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Original article

A method for tracing exogenous DNA uptake in live spermatozoa and embryos

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Abstract

Sperm-mediated gene transfer(SMGT) is a simple method for producing transgenic animals. Due to the lack of repeatability in spermatozoa binding and internalization of exogenous DNA, the efficiency of SMGT is still low. Considering this point, the present work aims to develop a method for evaluating the spermatozoa capacity of binding exogenous DNA after co-incubation with DNA. The main approach is using a Cy5-labelled DNA to trace the exogenous DNA and assess the ability of spermatozoa to take up exogenous DNA. Using this technique, we found that the percentage of spermatozoa that are binding and uptaking DNA is higher at concentration of 10 μg/mL and 100 μg/mL than 5 μg/mL, 1 μg/mL and 0 μg/mL after incubation with Cy5-DNA for 30min at 37°C. After fertilization, the DNA fluorescence signal was also detected in zygotes in groups where spermatozoa were incubated with 10 μg/mL and 100 μg/mL of Cy5-DNA. These results showed a simple and convenient method to trace the exogenous DNA in spermatozoa and zygote when compared to conventional methods of labeling DNA during fertilization, resulting in a real-time observation of the exogenous DNA in spermatozoa and zygote.

Key words: spermatozoa, SMGT, exogenous DNA, IVF

Introduction

Mammalian spermatozoa can spontaneously bind exogenous DNA molecules and transfer them to oocytes after fertilization (Brackett et al. 1971, Lavitrano et al. 1989, Atkinson et al. 1991). Based on this capability of spermatozoa, the sperm-mediated gene transfer (SMGT) method was developed to produce genetically modified animals (Oko et al. 1993, Kuznetsov et al. 2000, Osada et al. 2005). SMGT method is simpler, less expensive, and more user-friendly than other techniques such as DNA microinjection, nuclear transfer, and retroviral infection (Robl et al. 2007). SMGT have been successfully applied to a variety of animal species, including fish (Patil et al. 1996), chicken (Nakanishi et al. 1993), mouse (Maione et al. 1998), and swine (Lavitrano et al. 1997).

For SMGT to be successful, the critical step was that the spermatozoa should bind and internalize exogenous DNA into the head. In some reports, transgenesis can readily be achieved by spermatozoa co-incubated with exogenous DNA directly (Lavitrano et al. 1997, Maione et al. 1998). There were also some methods tested to improve uptake of exogenous DNA into spermatozoa. These include electropora-

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tion (Gagne et al. 1991), lipofection (Bachiller et al. 1991) and magnetofection (Campos et al. 2011) as well as addition of DMSO (Li et al. 2006, Shen et al. 2006) or protamine (Alderson et al. 2006).

Irrespective of the transfection method, many studies have employed different methods to confirm that the spermatozoa could uptake DNA. These include coincubation of spermatozoa with ^{3}H-DNA and evaluating the resulting with liquid scintillation counting , coincubation of spermatozoa with fluoresceintly labelled plasmids and evaluating the resulting with flow cytometry or using genomic PCR method to evaluate the ability of spermatozoa to incorporate exogenous DNA (Atkinson et al. 1991, Hoelker et al. 2007, De Cecco et al. 2010). These methods demonstrate that spermatozoa have the ability to uptake DNA, but the mechanism of binding and internalization of exogenous DNA is a question which still need to be solved. Some visible methods were also used to locate exogenous DNA in spermatozoa, such as autoradiography (Atkinson et al. 1991), wide-field (Anzar et al. 2006) and confocal laser scanning epifluorescence microscopy of fluorescently labelled spermatozoa (Chan et al. 2000) or transmission electron microscopy on thin sections (Parasassi et al. 1997). These studies have shown exogenous DNA binding to the spermatozoa surface, preferentially in the post-acrosomal region, but could not prove whether the exogenous DNA was located inside of the sperm.

Beside the spermatozoa uptaking DNA, the efficiency of spermatozoa to transfer DNA into the oocyte during fertilization remains to be defined. Quantitative Real-Time PCR (qRT-PCR) or genomic PCR were the most common method to detect the exogenous DNA in embryos after SMGT (Eghbalsaied et al. 2013). PCR was simple method, but its results were indirect and couldn't distinguish the position of the exogenous DNA in the embryo or surrounding culture medium. Another method to locate the exogenous DNA was using reporter gene such as green fluorescent protein (GFP) (Eghbalsaied et al. 2013). However, the expression of exogenous DNA was influenced by many factors, so this method was not sufficient to evaluate the ability of spermatozoa to incorporate exogenous DNA into the oocyte.

In the present study, we sought to investigate a simple and efficient method that quantify the ability of spermatozoa to uptake exogenous DNA and transfer its DNA into the oocyte during SMGT.

