Techniques for augmentation of exogenous DNA uptake by ovine spermatozoa

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Summary

Sperm mediated gene transfer can be an inexpensive and simple method in animal transgenesis; however its efficiency is poor, mainly due to the spermatozoa's lesser uptake of exogenous DNA. In the present study, the effects of lipofection and other augmentation techniques, such as sperm freezing and spermatozoa treatment with triton X100 and DMSO, on exogenous DNA uptake by sheep spermatozoa and motility of sperms with plasmid uptake were evaluated. In the first experiment, ram sperms were incubated with a complex of rhodamine labeled plasmid (p-EGFP) and Lipofectamine 2000^{TM}. In the second, spermatozoa were treated with Triton X-100^{TM} or DMSO or were frozen without cryoprotectant. The results indicated that there was no significant difference ($P<0.05$) in the transfection rates and in the uptake intensity of lipofected sperms with 300 and 600 ng of plasmid in comparison with control group, i.e. transfected without lipofectamine. Furthermore, lipofection could not improve sperm motility during true plasmid uptake. Almost all of triton X100 treated and frozen-thawed spermatozoa had absorbed foreign DNA, though all were immotile. In spermatozoa treated with 0.1% DMSO, plasmid absorption rate (69.40%) was significantly higher ($P<0.05$) than untreated spermatozoa (57.80%), but sperm motility was not significantly different from control group. In conclusion, lipofectamine® 2000 could neither improve transfection rate, nor support motility in transfected sperms. The methods inducing membrane disruption like, freeze-thaw and triton X100 treatment, can be used in ICSI-sperm mediated gene transfer without the need for sperm selection, provided that they cause no damage to sperm nucleus.

Key words: Lipofection, Sheep, SMGT, Sperm, Transfection

Introduction

Over the past three decades, several methods have been developed for gene transfer into animals. Foreign gene injection into egg pronucleus was the first and most common technique (Gordon and Ruddle, 1981). Nevertheless, due to both its high cost and low efficiency, (particularly in farm animals), this technique has been replaced by other methods.

Sperm mediated gene transfer (SMGT) for developing transgenic animals was first used by Lavitrano *et al*. (1989) in mouse, with a high rate of success (30%). Foreign DNA uptake by sperms has been shown in some other species such as ram and deer (Castro *et al*., 1990), bull (Atkinson *et al*., 1991) boar (Lavitrano *et al*., 2003) and tomcat (Pereyra-Bonnet *et al*., 2008). Anzar and Buhre (2006) found that bull spermatozoa absorbed approximately half of rhodamine-labeled plasmid. Although it was previously reported most of the foreign DNA were attached, possibly to the sperm membrane (Chan *et al*., 1996).

It has been reported that, sperm membrane usually prevents foreign DNA entry into the cytoplasm in SMGT (Maione *et al*., 1997). Efforts have been made to increase the uptake rate of foreign DNA by spermatozoa, such as electroporation (Rieth *et al*., 2000; Tsai, 2000), and use of DNA nanocarriers like liposomes (Collares *et al*., 2011) and magnetic nanoparticle (Kim *et al*., 2010). Besides, relative disruption of cell membrane by chemical treatments including dimethyl sulfoxide (DMSO) (Shen *et al*., 2006), triton X100 (Zhao *et al*., 2012) and also freezing-thawing of spermatozoa (Kurome *et al*., 2007; García-Vázquez *et al*., 2009; Shadanloo *et al*., 2010) can facilitate the entrance of foreign DNA to the cytoplasm.

In our previous study on transfection of sheep spermatozoa with rhodamine-labeled plasmid, no motile sperm with post acrosome absorption of plasmid (true uptake) was seen (Hoseini Pajooh *et al*., 2014).

In the present study, we report the use of lipofection to increase the absorption rate and production of motile sperms with post acrosome absorption of plasmids, as well as chemical treatments and freezing-thawing of ram spermatozoa for augmenting the exogenous DNA uptake.

Materials and Methods

pEGFP plasmid, digested by StuI (EcoR147, Fermentas Inc.) and labeled by tetramethyl-rhodamine-5-dutp (Roch Inc.), was utilized to track the plasmid in sperm transfection experiments.

