Optimal Condition for Sperm-mediated Gene Transfer by Liposome in Pigs

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ABSTRACT

Production of transgenic animals for studying specific gene has been limited due to a low efficiency, lack of skilled researchers and the need for expensive equipment. Currently, the boar spermatozoa as a vector to deliver exogenous DNA into the oocyte were used to improve the efficiency of transfection rate. In this study, we revealed that the optimal conditions for DNA uptake in spermatozoa by liposome were to 90 min of incubation, 17℃, 10^5 spermatozoa, 4 ng/ml of exogenous DNA and 0.5% (v/v) liposome, without damage to fertility. In addition, the developmental rate to the blastocyst stage of embryo in control group was significantly higher than those embryos with exogenous DNA and liposome, whereas there were no significant differences in embryo development between the liposome and type of DNA. The transfection rates of embryo using treated spermatozoa with both liposome and circular DNA were higher than those using linear DNA. These findings raise the possibility thattreated spermatozoa with liposome/DNA complexes could be used in *in vitro* fertilization, and the exogenous DNA transferred into the oocytes. Taken together, we demonstrated that liposome a vector for the uptake of exogenous DNA in boar spermatozoa could improve the efficiency of sperm-mediated gene transfer in creating transgenic pig and the other domestic transgenic animals.

(Key words : Sperm-mediated gene transfer, Liposome, Exogenous DNA, Pig)

INTRODUCTION

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, microinjection of an exogenous DNA solution and viral infection of exogenous DNA. However, producing transgenic animals has been limited due to low efficiency, lack of skilled researchers and expensive equipment. To resolve these problems, the new method using sperm-mediated gene transfer (SMGT) is based on the ability of spermatozoa to bind and internalize exogenous DNA and to transfer it into the oocyte during 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 internalizes in the sperm genome. The SMGT method appears to be a simple, efficient and applicable to all species that use spermatozoa for reproduction. The SMGT was first applied to rabbit spermatozoa (Brackett *et al.*, 1971), as well as in other species, such as chicken (Rottmann *et al.*, 1992), sea urchin (Arezzo 1989), zebra fish (Khoo *et al.*, 1992) and bovine (Perez *et al.*, 1991). However, inhibitory factor Ⅰ (IF-Ⅰ) has been isolated from the seminal fluid of mammals, which blocks the interaction between exogenous DNA and sperm. In addition, this method has revealed that successfully producing transgenic animals has been limited due to species specificity and a variety of experimental conditions.

Cationic liposome is currently investigated as possible carriers of nucleic acids in gene delivery (Felgner *et al.*, 1995; Liu *et al.*, 1995). Cationic liposome interacts with the negatively charged nucleic acid molecules and form complexes, which can then associate with the negatively charged cell membrane allowing the internalization of nucleic acid. In addition, Comparisons between circular and linear DNA have been performed demonstrating differences in the transfection efficiency (Chancham *et al.*, 2001). Although circular supercoiled DNA has demonstrated a higher efficiency of trans-

* This work was supported by a grant (Code# 20070401034015 and Code# 20070301034040) from BioGreen21 Program, Rural Development Administration, Korea.

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fection when analyzed by protein expressing cell number, linear DNA has been described as more stable in *in vivo* (Chen *et al.*, 2001) and *in vitro* (Chancham *et al.*, 2001) experiments.

To improve the efficiency of SMGT methods in pigs, the optimal conditions for DNA uptake in boar spermatozoa were tested under conditions such as incubation times, incubate temperature, number of spermatozoa, and quantity of exogenous DNA and liposome. Furthermore, boar spermatozoa treated with circular or linear DNA under optimal conditions was used in the *in vitro* fertilization to produce the transfected embryo. Then the developmental rate and transfection rate of the embryo was tested.

MATERIALS AND METHODS

Semen Collection

Semen-rich fractions were collected at the Young-Seo Artificial Insemination Center in the Republic of Korea. Semen was collected once per week from each of the five adult Duroc boars (15~22 months old) by the gloved-hand technique and filtered through cotton gauze into a pre-warmed cup to remove the gelatinous particles. Semen was extended with equal volumes of semen extender (Byu-Ri, SpermGene, Korea) and maintained for 20 min at room temperature. The extended semen was transported to the laboratory at 17℃ within 3 h. The spermatozoa samples used for this experiment exhibited progressive motility of more than 90% when placed on a warming plate at 37℃.