Materials and Methods

Animals

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 Cy-5 labelled DNA

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

Preparation and Incubation of Spermatozoa with Exogenous DNA

Male ICR mice (8-12 weeks old) were sacrificed by cervical dislocation. Spermatozoa were collected from epididymis ,diluted into 1 mL human tubal fluid (HTF) (Millipore, China) and incubated at 37°C for 40 min to induce the spermatozoa capacitation.

After capacitation, the concentration of spermatozoa was adjusted to 10^{7}/mL in HTF and they were incubated with 0 μg/mL, 1 μg/mL, 5 μg/mL, 10 μg/mL and 100 μg/mL of Cy-5-DNA for 30 min at 37°C. After DNA incubation, spermatozoa were washed three times with DPBS by centrifuging at 500 g for 5 min.

Assessment of Viability and DNA Internalization Rate of Spermatozoa

To examine the effect of DNA on spermatozoa viability, the spermatozoa were incubated with

different concentration DNA at 37° for 30 min. After incubation with exogenous DNA, the spermatozoa were stained by 0.5% trypan blue solution for 15 min. Incubated spermatozoa was applied to a sperm count plate. The experiment was performed 4 times independently. The average number of cells counted per sample was approximately 300. Live spermatozoa percent formula is: spermatozoa viability % = (live spermatozoa count / total spermatozoa) ×100%. Mean percentages (±S.D.) of spermatozoa viability were quantified after spermatozoa were co-incubated with 0, 1, 5, 10, 100 μg DNA. Data were devived from two independent experiments performed in triplicate.

To examine the fluorescence signal of spermatozoa binding labelled DNA. Spermatozoa were co-incubated with 0, 1, 5, 10, 100 μg DNA for 30min. After DNA incubation, spermatozoa were washed with PBS buffer for 3 times. Aliquots of 5 μL from the different experimental groups were sampled for assessment of the DNA internalization rate of spermatozoa. The spermatozoa samples were put on the glass slide and covered with the coverslip. Samples were directly analysed by fluorescence microscopy at excitation wavelength of 530 nm (OlympusBX51, Japan). The positive counting efficiency formula is: positive efficiency% = red fluorescent spermatozoa count / total spermatozoa × 100%. The fluorescence intensity of spermatozoa binding labelled DNA was analyzed by Image J software. The positive signal spermatozoa were counted under the fluorescence. The total number of spermatozoa was counted under bright field. Data are means ± standard deviation of at least two independent experiments performed in triplicate.

The DNase protection assays were conducted to investigate whether the labelled exogenous DNA could be internalized DNA into spermatozoa. After DNA incubation and DNase I treatment, spermatozoa were fixed, stained with Hoechst 33342 and analysed by confocal microscopy. After DNA incubation and washing 3 times, spermatozoa were treated with 10U DNase I at 37°C for 60 min. Then spermatozoa were washed two times with DPBS by centrifuging at 500 g for 5 min. Aliquots of 5 μL from the different experimental groups were sampled for assessment of the DNA internalization rate of spermatozoa. The spermatozoa samples were put on the glass slide and covered with the coverslip. Samples were directly analysed by fluorescence microscopy at excitation wavelength of 530 nm (OlympusBX51, Japan). The internalization of positive counting efficiency formula is: positive efficiency % = red fluorescent spermatozoa count / total spermatozoa × 100%. The fluorescence intensity of spermatozoa binding labelled DNA was analyzed by Image J software. After DNA incubation and DNase I treatment, the number of positive signal spermatozoa was counted under the fluorescence. The total number of spermatozoa was counted under bright field. Data are means ± standard deviation of at least two independent experiments performed in triplicate.

In Vitro Oocytes Maturation and *in Vitro* Fertilization

To detect whether spermatozoa could transport the labelled DNA into the oocyte, spermatozoa were incubated with fluorescently labelled DNA and used for IVF. Oocytes were fertilized with spermatozoa co-incubated with or without Cy5 labelled DNA. One day post-fertilization, zygotes were washed with PBS buffer and zona was removed by mechanical means. Then, zygotes were fixed, stained with Hoechst 33342 and analysed by fluorescence microscopy. Female ICR mice (8-12 weeks old) were used as oocyte donors and mice were superovulated by an intraperitoneal injection of 7.5 IU pregnant mare serum gonadotropin followed by 5 IU hCG 48 hours later. At 14 to 15 hours after hCG injection, females were euthanized by cervical dislocation and their oviducts were removed aseptically. Oocytes were washed 3 times in HTF and were transferred into IVF droplet (250 μL HTF, covered by paraffin oil). Spermatozoa co-incubated with or without exogenous DNA were added to the HTF at final concentration of 1×10^6 /mL. After 4-6 hours of coincubation, oocytes with second polar body were removed and washed four times. Then, the oocytes were cultured in KOSM medium (95 mmol/L NaCl, 2.5 mmol/L KCl, 0.35 mmol/L KH_2PO_4, 0.2 mmol/L $MgSO_4 \cdot 7H_2O$, 0.2 mmol/L glucose, 10 mmol/L sodium lactate, 25 mmol/L $NaHCO_3$, 0.2 mmol/L sodium pyruvate, 1.71 mmol/L $CaCl \cdot 2H_2O$, 0.01 mmol/L EDTA, 1 mmol/L L-glutamine, 0.1 mmol/L EAA, 0.1 mmol/L NEAA, 4 mg/mL BSA) at 37°C with addition of 5% CO_2. The embryos at the cleavage and blastocyst stage were observed by microscopy 1day and 4.5 days *in vitro* culture.

