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Sperm-mediated gene transfer in poultry

1. The relationship with cock sperm viability

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ABSTRACT

The cock spermatozoa were used in this study as vectors for gene transfer. The objectives of the study were to assess the efficacy of the sperm to uptake exogenous DNA in relation to sperm viability. Two trials were carried out. Trial 1, was achieved to assess the effects of semen dilution (4 µl diluent/1 µl semen), heat incubation (exposure of semen to 37°C for 30 minutes) and the addition of lipofectin on sperm characteristics. No significant effects of heat incubation or semen dilution were mostly observed in the percentages of live, dead and abnormal sperm. The addition of lipofectin at 5% concentration to the diluted semen then heat incubated, showed the lowest detraction in semen characteristics. Trial 2, was designed to assess the effects of lipofectin (5%) addition on the fusion of DNA into the sperm and sperm viability. The exogenous DNA used was the plasmid pUC18. The plasmid DNA was successfully internalized into the sperm treated and un-treated with lipofectin. However, the DNA fragment recognized in the sperm incubated with lipofectin was apparently of higher yield. It was concluded that lipofectin stabilizes and facilitates the fusion of DNA into the sperm.

Keywords: Sperm-mediated gene transfer, cock spermatozoa, lipofectin, sperm viability

INTRODUCTION

Sperm cells can spontaneously incorporate exogenous DNA and transfer it to the eggs during fertilization, thus the exogenous DNA integrates the germ line. The efficacy of binding the DNA molecules to the sperm dramatically increases when the DNA molecules are conjugated with liposomes, and the efficiency of DNA uptake correlates with concentrations of liposomes (Zoraqi and Spadafora, 1997; and Glick and Pasternak, 2003). The fluorescence *in situ* hybridization used by Nakanishi and Iritani (1993), showed that 51.6% of the exogenous DNA-lipofectin-treated sperm retained the exogenous DNA. Fellgner *et al.* (1987) and Sato *et al.* (2003) indicated that lipofectin stabilizes exogenous DNA and keeps it intact. The fertility of cock spermatozoa was detracted when treated with lipofectin (Rottman *et al.*, 1992; and Squires and Drake, 1993). However, Trefil *et al.,* (1996) observed continuous egg fertility for three weeks of hens inseminated with lipofectin-treated spermatozoa. Although the sperm-mediated gene transfer seems applicable in avian species, it would be presumed to apply the artificial insemination to make the sperm mediation of gene transfer practicable. Therefore, the objectives of this study are to examine the efficacy of the sperm to uptake exogenous DNA and to investigate the effects of DNA incubation with sperm on their viability.

MATERIALS AND METHODS

Semen collection and exogenous DNA

Adult males, 5 individuals of 10 months old, of a local broiler male line were used in this experiment. Individual semen samples were collected five times, three days apart. In each, the samples were immediately mixed after collection forming pooled semen.. The exogenous DNA used was the plasmid pUC18 described by Yanisch-Perron *et al.* (1985).

Experimental design

Two trials were carried out to achieve the aims of the study. Trial 1, was proposed to study the effects of semen dilution (4 µl diluent/1 µl semen), heat incubation (exposure of semen to 37°C for 30 minutes using water path) and the addition of lipofectin (5, 10 and 20 µg/100 µl diluted semen) and their combinations on sperm characteristics. The diluent used was Beltsville Poultry Semen Extender (BPSE) developed by Sexton (1977). Trial 2, was designed based on the results obtained in trial 1. So, trial 2, was proposed to assess the effects of lipofectin on the DNA incorporation into the sperm and sperm viability. In this trial the pooled semen was divided, as equally as possible, into three parts. Part 1, was kept un-treated (control). Parts 2, and 3, were diluted with the BPSE (4 µl BPSE/1 µl semen). Part 2, was then mixed with the pUC18 plasmid (2.5 µg plasmid/100 µl diluted semen), then heat incubated. Part 3, was incubated with a mixture of the pUC18 plasmid and lipofectin (2.5 µg plasmid + 5 µg lipofectin/100 µl diluted semen), then heat incubated. The sperm of all treatments were washed twice by adding 500 µl of BPSE to each treatment and centrifuged at 4000 rpm for 5 minutes.

DNA Analysis

DNA extraction: DNA was extracted from the washed sperm of all treatments of trial 2, as well as from the un-treated sperm (control), according to the procedures of Jerzy *et al.* (2003).