Spermatozoa preparation

Epididymal sperms were obtained from ram testes, prepared from a local abattoir, according to Blash meth-

od (Blash *et al*., 2000). After transferring testes to the lab in a cooling flask, sperms were obtained by excision of caudal epididymis and transferred to 2-ml tubes containing 500 µL of TCM199 (Gibco Inc.) by a pipette. The sperms were observed under a microscope (NIKON-ECLIPSE E600) and samples with more than 95% motility were selected for experiments. Ejaculated sperms were prepared from four 3–4-year-old rams by electro ejaculator. Samples carried in previously warmed tubes (37°C) were then submitted to the lab, in less than 10 min. The samples with more than 90% motile sperm cells were selected, and washed three times in TCM199 (500 g, 5 min). Sperm cells were then counted and motility was assessed. Eventually, 1×10^6 motile spermatozoa were added to 0.5 ml tubes containing TCM and labeled plasmid in a final valium of 25 µL.

Sperm analysis

Sperm motility was determined under a NIKON microscope (ECLIPSE E600) in ×40 magnification with visible light after incubating with plasmids. To assess the rate, intensity and pattern of DNA uptake, spermatozoa were observed by the same microscope by green filter (510-580 nm) and ×100 magnifying (at least 3 view and 100 spermatozoa). DNA uptake was confirmed by emitting a red light. Uptake intensity was classified as very weak (hardly detectable), weak (well detectable), strong (well bright), and very strong (very bright and shiny).

Statistical analysis

Data were analyzed using statistical software SPSS® (ver. 16) by ANOVA, expressed as the mean±SEM and when ANOVA revealed significant effects, values were compared by the least significant difference pair wise multiple comparison post hoc test. Differences were considered statistically significant at $P<0.05$.

Experimental design

Method 1: Use of lipofectamine in sperm transfection

100, 300 and 600 ng of plasmid with 0.4, 1.2 and 2.4 µL of lipofectamine™ 2000 (invitrogen), respectively, were incubated in 10 µL TCM in RT for 20 min in 1.5 ml tubes. A concentration of 1×10^6 washed ejaculated spermatozoa in 10 µL TCM from each ram was added to these tubes and incubated at 37°C, 5% CO_2 and 95% humidity for 60 min. To assess the effect of incubation time, spermatozoa were incubated with DNA/liposome complex (100 ng/0.4 µL) for 60 and 120 min. After incubating, sperms were examined for DNA uptake rate, intensity and patterns.

To find the effect of presence of BSA in transfection media on DNA uptake and sperm motility, sperms were incubated with DNA/liposome complex (200 ng/0.8 µL) with (6 mg/ml) or without BSA (Bovine Serum Albumin-Fraction V, Sigma) for 60 and 120 min and then were observed.

To detect whether plasmid is absorbed by sperms in lipofection or only attached to the sperm membrane capsulated in liposome, lipofected spermatozoa and transfected spermatozoa with naked DNA (as controls) were washed with PBS and treated by triton X 0.5% in 37°C for 10 min, washed with PBS again, treated with DNase I and observed under fluorescent microscopy.

To find the effect of lipofection in epididymal spermatozoa, sperms from 13 testes were also incubated with DNA/liposome complex (200 ng/1 µL) for 60 to 180 min.

In all of the above experiments, the controls were incubated spermatozoa with plasmid and without lipofectamine.

Method 2: Treatment of spermatozoa with triton X100 and DMSO

Epididymal spermatozoa (n=8) and ejaculated spermatozoa from 4 rams (n=4×2) were washed and treated with 0.05% and 0.1% triton™ X-100, (Sigma-Aldrich) for 10 min in room temperature (RT). In another experiment, epididymal spermatozoa (n=5) were treated with 3% and 0.1% DMSO (Sigma-Aldrich) for 10 min in RT. Non treated spermatozoa from each sample were as controls. After the treatments, sperms were washed in TCM 3 times, incubated with 100 ng plasmid, for 30 min in RT and then examined for rate, intensity and patterns of DNA uptake.

Method 3: Freezing-thawing of spermatozoa without cryoprotectant

Epididymal (n=5) and ejaculated (n=4×2) spermatozoa were suspended in TCM and aliquoted to three tubes. Sperms in one tube were frozen-thawed without cryoprotectant for three cycles. Sperms in the second tube were diluted with the same volume of distilled water to induce membrane disturbance. The third tube was not treated at all (as control). The samples were incubated with 100 ng plasmid for 30 min in RT and examined under fluorescent microscope.