Hoechst 33342 staining and fluorescence microscopy

Spermatozoa co-incubated with or without exogenous DNA and zygotes co-incubated with spermatozoa for 4 hours were stained using Hoechst 33342. Spermatozoa were washed three times in DPBS and were fixed in 4% (w/v) paraformaldehyde/4% (w/v) sucrose in DPBS for 40 min at room temperature. Then spermatozoa were stained with

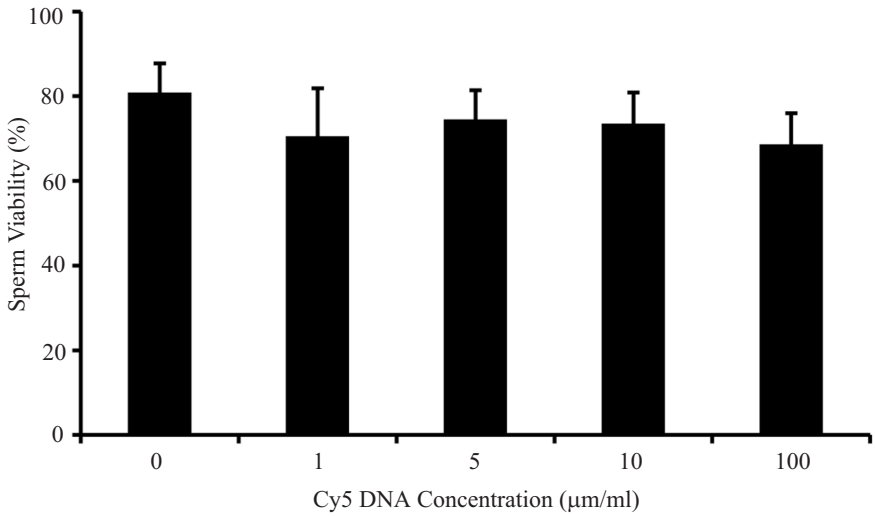

Fig. 1. Effect of DNA concentration on spermatozoa viability after spermatozoa incubated with DNA. Mean percentages (±S.D.) of spermatozoa viability were quantified after spermatozoa were co-incubated with 0, 1,5,10, 100 μg/mL DNA. Data of two independent experiments performed in triplicate. **p<0.05 means differ significantly.

5 mg/ml Hoechst 33342 for 8 min and then washed twice in DPBS. The spermatozoa were put on the glass slide, antifade mounting medium was added and sealed with nail polish. Zygotes were processed the same way, except that the zona pellucida was removed by mechanical means in FHM Hepes-buffered medium (Millipore, China) as previously described (Cieslak et al. 1999).Then, the zygotes were fixed in 4% (w/v) paraformaldehyde/4% (w/v) sucrose. Samples were assessed by fluorescence microscopy at excitation 530 nm and 480 nm wavelength (OlympusBX51, Japan).

PCR of single blastocyst

Blastocysts were washed three times in DPBS. The zona pellucida was removed by mechanical means in FHM Hepes-buffered medium (Cieslak et al. 1999). Then blastocysts were washed three times in DPBS and incubated with 5U DNaseI at 37°C for 1 hour. Blastocysts were washed three times in DPBS. A single blastocyst was transferred to the wall near the bottom of a 0.2-mL PCR tube using a glass micropipette. Thereafter, 5 μL of blastocyst lysis buffer (Beyotime, China) was gently added to each tube. Each PCR tube was incubated at 95°C for 10 min in a PCR machine. The blastocysts DNA solution was stored at -20°C until used.