Recognition of exogenous DNA in the sperm: The polymerase chain reaction (PCR) was applied to the sperm treatments of trial 2, to recognize the existence of the plasmid DNA in the sperm. Two specific primers, forward (5'-TGACGCCGGGCAAGAGCA-3') and reverse (5'-GGCCGAGCGCAG AAGTGGTC-3'), were used to prime a region of 420 bp long, on the plasmid.

Sperm Motility and Viability

Individual motility of the sperm was assessed, according to Morisson *et al.* (1997). The parameters of spermatozoa viability included percentages of live, dead and live but abnormal sperm were calculated according to Sorensen (1979).

Statistical analysis

The statistical analysis system (SAS, 1999), was applied to the data set of each trial for the analysis of the effect of different treatments on semen characteristics. Multiple range test of Duncan (1955) was used to separate means, whenever significance of treatment effect was shown.

RESULTS AND DISCUSSION

Trial 1

The sperm in un-treated semen (control) showed 90% motility and the percentages of live, dead and abnormal sperm were 94.8, 1.6, and 3.4%, respectively (Table1). The sperm motility in the heat-incubated semen was 79%, and was significantly less than that of the control treatment. However, no significant differences were found in the percentages of live, dead and abnormal sperm between both treatments. The addition of BPSE significantly reduced the individual motility to 79%, however it did not significantly influence the percentages of live, dead and abnormal sperm. The estimates of live, dead and abnormal sperm percentages obtained for all treatments that have undergone incubation and/or dilution, although were, significantly different from those of the control, they were still in agreement with those reported by Morisson *et al.* (1997).

The addition of lipofectin to the BPSE-diluted and heat-incubated semen treatments significantly detracted the motility, and the detraction was positively associated with lipofectin concentration. The motility was 53.3% for the BPSE-diluted, 5% lipofectin-, and heat-incubated semen and reached to 20% for the BPSE-diluted, 20% lipofectin, and heat-incubated semen. Also, the percentage of live sperm has significantly been reduced and that of dead sperm has been increased. The more the concentration of lipofectin, the less the percentage of live sperm and obviously the more the percentage of dead sperm. The percentages of live and dead sperm reached 28.3 and 65.3% in the BPSE-diluted, 20% lipofectin-, heat-incubated semen *vs* 94.8, and 1.6% in the un-treated semen. The percentages of abnormal sperm for semen treated by different lipofectin concentrations varied from 6.1 to 7.2%. These estimates were significantly much higher than that of the control, and were also somewhat higher than the sperm abnormalities estimated for semen samples not treated with lipofectin. The results denote that the heat incubation of semen and/or the dilution with BPSE did not result in much depression in the sperm characteristics. Because of the necessity of using lipofectin to enhance the fusion of DNA into the sperm, therefore, it may be used at low concentration (5%). Bachiller *et al.* (1991) reported a reduction in toxicity of lipofectin on mouse spermatozoa when used with low concentrations.

Trial 2

1. Recognition of exogenous DNA in sperm cells and the effect of lipofectin

Figure (1) shows that the primers successfully primed the DNA substrate. Therefore, the plasmid was used as a positive control and the un-treated sperm treatment was used as a negative control. The

products of PCR for the two experimental treatments were electrophorated on a gel along with the PCR products of the two control treatments (Fig. 2). It is observed that the plasmid DNA was successfully internalized into the sperm in both treatments. However, the fragment recognized in the DNA isolated from the sperm incubated with the plasmid DNA-lipofectin mixture was apparently of higher yield than that recognized in the DNA of the sperm incubated with the plasmid not treated with lipofectin, although the DNA concentration used in both treatments was high (100 ng/ml). This suggests that more molecules of the plasmid DNA were fused into the sperm cells in the presence of lipofectin. Nakanishi and Iritani (1993) reported that the treatment of sperm cells with lipofectin to enhance the exogenous DNA uptake, resulted in having 51.6% of the sperm retained exogenous DNA *vs* 6.3% for the sperm not treated with lipofectin. It was suggested that lipofectin molecules interact spontaneously with DNA forming a lipid-DNA complex that can be easily fused in the plasma membrane. Sato *et al.* (2003) presumed that seminal plasma of chicken males contains DNase activity, that degrades the exogenous DNA, unless the DNA is conjugated with the lipofectin to sustain the mediation of the sperm as vectors for gene transfer. It is concluded that lipofectin stabilizes and facilitates the fusion of DNA into the sperm cells through protecting it from the digestion by DNase. However, the addition of lipofectin may cause retardation on sperm viability. Therefore, the use of lipofectin at a concentration as low as possible, just enough to protect the DNA molecules may not influence the stability of the sperm population viability, which is still of much interest to retain the fertilizing capacity.