Optimal Conditions for DNA Uptake on Spermatozoa

DNA uptake on spermatozoa was performed by using DNA solution alone or liposome/DNA complexes under different conditions. The exogenous DNA was the pCX-EGFP/NEO transgene encoding the enhanced green fluorescent protein (GFP) reporter gene, cytomegalovirus (CMV) enhancer, chicken β-actin promoter, and rabbit β-globin poly A signal. Liposome/DNA complexes were composed of liposome (Sigma). 10^6 spermatozoa were washed twice in an Androhep extender contained 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 l of distilled H_2O by brief centrifugation (500 ×g, 5 min). The pellet was mixed in the DNA solution or the liposome/DNA complexes depend on different conditions such as period of incubation (from 10 to 180 min), temperature (4, 17, 25, 38.5 and 40℃), exogenous DNA (from 40 to 4×10^5 pg/ml), liposome (from 0 to 5% (v/v)) and number of spermatozoa (from 10^4 to 10^7 spermatozoa). The final volume of the treatment solution was 100 μl.

Assessment of DNA Uptake of Sperm

A transfected spermatozoa under different conditions was washed twice by centrifuging at 500×g for 5 min in an Eppendorf centrifuge tube. The pellet was re-suspended with DNase (15U) and was incubated at 37℃ for 1 hr to remove the exogenous DNA bounded cell membrane of the 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 the forward primer 5′-TGAACCGCATCGAGCTGAAGGG-3′ and reverse primer 5′-TCCAGCGGACCATGTGATCGC-3′. The primers were designed to amplify a 307 bp fragment from the EGFP (GeneBank accession no. AY491379). 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. The negative and positive control was non-treated spermatozoa and 1.2 kb plasmid DNA fragment, respectively. 10 μl of the reaction mixture was then analyzed on a 1.0% agarose gel. The gels were stained with ethidium bromide, and amplified DNA bands were visualized by ultraviolet transillumination. The band intensity was quantified using NIH Image 1.62 (Center for information Technology National Institutes of Health, Maryland, USA). To analyze the quantitation of exogenous DNA on spermatoaoza under different conditions, quantitation of exogenous DNA using band intensity by PCR was estimated by visual comparison of the band intensity with the standards.

Preparation of Oocytes

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory in 0.9% NaCl maintained at 25~30℃. Immature oocytes were obtained by aspirating medium-sized follicles (3~6 mm in diameter) with an 18 gauge needle fixed to a 10 ml disposable syringe. Contents were collected a 50 ml tube (NUNC) and allowed to settle. The supernatant was discarded and the sediment was re-suspended with Dulbecco's PBS (D-PBS; Gibico, USA) containing 0.1(w/v) polyvinyl alcohol (Sigma, USA), 0.1 (w/v) glucose, 0.36 g/l sodium pyruvate and 0.005 g/l phenol red and allowed to settle. This was repeated twice, and then the contents were observed under a stereomicroscope. The oocytes were surrounded by a compact cumulus mass and with an evenly granulated cytoplasm and were selected and washed three times in oocyte maturation medium. The medium used for oocyte maturation was TCM199 supplemented with 0.57 mM L-cystein, 0.5 μg/ml LH, 0.5 μg/ml FSH, 10 ng/ml EGF and 10% (v/v) porcine follicular fluid (pFF).

Then, 20~22 oocytes were transferred into 100 μl of the same medium which had been previously covered with mineral oil (Sigma, USA) in a polystyrene culture dish (35×10 mm) and equilibrated in an atmosphere of 5% CO_2 in air for about 5 hr. After 20~22 hr of maturation culture, oocytes were washed two times and transferred into the same maturation medium but without hormonal supplements for an additional 20~22 hr of culture.