Reaction system included 12.5°C μl of Ex Taq Premix, 1°C μl of upstream primer, 1°C μl of downstream primer and 4°C μl of blastocysts DNA, then ddH2O was added to 25°C μl. PCR conditions were as follows: 94° denaturation for 5 min; 94°C denaturation 30 s, 60.5°C annealing 30 s, 72°C extension 30 s, 33 cycles; 72°C final extension 10 min. Primers sequence was: upstream 5'-TGG GCG TGG ATA GCG GTT TGA CT-3'; downstream 5'-CCG TCG TCC TTG AAG AAG ATG GT-3'.

Statistical Analysis

Experimental data were expressed as mean ± SEM. Statistical analysis was performed using statistical SPSS19 software (Somers, NY). Statistical significance was determined using the ANOVA regression table. $P<0.05$ was considered to be statistically significant.

Results

Effect of Exogenous DNA Concentration on Spermatozoa Viability

Obstained results showed that the viability of spermatozoa were 80.08 ± 6.86%, 70.58 ± 11.27%, 74.51± 6.86%, 73.52 ± 7.35%, 68.63 ± 7.35% after incubation with 0, 1.0, 5.0, 10.0 and 100 μg/ml DNA, respectively (Fig. 1). The viability of spermatozoa was slightly lower in 1.0, 5.0, 10.0 and 100 μg/ml DNA group than it in 0 μg/ml group. However, the viability of spermatozoa was not significantly different between the 0, 1.0, 5.0, 10.0 and 100 μg/ml DNA groups.