2. The interaction between sperm uptake of DNA and sperm viability

The incubation of plasmid DNA with the BPSE-diluted semen significantly detracted motility to 75%, compared to 90% for the control (Table 2). But the detraction was significantly similar to that caused by the incubation of semen at 37°C for 30 minutes (79%). However, the reduction in motility was even more and reached to 56.7% when the plasmid DNA-lipofectin (5%) mixture was heat incubated with the BPSE-diluted semen. The results indicate that the introduction of DNA to the sperm did not disturb their motility and the depression in motility was obviously attributed to the heat incubation of the semen. However, the drastic reduction in sperm motility in the lipofectin-incubated semen obviously transcended the heat incubation effect to include the effect of lipofectin as well. Castro *et al.* (1990) demonstrated a positive association between sperm motility and the incorporation of DNA into spermatozoa.

The percentage of live sperm in the BPSE-diluted, plasmid DNA-incubated semen (90.6%) was significantly less than that in the control (94.8%), and also less than that in the heat- incubated semen (95.7%). The treatment of the semen with lipofectin significantly resulted in much less percentage of live sperm (65.1%). This indicates that the introduction of DNA into the sperm depressed their viability and the depression was more pronounced with the addition of lipofectin. The percentage of abnormal sperm in the semen diluted with BPSE and heat incubated with plasmid DNA was not significantly

different from that of the control (4.0% *vs* 3.4%). However, it was significantly much higher (9.5%) when lipofectin accompanied the exogenous DNA during the incubation with the BPSE-diluted semen. This again indicates the relative damage in the sperm due to the incorporation with exogenous substrates. The results reported in this study may explain the causes of the reduction in fertility of cock spermatozoa reported by Rottmann *et al.* (1992), when liposomes were incubated with sperm cells. However, Nakanishi and Iritani (1993) stated that lipofectin addition to chicken sperm did not affect fertility, although it slightly reduced motility.

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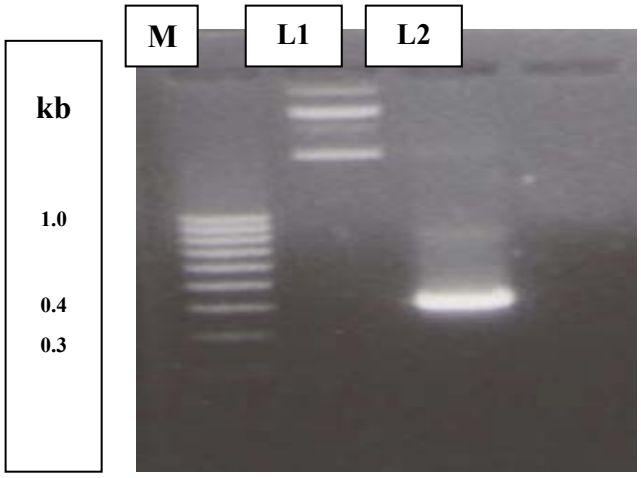

Fig. (1): Profile of the gel electrophoresis of the positive control treatment used in the experiment. Lane 1: the plasmid pUC18, not subjected to PCR analysis. Lane 2: the PCR product of the plasmid pUC18, where the specific primers primed the DNA substrate (420 bp) of the pMB1 replicon. M appriviates for the DNA marker with known molecular size.

