concentration of sperm and DNA, temperature and BSA (Lavitrano *et al.*, 2003).

On the other hand, efficiency of transfection using liposome in spermatozoa was higher than that in DNA solution only. This is in agreement with the report of others studies that liposome is an effective agent for transferring DNA into spermatozoa varying species including mice, bovine and chicken (Rottmann *et al.*, 1996; Shemesh *et al.*, 2000). However, fertilization rate using liposome treated mouse spermatozoa (16%) was lower than that of untreated spermatozoa (53%) (Bachiller *et al.*, 1991). These interpretations could explain that liposome is a good vector for delivering DNA to spermatozoa, but spermatozoa is damaged due to toxicity of high concentration of liposome.

Interaction of exogenous DNA with spermatozoa was also affected by incubation period. In present study, transfection efficiency in the incubation for 90 min was higher than that in other periods of incubation (10, 30 and 60 min). It coincides with the beginning capacitation of spermatozoa. This finding raises the possibility that fertilization by SMGT was first as considered capacitation and acrosome reaction because both are related with fertility and pregnancy (Tardif *et al.*, 1999). Both viability and motility of transfected spermatozoa were lower than those of untreated spermatozoa. However, there were no significant differences of transfection efficiency in tranfected spermatzoa under different treatment. These results were different comparing with other study, because damage of spermatozoa was associated with concentration exogenous DNA and liposome.

In conclusion, liposome could be an efficient agent introducing exogenous DNA into the spermatozoa, and trnsfected spermatozoa could be used in insemination and *in vitro* fertilization to generate transgenic animals.

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