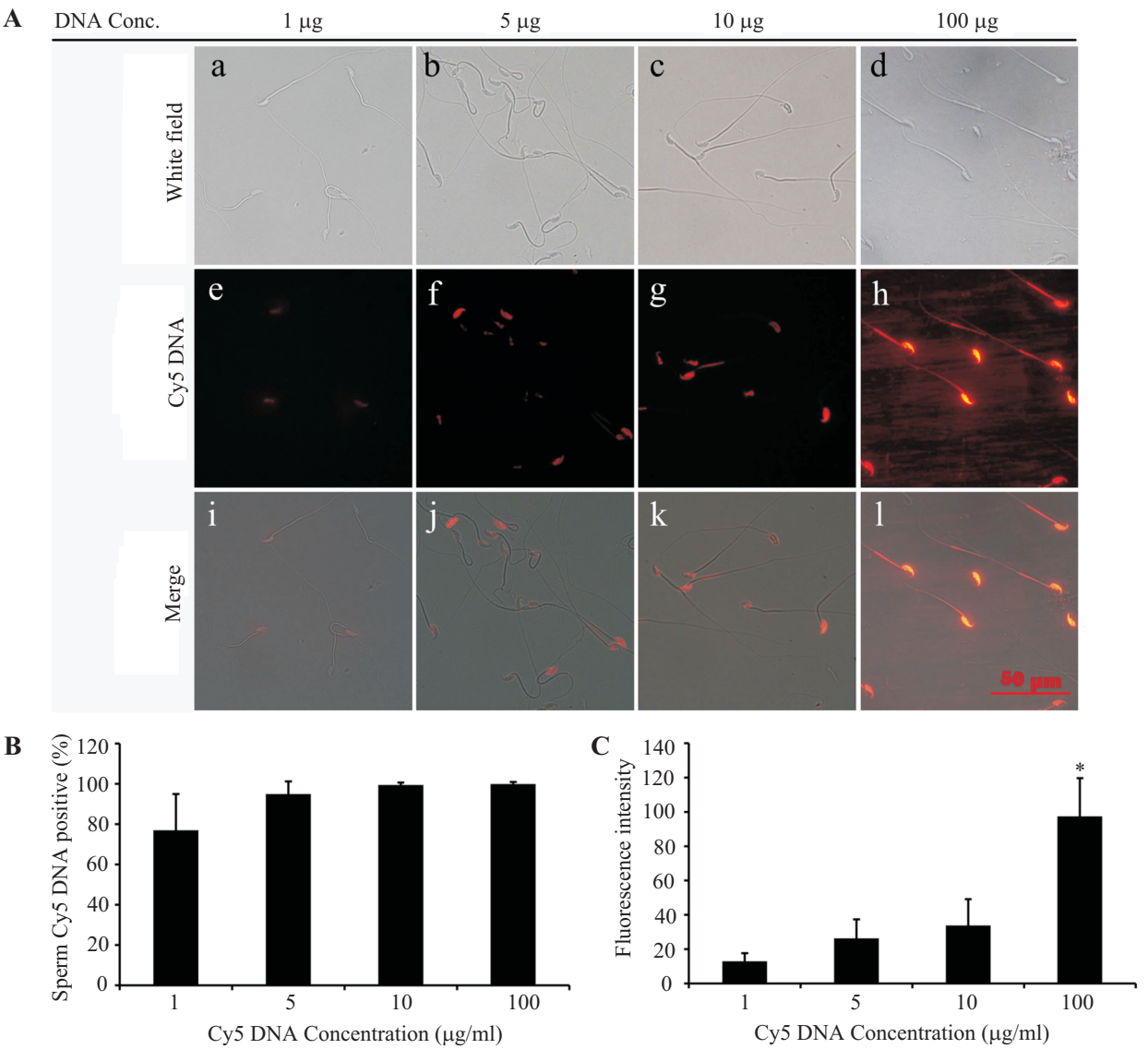

Fig. 2. Binding of Cy5-labelled DNA to spermatozoa. (A) Fluorescence signal of spermatozoa binding labelled DNA. Spermatozoa were co-incubated with 0, 1, 5, 10, 100 µg/mL DNA for 30 min. (e, f, g, h) fluorescence signal in spermatozoa after co-incubation with DNA, (a, b, c, d) corresponding bright field and (i, j, k, l) merged images. (B) The percent of positive signal in spermatozoa after incubation with DNA. Data are means+standard deviation of at least two independent experiments performed in triplicate. **P<0.05 means differ significantly. (C) Fluorescence intensity of spermatozoa binding labelled DNA. The intensity of fluorescence signal in spermatozoa was analyzed by Image J software. Data are means±standard deviation of at least two independent experiments performed in triplicate. **p<0.05 means differ significantly.

Live spermatozoa bind labelled DNA and fluorescence intensity increase with the DNA concentration

The results of fluorescence microscopy revealed that the presence of labelled DNA in spermatozoa were 76.94 ± 17.96%, 94.91 ± 6.29%, 99.40 ± 1.19%, 99.85 ± 1.023% after incubation with 1.0, 5.0, 10.0 and 100 µg/ml labelled DNA, respectively (Fig. 2A). The percent of spermatozoa showing the labelled DNA was significantly higher in 10.0 and 100 µg DNA groups than in 1.0 and 5.0 µg groups. The mean percent of spermatozoa showing the labelled DNA did not differ between 10.0 and 100 µg groups (Fig. 2B). The fluorescence intensity of Cy-5 DNA in spermatozoa were significantly higher in 100 µg DNA group than those in other groups (Fig. 2C). This means that there was a higher degree of fluorescent signal in spermatozoa when more DNA was added to the assay.