Results

Use of lipofectamine in sperm transfection

In ejaculated spermatozoa, DNA/liposome complex was attached to many sperms throughout the heads (mostly in acrosome and post acrosome regions) and tails then remained for at least 90 to 120 min (Fig. 1). In addition, some sperms absorbed plasmids, merely as non granulated DNA, just like sperms absorbing naked DNA, i.e. homogeneous illumination in acrosome and post acrosome regions (Fig. 2). In lipofected spermatozoa with 100 ng for 60 min, the transfection rate was less than control group, ($P<0.05$), also there was no significant difference between control and experiment groups in experiments with 300 and 600 ng DNA. There was also no significant difference between groups in the rate of plasmid attached to sperms (uptake intensity) and sperm motility (Table 1).

Incubating spermatozoa with the DNA/liposome complex (100 ng/0.4 µL) for 60 and 120 min, resulted in the uptake rate of 25.16 ± 3.26 and 49.83 ± 8.49%, respectively. This showed significant difference between the two groups ($P<0.05$), while there was no significant

difference in uptake intensity. No motile sperm with post acrosome absorption of plasmid was seen in lipofection. However, the sperms with just acrosome absorption of plasmid were motile.

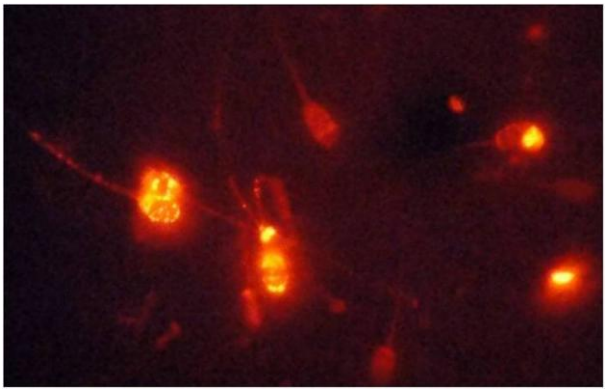

Fig. 1: DNA/liposome complex as microscopic granules attached to sperms through the head and tail

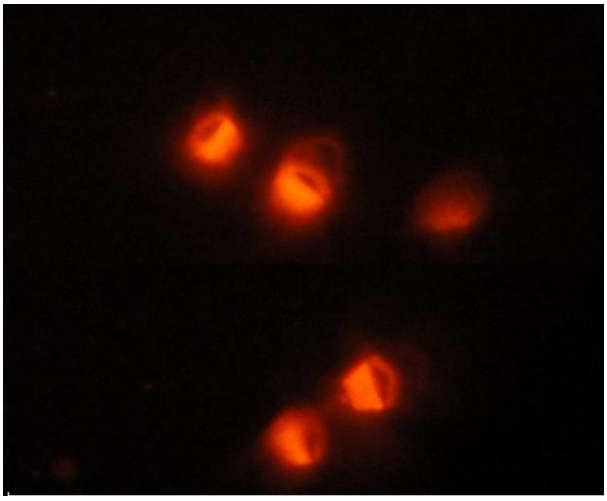

Fig. 2: Transfected sperms with naked DNA (homogeneous illumination in acrosome and post acrosome region)

The uptake rates in spermatozoa incubated with DNA/liposome complex with or without BSA were 35.33 ± 5.71 and 39.50 ± 7.78%, respectively. Likewise, the uptake intensities were 2.58 ± 0.20 and 2.33±0.33%. Addition of BSA did not have a significant effect on the rate and intensity of plasmid uptake. However, motility was significantly higher in BSA group (55.50 ± 4.08 and 46.50 ± 6.04%, respectively). Although addition of BSA did not produce motile absorbed sperms.

Treatment of DNA/liposome complex incubated spermatozoa with DNase I: after PBS washing of spermatozoa incubated with DNA/liposome complex and naked DNA, the fluorescence in the acrosome was gloomy, though strong and shiny in the post acrosome. After treatment with triton X100 0.5% and DNase I, the fluorescence in the post acrosome decreased again and this was more remarkable in spermatozoa incubated with DNA/liposome, so emission turned out to be very weak. The fluorescence in acrosome was quite gloomy and homogeneous.