***In Vitro* Fertilization and Culture of Embryos**

After the completion of culture of oocytes for IVM, cumulus cells were removed with 0.1% hyaluronidase in mTBM and washed two times with mTBM containing 0.4% BSA (fatty acid free). The basic medium used for *in vitro* fertilization was modified Tris-buffered medium (mTBM) which consists of 113.1 mM NaCl, 3 mM KC1, 7.5 mM $CaCl_2 \cdot 2H_2O$, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate, 100 IU/ml penicillin G and 50 μg/ml streptomycin. After washing, 15 denuded oocytes were placed in 50 μl drops of the same medium that had been covered with warm mineral oil in a polystyrene culture dish 35×10 mm. The dishes were kept in the incubator for about 30 min until spermatozoa were added for fertilization. The transgene used was pCX-EGFP/Neo encoding green fluorescent protein and contained with CMV-IV enhancer, chicken β-actin promoter and rabbit β-globin poly A. Circular DNA was digested with *Sal* I and run low melting agarose gel. Linear DNA was recovered using the phenol extraction methods. The spermatozoa transfected with or without the liposome under optimal conditions were washed two times by centrifugation at 1,900 ×g for 4 min in Dulbecco's PBS (Gibco, USA) supplemented with 0.1% BSA, 75 μg/ml potassium penicillin G, and 50 μg/ml streptomycin sulfate (pH 7.2). At the end of the washing procedure, the sperm pellet was re-suspended in mTBM medium containing 1 mM caffeine and 0.4% BSA (Fraction V) After appropriate dilution, 50 μl of this sperm suspension was added to 50 μl of the medium that contained oocytes to give a final sperm concentration of 1.5×10^5 cells/ml. Oocytes were co-incubated with spermatozoa for 5~6 h at 39℃ in an atmosphere with 5% CO_2 in air. After sperm-oocyte coincubation, putative zygotes were washed two times in IVP medium, transferred (20~22 zygotes) into 100 μl of the same medium which had been previously covered with mineral oil (Sigma, USA) in a polystyrene culture dish (35×10 mm), and then incubated at 38.5℃ with 5% CO_2 in air. The IVP medium for embryo development was porcine zygote medium-3 (PZM-3) (Yoshioka *et al.*, 2002) containing 0.3% fatty acid free BSA. At 48 hr and 144 hr after IVF, cleavage rate and blastocyst formation were evaluated under a stereomicroscope.

Polymerase Chain Reaction (PCR) of Embryo

The embryos were prepared as previously described (Carballada *et al.*, 2000). Briefly, the zona pellucida of the blastocyst or morulae stage-embryos were removed by repetitive pipetting, and then the cytoplasms of the blastocyst or morulae stage-embryos were washed three times with PBS and transferred into PCR tubes with 10 μl of deionized water. The cytoplasm incubated at 100 ℃ for 5 min and 10 pmol of each primer were added to a PCR mixture tube (AccuPower PCR PreMix; Bioneer, Daejeon, Korea), which contained 1 U of *Taq* DNA polymerase, each deoxynucleosidetriphosphate at a concentration of 250 μM, 50 mM Tris-HCl (pH 8.3), 40 mM KCl, 1.5 mM $MgCl_2$, and gel loading dye. The final volume was adjusted to 20 μl with distilled water. The PCR amplification was performed according to the standard protocol using the forward primer 5′-TGAACCGCATCGAGCTGAAGGG-3′ and reverse primer 5′- TCCAGCGGACCATGTGATCGC-3′. The primers were designed to amplify a 307 bp fragment from the EGFP sequence (GeneBank accession no. AY491379). 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 and extension at 72℃ for 30 sec. Cycle 35 contained an additional extension at 72℃ for 5 min. Normal embryo and DNA fragments were used as a negative and a positive control, respectively. 10 μl of the reaction mixture was then analyzed on a 1.0% agarose gel. The gels were stained with ethidium bromide, and amplified DNA bands were visualized by ultraviolet transillumination.