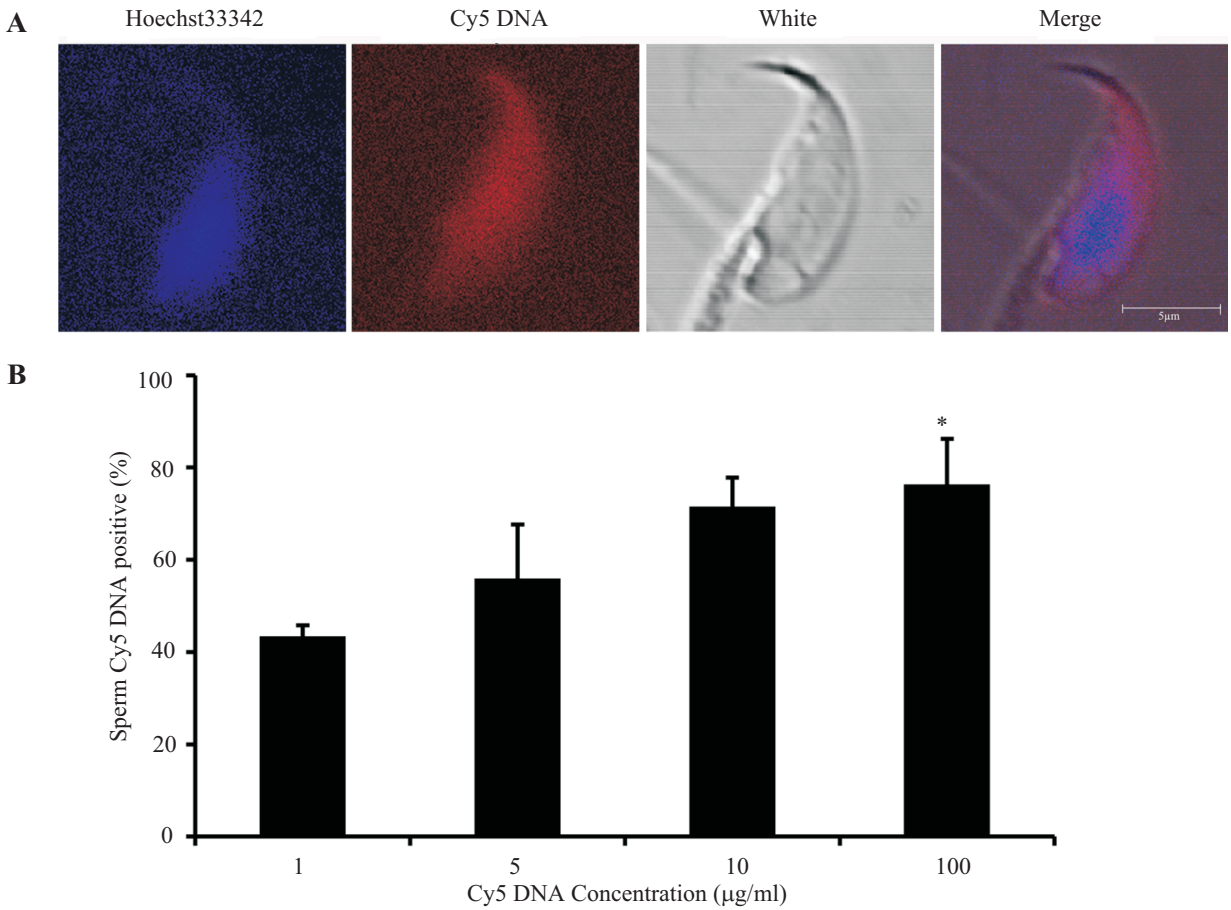

Fig. 3. Uptaking of Cy5-labelled DNA to spermatozoa. (A) Position of exogenous DNA in spermatozoa. (B) The percent of positive signal in spermatozoa after DNase I treatment. Data are means+standard deviation of at least two independent experiments performed in triplicate. **p<0.05 means differ significantly.

Spermatozoa uptakes exogenous DNA and protects exogenous DNA from DNase I

Obtained results showed that the location of labelled exogenous DNA was at the nucleus of spermatozoa head (Fig. 3A). After DNase I digestion, the proportion of spermatozoa which still had fluorescent signal were 43.41 ± 2.40%, 55.98 ± 11.67%, 71.56 ± 6.28% and 76.34 ± 9.88% in 1.0, 5.0, 10.0 and 100 μg/ml DNA groups, respectively (Fig. 3B). The highest proportion of DNase-resistant DNA was present in the 10.0 and 100.0 μg group, which did not differ significantly (71.56 and 76.34% respectively).

DNA-associated spermatozoa carry DNA into oocytes

After fertilization, the fluorescence signal could be observed in the zygotes in groups where spermatozoa were incubated with 10.0 and 100.0 μg DNA (Fig. 4A). There was no fluorescence signal detected in 1.0 and 5.0 μg group at zygotes stage (data not show). In Fig. 4A, one oocyte was fertilized and the another was not fertilized. In the unfertilized oocyte, there was no fluorescence signal in the oocyte while there was obvious fluorescent signal in fertilized oocyte. This means that the fluorsecent signal was derived from labelled DNA, but not background fluorescence. The percentage of fluorescence signal-positive zygotes was 10.00 ± 7.78% and 28.79 ± 8.14% in the 10.0 and 100.0 μg group (Table 1). The positive rate of fluorescence signal in zygotes was significantly higher in the 100.0 μg group than in 10.0 μg group. Following in vitro development, the cleavage and blastocyst rates were not significantly different between 0, 10.0 and 100.0 μg groups. No fluorescence signal was detected in blastocysts which came from oocytes fertilized with spermatozoa co-incubated with any concentration of labelled DNA (Table 2). However, by single blastocyst PCR testing, the DNA positive rate of blastocysts was 10.37 ± 5.42% and 25.50 ± 8.77% in the IVF blastocysts derived from spermatozoa that were co-incubated with 10.0 and 100.0 μg/ml of DNA (Table 2).