In epididymal spermatozoa incubated with DNA/ liposome complex (200 ng/µL) for up to 180 min, the uptake rate was negligible, as no plasmid uptake was seen in 7 out of 13 samples even after 2-3 h incubation. Likewise, in sperms with plasmid uptake, the absorption rate was at a maximum of 28%, however this was up to 66% in samples treated with liposome-free plasmids. There was no significant difference in sperm motility between treatment and control groups (30.40 ± 6.75 and 34.00 ± 3.33%, respectively).

Treatment of spermatozoa with triton X100 and DMSO

The pattern of DNA absorption in treated sperms with triton X100 or DMSO was the same as in the untreated or freeze-thawed sperms in which high uptake of foreign DNA in post acrosome and low uptake in acrosome were seen. In triton X100 treated groups, either in ejaculated or epididymal spermatozoa, almost all of sperm cells had absorbed foreign DNA which was significantly different compared with untreated spermatozoa. All spermatozoa treated with triton X100 were immotile (Table 2).

Treatment of epididymal spermatozoa with 0.1% DMSO significantly ($P<0.05$) increased plasmid absorption but sperm motility was not significantly different from control group (Table 3), although all of the plasmid absorbed spermatozoa were non-motile. Spermatozoa treated with 3% DMSO were thoroughly destroyed.

Table 2: Rate of DNA absorption and sperm motility after treatment of spermatozoa with triton X100

Sperm treatment	Spermatozoa	Absorption rate (%)	Motility (%)
Treated	Ejaculated	99.25 ± 0.52	0
	Epididymal	96 ± 1.35	0
Control	Ejaculated	54.25 ± 3.36	32.37 ± 2.77
	Epididymal	40.25 ± 40.20	46.25 ± 3.28

Table 1: Rate and intensity of DNA absorption and sperm motility after 30 min incubation with 100, 300 and 600 ng plasmid with or without liposome

DNA/liposome complex	Liposome	100 ng	300 ng	600 ng
Absorption rate (%)	-	48.75 ± 2.83	54.75 ± 1.70	62.25 ± 1.10
	+	18.75 ± 9.81	32.50 ± 19.16	52.50 ± 14.20
Absorption intensity (%)	-	2.87 ± 0.12	3.12 ± 0.31	4.00 ± 0.00
	+	2.25 ± 0.25	2.50 ± 0.50	3.00 ± 0.40
Motility (%)	-	33.00 ± 11.09	26.00 ± 8.82	21.78 ± 7.18
	+	29.25 ± 11.10	24.00 ± 10.60	25.00 ± 11.36

Table 3: Rate of DNA absorption and sperm motility after treatment of spermatozoa with DMSO

Sperm treatment	Absorption rate (%)	Motility (%)
Treated	69.40 ± 2.89	25.00 ± 3.74
Control	57.80 ± 2.45	27.60 ± 4.20

Freezing-thawing of spermatozoa without cryoprotectant

All of the frozen-thawed sperms incubated with labeled plasmid absorbed it into acrosome and post acrosome as well as sperms that died due to decreased osmotic pressure. Absorption rate in non-treated epididymal and ejaculated sperms were 47.60 ± 2.11 and 55.16 ± 1.85%, respectively.

Discussion

Some studies have shown that DNA absorption rate may rise by use of liposome as a DNA carrier (Harel-Markowitz *et al*., 2009; Thomas *et al*., 2012). Ball *et al*. (2008) showed transfection of equine sperm by liposome increased p32 labeled DNA uptake, while no GFP expression was observed in obtained embryos (Ball *et al*., 2008).

In the present study, we prove that lipofectamine does not enhance sperm transfection. In our method, labeled DNA attached thoroughly to the membrane of sperms as granules, probably enclosed in liposomes. In lipofection, absorption rate increased even after 60 min of incubation, whereas in sperms incubated with DNA without liposome the absorption rate reaches to its maximum rate after 30 to 60 min (Hoseini Pajooh *et al*., 2014). It seems the condensation of attached DNA-liposome granules decreases after 120 min, which is due to their detachment of and/or absorption into sperms. Although, the uptake of plasmids as non granular and homogenous forms by sperms might be due to the absorption of non-liposome enclosed DNA. Hence, liposomes may have no effect on plasmid uptake. In epididymal sperms incubated with DNA/lipofectamine complex, DNA absorption rate was even less than that of ejaculated sperms, as in many experiments there was no visible absorption. Simões *et al*. (2015) evaluated four methods of DNA uptake for sperm mediated gene transfer in bovine including:

1) Incubation with naked DNA without treatment of sperms
2) Plasma membrane alteration induced by calcium ionophore
3) Electroporation
4) Lipofection

with no significant difference between these four expermental groups.