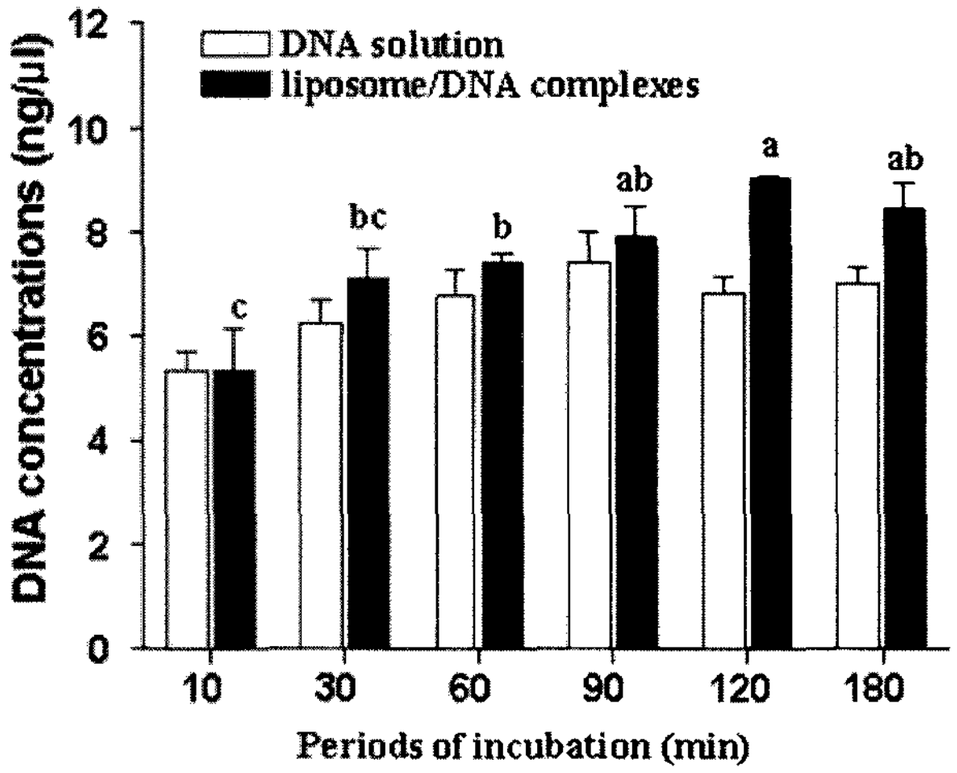
Statistical Analysis

Each experiment was replicated three or 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) using the Statistical Analysis System (SAS version 8.2, SAS Institute Inc., Cary, NC, USA). $p<0.05$ was considered to be statistically significant.