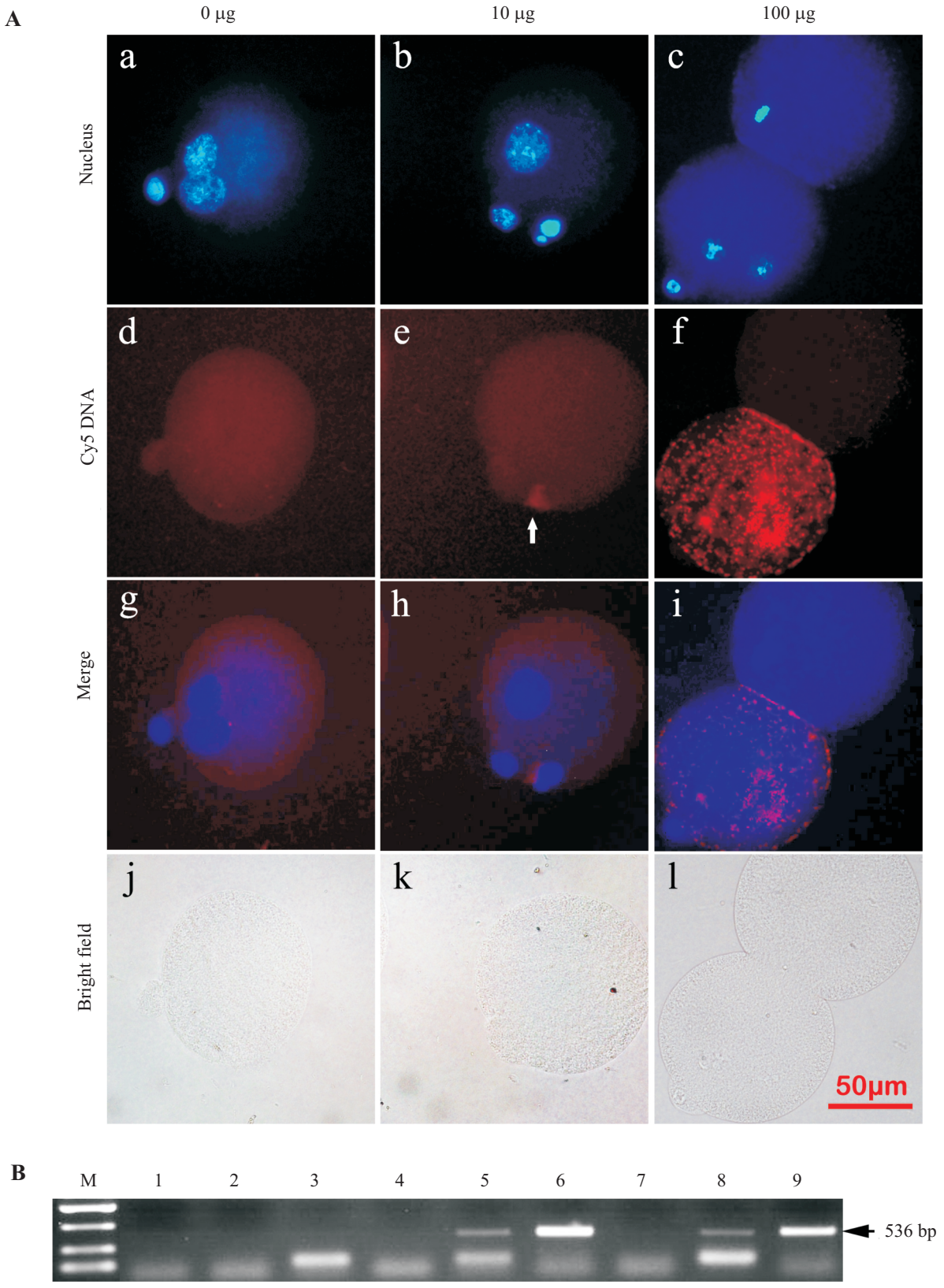

Fig. 4. Transfering of Cy5-labelled DNA into oocyte by spermatozoa. (A) Detection of labelled DNA in zygote by fluorescence microscopy. (a, b, c) fluorescence signal of nucleus in zygotes, (d, e, f) fluorescence signal of Cy5-labelled DNA in zygotes, (g, h, i) corresponding merged images and (j, k, l) corresponding bright field. (B) Detection of labelled DNA in blastocyst by PCR. (1, 2) no template controls, (3, 4, 5, 6, 7, 8) the blastocyst samples, (9) the positive control.

Table 1. The effect of the concentration of exogenous DNA on sperm-mediated gene transfer efficiency.

Treatment	Oocytes	Fertilization(%)	Cy5 signal positive zygote (%)
0 μg/mL	94	61 (63.81 ± 10.76)[a]	0(0)[a]
10 μg/mL	117	77 (66.28 ± 11.34)[a]	6 (10.00 ± 7.78)[a]
100 μg/mL	123	88 (71.61 ± 9.73)[a]	23 (28.79 ± 8.14)[b]

The same row, different letters indicate significant differences ($p<0.05$).

Table 2. Embryonic development and the efficiency of transgenesis after the sperms were incubated with Cy5-DNA for IVF.

Treatment	Oocytes (%)	Fertilization (%)	Cleavage (%)	Blastocyst (%)	DNA positive blastocyst (%)	Cy5 signal positive blastocyst (%)
0 μg/mL	313	247(79.35±8.65)	220(89.17±6.63)	184(84.19±8.99)	0/20 (0)	0(0)
10 μg/mL	316	240(76.12±7.23)	218(91.04±7.10)	178(82.76±6.85)	14/135(10.37±5.42)[a]	0(0)
100 μg/mL	321	237(74.54±7.21)	210(89.37±6.31)	170(81.16±8.42)	41/165(25.50±8.77)[b]	0(0)

The same row, different letters indicate significant differences ($p<0.05$).