In the present study, treatment of lipofected sperms with DNase I and triton X100 showed that although most of the DNA attach to the sperm membrane as granules, probably enclosed in liposomes, but some DNA can also be absorbed directly by sperms. This finding was somewhat similar to the findings of Cho *et al*. (2002) and Eghbalsaied *et al*. (2013). In their experiments, foreign DNA just attached to sperm membrane (Cho *et al*., 2002; Eghbalsaied *et al*., 2013). Eghbalsaeid *et al*. (2013) also showed only dead or immobile sperms absorbed DNA. Hoelker *et al*. (2007) reported lipofectamine contrary to Fugene6® could not increase the amount of DNA absorption. Surprisingly, like sperm transfection by naked DNA, all of the sperms with DNA adsorption and/or absorption were immotile in transfection by liposome. The presence of BSA had no significant effect on neither transfection rate nor sperm motility.

To increase the DNA absorption rate by spermatozoa, DMSO and triton X100 have been used (Lavitrano *et al*., 2002; Shen *et al*., 2006; Hoelker *et al*., 2007; Yin *et al*., 2009; Zhao *et al*., 2012). Treatments that damage the sperm membrane cause greater capacity of sperm to bind exogenous DNA (García-Vázquez *et al*., 2009). Altered plasma membranes facilitate interactions between exogenous DNA and the sperm chromatin (García-Vázquez *et al*., 2009). However, severe sperm treatments such as quick freeze and TX-100 may damage the sperm nucleus, induce DNA fragmentation, and/or lead to chromosomal breakage with a detrimental effect on next embryonic development (García-Vázquez *et al*., 2009).

In the present study, almost all of the triton X100 treated sperms showed DNA absorption. However, they lost their motility, indicating extreme membrane damages. Shen *et al*. (2006) show GFP expression in embryos by treatment of mouse and rabbit spermatozoa with 3% DMSO for 10-15 min in 4°C followed by IVF. Collares *et al*. (2011) showed that DMSO (3%) treatment of spermatozoa can be an efficient method for transfection in chickens. In the present study however, treatment with DMSO 3% destroyed all of the sperms. Consequently, sperms were treated with lower (0.1%) concentration of DMSO for 10 min. Although no sperm destruction was seen, all of the spermatozoa with plasmid absorbance appeared non-motile. Transfection rate was less than that of triton X100 treatment and more than that of non-treated sperms.

Garcia-Vazquez *et al*. (2009) could produce embryos expressing GFP with the use of sperms treated whit triton X100 or quick freezing (29.3% and 80.43%, respectively). Moreover, Shim showed treating with triton X100 had no harmful effect on the rate of GFP-expressing embryos (Shim *et al*., 2008).

The DNA uptake increases by freezing spermatozoa due to bringing about damages to the membrane (Anzar and Buhr, 2006). In this study, all of the treated sperms had absorbed foreign DNA in post acrosome region. In this method, although DNA absorption is more than the others, the rigorous effect of fragmentation of sperm DNA is to be considered. García-Vázquez *et al*. (2009) could produce GFP expressing embryos with the application of sperm freezing.

Severe sperm treatments such as quick freezing and TX-100 may cause damage to the sperm nucleus, induce DNA fragmentation, and/or lead to chromosomal breakage with a detrimental effect on further embryonic development (García-Vázquez *et al*., 2009). Some

researchers have achieved transgenic embryos by ICSI with the use of sperm membrane damaging methods (García-Vázquez *et al*., 2009; García-Vázquez *et al*., 2010; Li *et al*., 2010) but not by IVF (Wu *et al*., 2009).

In our experiments on ovine spermatozoa, lipofectamine® 2000 could neither improve transfection rate, compared to untreated group, nor support motile transfected sperms. The maximum transfection rates were observed with freezing thawing, as well as with triton X100 treated sperms. However, the detrimental effect of fragmentation on sperm DNA and serious damages are to be considered. The membrane disrupting methods can be used in ICSI-sperm mediated gene transfer, without the need for sperm selection, provided that they cause no damage to sperm nucleus.

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Conflict of interest

There is no financial and personal relationship with other people or organizations that inappropriately influenced or biased our study.

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