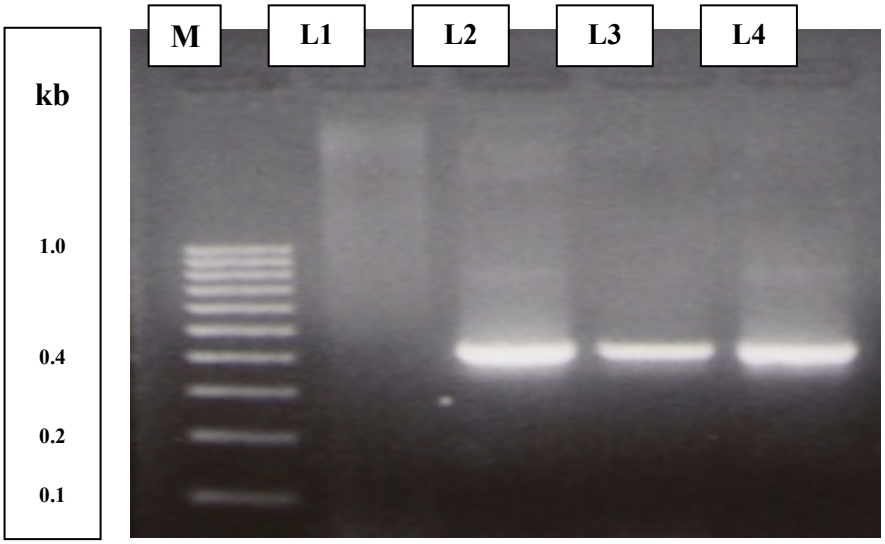

Fig. (2): photograph of the electrophoretic gel of the DNA in the different treatments. Lane 1: the un-treated sperm DNA (negative control). Lane 2: the PCR product of the un-treated plasmid DNA, where the specific band (420 bp) on the *rep* pMB1 was amplified (positive control). Lane 3: the PCR products of sperm incubated with the plasmid DNA. Lane 4: the PCR products of sperm incubated with the plasmid DNA-lipofectin complex. M appriviates for the DNA marker with known molecular size.

Table (1): Summary of fowl sperm characteristics in different treatments (Trial 1).

	$\bar{X}$ ±SE			
Treatment	**Motility, %**	**Live, %**	**Dead, %**	**Abnormal, %**
Non-treated semen (control)	90.0 ±1.6 [a]	94.8 ±0.8 [ab]	1.6 ±0.4 [e]	3.4 ±0.5 [cb]
Heat-incubated semen	79.0 ±2.9 [b]	95.7 ±0.7 [a]	1.2 ±0.5 [e]	3.0 ±0.4 [c]
BPSE [1]-diluted semen	79.0 ±3.3 [b]	93.1 ±0.9 [ab]	2.6 ±0.5 [de]	4.3 ±0.5 [abc]
BPSE-diluted, heat-incubated semen	70.0 ±2.7 [c]	91.8 ±1.4 [b]	3.2 ±0.6 [d]	4.9 ±1.5 [abc]
BPSE-diluted, 5% lipofectin-, heat-incubated semen	53.3 ±3.3 [d]	69.8 ±0.2 [c]	22.9 ±0.5 [c]	7.2 ±0.4 [a]
BPSE-diluted, 10% lipofectin-, heat-incubated semen	33.3 ±3.3 [e]	65.2 ±0.5 [d]	28.7 ±0.2 [b]	6.1 ±0.2 [ab]
BPSE-diluted, 20% lipofectin-, heat-incubated semen	20.0 ±0.0 [f]	28.3 ±0.6 [e]	65.3 ±0.6 [a]	6.3 ±1.2 [ab]

[1], BPSE = Beltsville poultry semen extender.
[a-f], means of different treatments, within trait, with different superscripts are significantly different ($P \leq 0.05$).

Table (2): Summary of fowl sperm characteristics for samples incubated with plasmid DNA (Trial 2).

	$\bar{X}$ ±SE			
Treatment	**Motility, %**	**Live, %**	**Dead, %**	**Abnormal, %**
Non-treated semen (control)	90.0 ±1.6 [a]	94.8 ±0.8 [a]	1.6 ±0.4 [c]	3.4 ±0.5 [b]
Heat-incubated semen	79.0 ±3.0 [b]	95.7 ±0.7 [a]	1.2 ±0.5 [c]	3.0 ±0.4 [b]
BPSE-diluted, plasmid DNA, heat-incubated semen	75.0 ±2.9 [b]	90.6 ±0.9 [b]	5.4 ±0.7 [b]	4.0 ±0.3 [b]
BPSE-diluted, 5% lipofectin-DNA mixture, heat-incubated semen	56.7 ±3.3 [c]	65.1 ±0.9 [c]	25.4 ±0.5 [a]	9.5 ±0.4 [a]

[1], BPSE = Beltsville poultry semen extender.
[a-c], means of different treatments, within trait, with different superscripts are significantly different ($p \leq 0.05$).