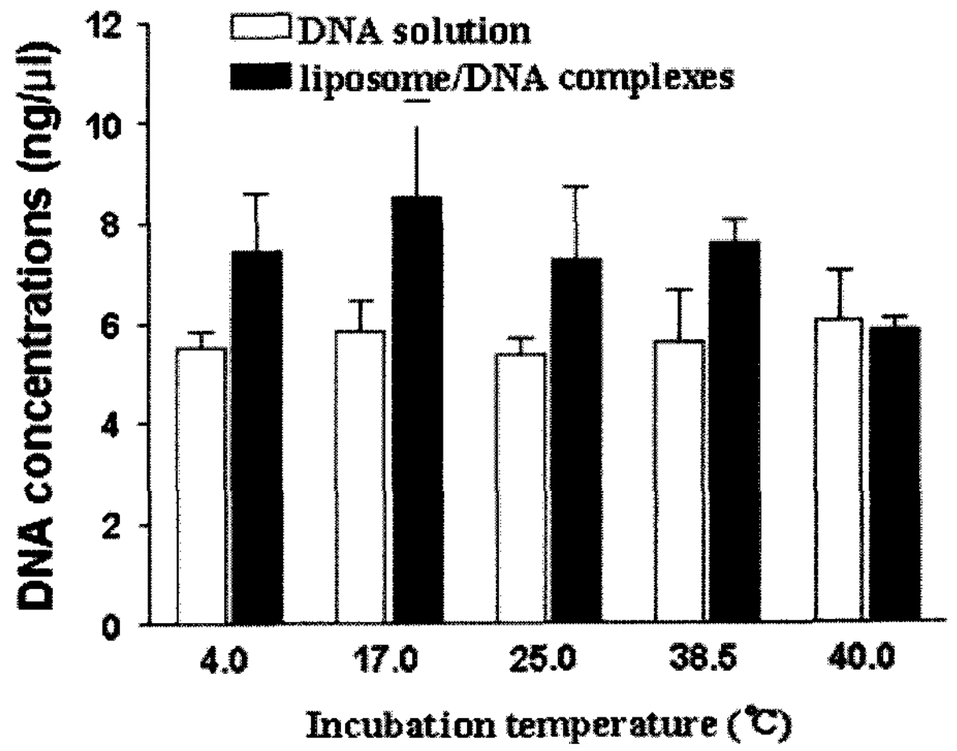
RESULTS

Optimal Condition for DNA Uptake on Spermatozoa

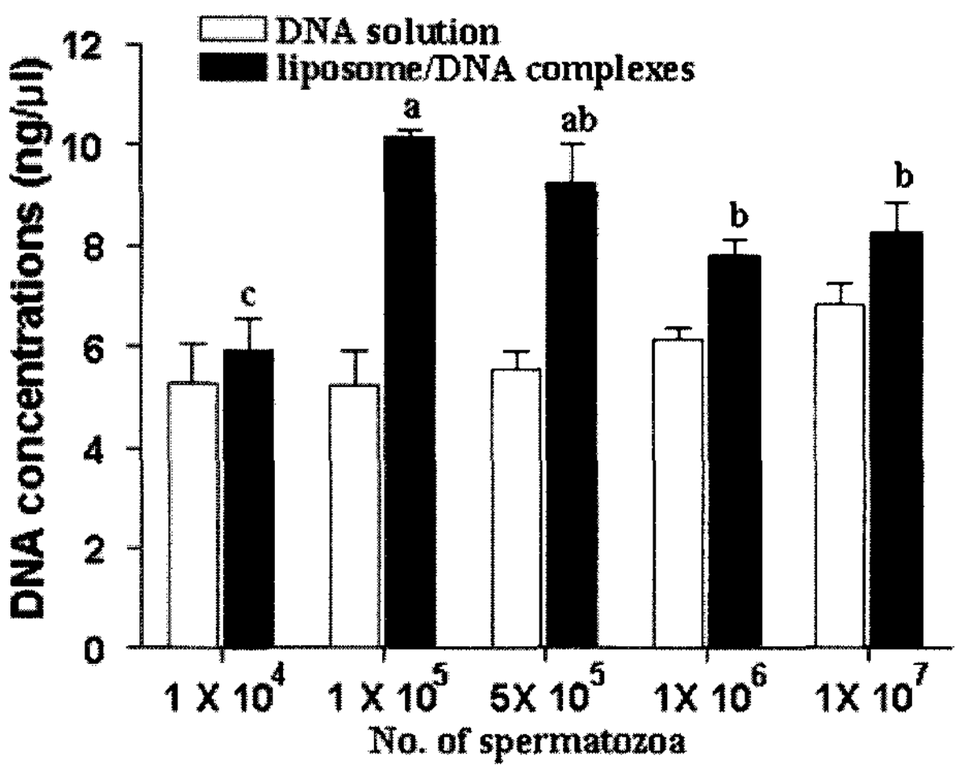
DNA uptake in boar spermatozoa according to incubation time is shown in Fig. 1 (A). Both DNA solution and liposome/DNA complexes rapidly take up most exogenous DNA for the 30~90 min, followed 90 min later by a plateau. DNA uptake in spermatozoa at 90 min of incubation in liposome/DNA complexes was significantly ($p<0.05$) higher than that at 10 min of



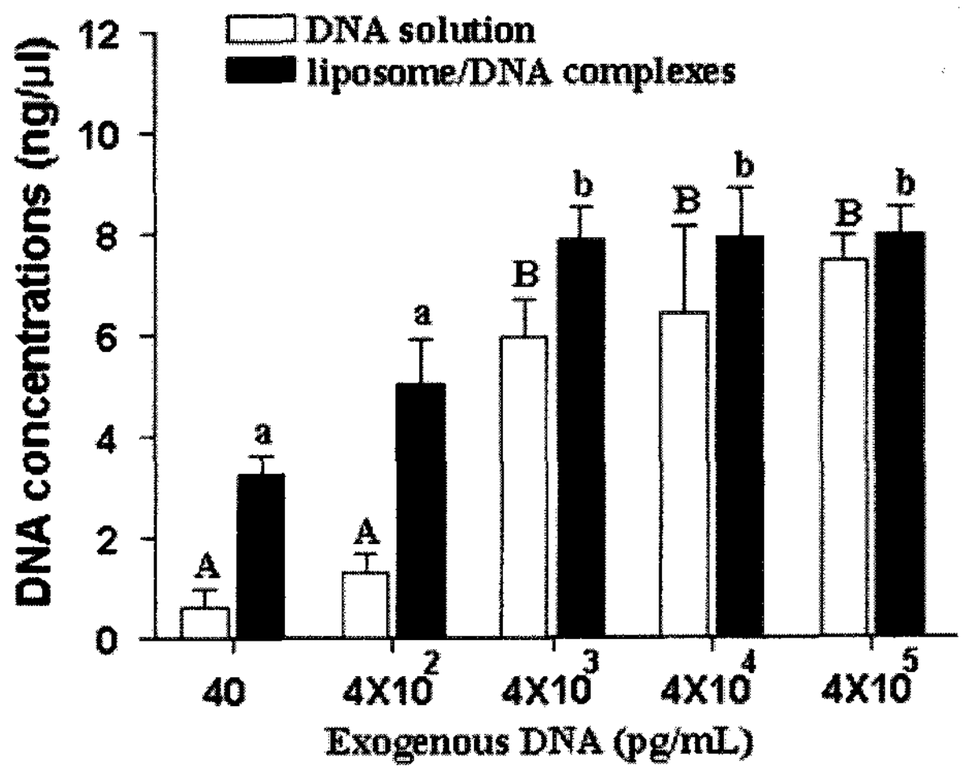
(A)