Discussion

A simple and reliable method to trace the DNA during IVF is necessary to evaluate the efficiency of SMGT method. The most common method for tracing DNA was DNA labeling. In the previous study, many markers were used to label DNA such as ^{3}H, digoxigenin (DIG) and fluorescent dye, etc. ^{3}H-labelled DNA was determined by liquid scintillation counting and required protective measures for worker (Atkinson et al. 1991, Fernandeza et al. 1999). DIG-labeled DNA was usually detected by FISH or Southern blot method and required a corresponding digoxin antibody (Sciamanna et al. 2000, Garcia-Vazquez et al. 2011). Compared to other DNA markers which required specialized equipment or reagents, fluorescent dye labeled DNA could be directly detected by fluorescence microscopy in live sperm, (De Cecco et al. 2010, Eghbalsaied et al. 2013). In this method, fluorescent dye Cy5 was used to labell DNA. After spermatozoa were incubated with Cy5-labelled DNA, the position of the DNA in the sperm and zygote could be observed directly under a fluorescence microscopy.

Although many studies had concern SMGT during the last 30 years, the effect of exogenous DNA on spermatozoa viability and motility remains unclear (Garcia-Vazquez et al. 2011, Eghbalsaied et al. 2013). There were many studies showing that the exogenous DNA had a negative effect on the motility of bovine spermatozoa (Rieth et al. 2000, Anzar et al. 2006), while others did not show any effect (Rieth et al. 2000, Alderson et al. 2006, Feitosa et al. 2010). Transgenic pigs have been repeatedly reported after using in utero insemination with sperm which was co-incubated with 50.0 μg/mL of exogenous DNA (Lavitrano et al. 2002, Lavitrano et al. 2003, Manzini et al. 2006). In our study, we showed that incubation with high concentration, such as 10.0 and 100.0 μg/mL of exogenous DNA had a slight negative effect on spermatozoa viability but it was not statistically significant as compared to other groups. After IVF, the cleavage rate and blastocyst rate of spermatozoa were not statistically significantly different between those of different concentration of exogenous DNA groups.

This method could assess the ability of spermatozoa to uptake exogenous DNA. In previous studies, exogenous DNA binding to the sperm surface, preferentially in the postacrosomal region, has been extensively reported (Eghbalsaied et al. 2013, Spadafora 1998). In this study, almost all the spermatozoa associated with Cy5-labelled DNA at the location of the sperm head after incubation with those of different concentration of exogenous DNA groups. Following DNase treatment, less than half of these spermatozoa retained their fluorescent label in 1 μg/mL DNA group. This reduction is in agreement with the previous studies using radioactively (Anchordoguy et al. 1991) or fluorescently labelled DNA (Carballada et al. 2001). However, there were still more than 70% spermatozoa retained their fluorescent label in 10.0 and 100.0 μg/mL DNA groups. These results indicated that the spermatozoa-associated DNA was protected from DNase digestion. Our finding is consistent with previous studies in which exogenous DNA was protected from DNase digestion via internalisation (Anchordoguy et al. 1991, Numata et al. 2010).

Besides addressed labelled DNA uptake into spermatozoa, this method could also evaluate the ability of spermatozoa to transfer DNA into the oocyte upon fertilization. In previous studies, using sperm co-incubated with fluorescently labelled DNA for IVF, the

results showed that 30% of all embryos produced the expected exogenous DNA band after genomic PCR (Eghbalsaied et al. 2013). However, this fluorescent signal disappeared in zygote after IVF. The possible reason was that the concentration of DNA incubated with spermatozoa was low with 1 or 5 μg/ml DNA, so fluorescence signal could not be detected in zygote after fertilization under this concentration (Eghbalsaied et al. 2013). In our study, when we used 10 and 100 μg/ml DNA to incubate with spermatozoa, the fluorescence signal could be observed in zygote after fertilization. After repeated washing, removed zona pellucida and DNase treatment of zygote, the fluorescence signal still could be detected and high-intensity signals appeared around the male pronucleus. These results suggested that DNA-associated spermatozoa could take DNA into oocytes by IVF and the fluorescence intensity was dose-depended. However, the fluorescence signal could not be detected at blastocyst stage: the possible reason may be the exogenous DNA was degraded by nucleases or diluted by cell division and the fluorescence signal became weakened.

In conclusion, we established a simple method to evaluate the capacity of spermatozoa to bind, internalize and transport exogenous DNA. The major advantages of this approach were to evaluate the co-incubation system of spermatozoa with DNA and establish a high efficiency co-incubation system to improve spermatozoa uptake of DNA. This method is helpful to set up a reliable transfection methods during SMGT and have practical value of efficiently producing transgenic blastocysts.

Acknowledgements

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