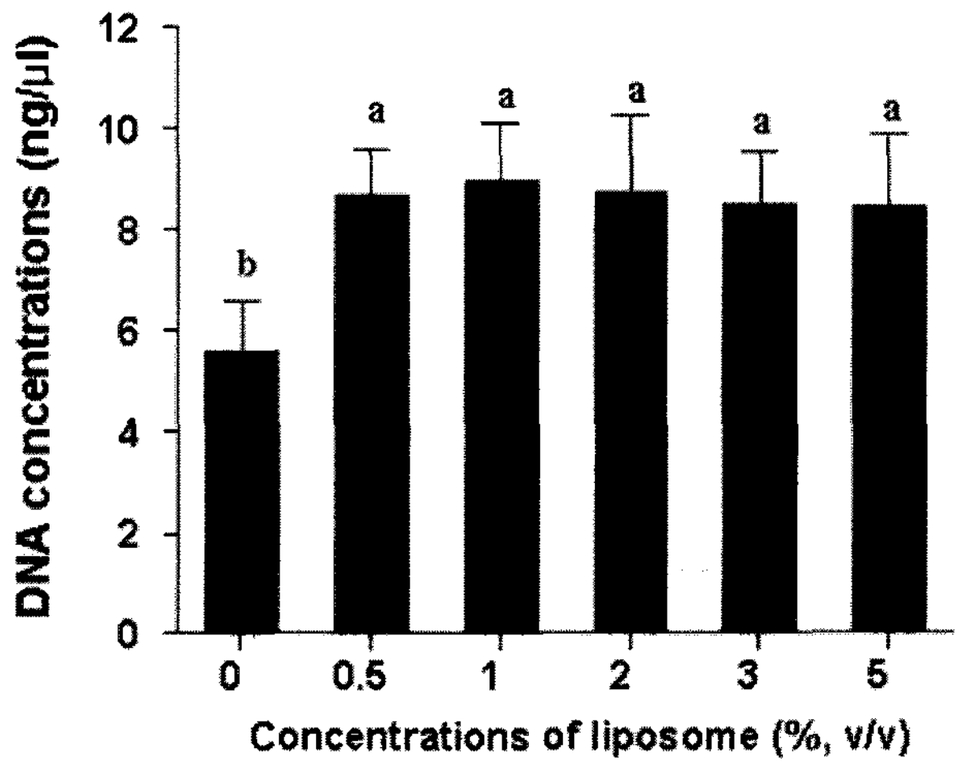
(B)



(C)



(D)



(E)

Fig. 1. Optimal conditions of DNA uptake on spermatozoa by DNA solution (white bar) and liposome (black bar). The boar spermatozoa were treated under different conditions such as incubation times (A), incubation temperature (B), number of sperm (C), exogenous DNA (D), and liposome (E), and then they were incubated with spermatozoa under different conditions. Data are presented as means ± SEM from three replication (n = 3). $^{A,B;a,b,c}$ Different superscript letters above each bar indicate significant differences ($p<0.05$), as determined by ANOVA followed by a Fisher's PLSD as a multiple comparison test.

incubation (7.9±0.59 ng/μl versus 5.3±0.83 ng/μl, respectively). Uptake of spermatozoa by the DNA solution gradually increased for 10 to 90 min. However, there were no significant differences between incubation times.

DNA uptake in boar spermatozoa by incubation temperature is shown in Fig. 1 (B). Although DNA uptake in spermatozoa by DNA solution did not effect by incubation temperature, DNA uptake in spermatozoa by liposome at 17℃ (8.5±1.41 ng/μl) was higher than those at 4, 25, 38.5, and 40℃ (7.4±1.20, 7.2±1.14, 7.6±0.48 and 5.8±0.23 ng/μl, respectively).

Additionally, DNA uptake in spermatozoa by the number of spermatozoa is shown in Fig. 1 (C). DNA uptake of spermatozoa by liposome/DNA complexes was significantly ($p<0.05$) higher in 10^5 spermatozoa (10.2±0.13 ng/μl) than those in 10^4, 10^6 and 10^7 spermatozoa (5.9±0.60, 7.8±0.33, and 8.3±0.64 ng/μl, respectively).

DNA uptake in spermatozoa by quantity of exogenous DNA in both treatments gradually increased from 40 to 4×10^3 pg/ml of exogenous DNA, and then they followed the plateau. DNA uptake of spermatozoa by both treatments were also significantly ($p<0.05$) higher in over 4×10^3 pg/ml of exogenous DNA than those in 40 and 4×10^2 pg/ml of exogenous DNA (Fig. 1 (D)). In addition, DNA uptake of spermatozoa by liposome was significantly ($p<0.05$) higher than that by DNA solution, but there was no significant difference betweens varying concentration of liposome (Fig. 1 (E)).

Developmental Rate of Embryo after IVF using Transfected Spermatozoa

Developmental rates of embryo after IVF with treated spermatozoa with DNA solution or liposome/ DNA complexes are shown in Table 1. There were no significant differences in cleavage rates of embryos between control and treatment groups. However, developmental rates of embryos using treated spermatozoa with liposome/DNA complexes were lower than those of embryos using treated spermatozoa with DNA solution. Additionally, developmental rates of embryo to blastocysts stage was significantly ($p<0.05$) higher in the control group than those by other treatments. However, developmental rates of embryo to four-cell and morulae stage embryo were not significant difference.

Table 2. Transfection efficiency of embryo using SMGT

Type of DNA	Liposome	No. of blastocyst	No. of GFP-positives blastocyst (%)
Circular DNA	-	40	16 (37.8)
	+	37	18 (57.3)
Linear DNA	-	38	11 (34.9)
	+	50	25 (48.3)

Transfection Rate of Blstocyst-Stage Embryo

The transfection rates of blstocyst-stage embryo by PCR assay are shown in Table 2. Transfection rates of embryo after IVF using treated spermatozoa with liposome/DNA complexes (57.3% for circular DNA and 48.3% for linear DNA) were higher than those by DNA solution (37.8% for circular DNA and 34.9% for linear DNA). However, there were no significant differences in transfection rates between the treatment groups.

DISCUSSION

A major goal of this research was to establish, increasing exogenous DNA uptake of spermatozoa by liposome as a novel way of creating transgenic animals. The optimal conditions of DNA uptake for boar spermatozoa were analyzed using liposome under different factors such as incubation time, incubation tem-

Table 1. *In vitro* developmental rates of embryo using SMGT

Treatment	Liposome	No. of oocytes inseminated	No. of oocytes cleaved (%)	No. of embryos development to (%)			
				≤ 2 cell	4cell ≤	≤ Morulae	≤ Blastocysts
Control	-	126	86 (68.3)^{ab}	12 (14.0) ^{a}	55 (64.0) ^{a}		19 (22.0) ^{a}
Circular DNA (7.4 kb)	+	160	90 (56.3)^{b}	11 (12.2) ^{a}	71 (78.8) ^{a}		8 (8.9) ^{b}
	-	128	81 (63.3) ^{ab}	9 (11.1) ^{a}	63 (77.8) ^{a}		9 (11.1) ^{b}
Linear DNA (7.4 kb)	+	180	111 (61.7) ^{ab}	15 (13.5) ^{a}	90 (81.1) ^{a}		6 (5.41) ^{b}
	-	138	103 (74.6) ^{a}	14 (13.6) ^{a}	84 (81.6) ^{a}		5 (4.9) ^{b}

^{a,b} Means with different superscripts were significantly different ($p<0.05$).

perature, number of spermatozoa, and quantity of exogenous DNA, and liposome. Additionally, the developmental rate and transfection rate of embryos fertilized with transfected spermatozoa by not only circular or linear DNA but present or absent liposome were tested.

Because the DNA uptake of spermatozoa was affected by the varying conditions, first of all, optimal conditions for DNA uptake of spermatozoa were investigated. These included incubation time, incubation temperature, number of spermatozoa, and quantity of exogenous DNA and liposome. The results revealed that optimal conditions for DNA uptake of boar spermatozoa were 90 min of incubation, 17℃, 10^5 spermatozoa, and 0.5% (v/v) liposome (Fig. 1). The DNA uptake of spermatozoa also gradually increased according to the increasing exogenous DNA. Furthermore, liposome induced the sufficient DNA uptake of spermatozoa during incubation times. This result is in agreement with previous reports in that an incremental increase in efficiency in DNA binding accompanied by the transfer of exogenous DNA into chicken fetus was obtained using liposome to mediate the DNA association to spermatozoa (Rottmann *et al.*, 1992). In the previous report, the subacrosome region of spermatozoa heads could spontaneously bind with foreign DNA. The specific molecules of spermatozoa head might have been structural substrates for interaction between exogenous DNA and spermatozoa. Some DNA fragment binding the spermatozoa could be internalized into the genome of spermatozoa, which was associated with the CD4 molecules. Spermatozoa from CD-4 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 part of the mechanism of between spermatozoa and exogenous DNA. Spermatozoa from MHC class Ⅱ knockout mice showed a reduced ability to bind DNA compared to spermatozoa from wild-type animals (Lavitrano *et al.*, 1997). This factor, called inhibitory factor Ⅰ (IF-Ⅰ), acts as blocking to bind spermatozoa with exogenous DNA. IF-Ⅰ could be isolated from the seminal plasma of variety animals as DNA-binding glycoproteins (DBPs; 37 - kDa) (Zani *et al.*, 1995). Thus binding and uptake of between spermatozoa and exogenous DNA could be controlled by a variety conditions, i.e., concentration of sperm, DNA, temperature and BSA. These results demonstrated that DNA uptake in spermatozoa by liposome is more efficiently bounded compared to that of DNA alone, because liposome can bind to all surfaces of the spermatozoa, while DNA solution can bind to the DBPs of sperm heads only. DNA uptake in spermatozoa by liposome could be a more sufficient vector in order to transfer genes into the oocyte.

In addition, the fertility of treated spermatozoa with DNA solution and liposome/DNA complexes such as survivability, motility and capacitation was also analyzed, which is not different compared to normal spermatozoa (data not shown). Therefore, we used treated spermatozoa with DNA solution and liposome/DNA complexes for *in vitro* fertilization. Developmental rate to the blastocyst stage of embryo in the control group was significantly higher than those of embryo fertilized with treated spermatozoa, whereas there were no significant differences in embryo development between treatments (Table 1). However, other researcher demonstrated that the rate of *in vitro* fertilization using treated mouse spermatozoa with liposome (16%) was lower than that of untreated spermatozoa (53%) (Bachiller *et al.*, 1991). These results indicate that the 0.5% (v/v) of liposome does not affect the *in vitro* development of embryo without cell damage.

In transgenesis, integration of exogenous DNA into oocytes is very low, this phenomenon generally described to nuclease activity (Hogan *et al.*, 1994). Nuclease destruction is probably more likely within the oocyte rather than the sperm cell, given the generally inert nature of sperm cells. In this study, the transfection rate of the embryo using treated spermatozoa with circular DNA was higher than that using spermatozoa transfected linear DNA. The transfection rates of embryo by liposome using circular (57.3%) or linear DNA (48.3%) was higher than those using circular (37.8%) or linear DNA (34.9%) alone. Thus the transfection of embryo by treated spermatozoa with both liposome and circular DNA was more efficient than liner DNA alone. These interpretations could explain that liposome is good reagent for delivering DNA to spermatozoa, and circular DNA was easily integrated into the genome of an embryo. This is in agreement with the reports from others studies that liposome is an effective agent for transferring DNA to spermatozoa varying species including mice, bovine and chicken (Bachiller *et al.*, 1991; Rottmann *et al.*, 1996; Shemesh *et al.*, 2000).

In conclusion, exogenous DNA under optimal conditions could be sufficiently introduced into spermatozoa by liposome, and then transfected spermatozoa could be used in insemination or *in vitro* fertilization to generate transgenic animals. These findings will provide a novel and efficient method for a generation of transgene-based animal models.

ACKNOWLEDGMENTS

We thank the Director, Young-Seo Artificial Insemination Center, Wonju-si, Republic of Korea for providing the boar semen during the course of the study.

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(Received; 15 April 2008/ Accepted: 11 June 2008)