Sperm-mediated gene transfer into oocytes of the golden hamster: Assessment of sperm function

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The possibility of sperm as a vehicle to deliver foreign DNA to oocytes was tested in hamsters. Epididymal spermatozoa, incubated with linearized plasmid DNA encoding ovine growth hormone (pCMXoGH), showed a spontaneous tendency to interact with DNA. Kinetics of sperm uptake of DNA was determined by using \(^{32}\)P-labeled DNA. Spermatozoa took up the added DNA by 15-30 min and the uptake was inhibited by human seminal fluid in a dose-dependent manner. Addition of DNA did not affect the functional competence of spermatozoa, in terms of their ability to undergo capacitation and acrosome reaction (34.5\%±2.2 vs 35\%±1.5). The fertilizing ability of DNA treated-spermatozoa from hamsters and humans was assessed by zona-free hamster egg penetration assay. Number of sperm penetrated per oocyte were 23±4.5 and 1.4±1.3 for hamster and human spermatozoa, respectively. Penetrated oocytes harbored sperm-treated DNA both with hamster (30.2 cpm/oocyte) and human (19.2 cpm/oocyte) spermatozoa. These results show that the hamster and human spermatozoa have a strong tendency to interact with exogenous (foreign) DNA and are able to transfer DNA to oocytes. Sperm may be used as a vector for DNA transfer and this approach has potential in the production of transgenic animals.

For producing transgenic animals, particularly in the mouse, DNA microinjection of pronuclear-stage eggs has been successfully used\textsuperscript{1,2}. However, given the difficulties encountered with this technique, alternate methods to produce transgenic animals have been attempted with varying degrees of success\textsuperscript{3}. The sperm-mediated delivery of DNA into oocytes has been demonstrated in the mouse by the pioneering work of Lavitrano and co-workers\textsuperscript{3,4}, resulting in about 30\% of the offspring being transgenic. This sparked off research on sperm-mediated gene transfer\textsuperscript{6,7}. There has also been a report on the unsuccessful attempts to reproduce the results\textsuperscript{7}. However, subsequent studies carried out by several other groups confirmed and extended the findings of Lavitrano in a variety of species from echinoids to mammals\textsuperscript{6-12}.

The use of spermatozoa as natural vectors for genetic transformation offers a unique route. This would be particularly attractive, since it requires neither expensive, sophisticated equipments nor the high level of technical expertise associated with the conventional genetic transformation methods, including micromanipulation technique. The sperm-mediated DNA delivery method has been successfully employed in mammals such as mice\textsuperscript{14}, rabbits\textsuperscript{12}, cattle\textsuperscript{14} and pigs\textsuperscript{15}.

While information is available on the basic studies on sperm-DNA interaction, there is an urgent need to obtain detailed comparative data on the sub-cellular localization of exogenous DNA in spermatozoa and study molecular regulation of its uptake by spermatozoa in mammals. It is believed that there is a set of secreted ( seminal component, IF-1) and sperm-bound proteins which modulate the interaction between sperm chromatin and the added DNA\textsuperscript{6,16,17}. These have to be studied in detail using a variety of mammalian species. Comparative data is lacking on the cellular and molecular mechanisms involved in the sperm-mediated delivery of foreign DNA. This approach could potentially be applied in different animal species and it is of extreme value in animal biotechnology. Moreover, it is advantageous in species which are refractory to microinjection and it is useful in carrying out mass transgenesis which can be performed at the field level as well.

In this study, we report sperm-mediated gene delivery to oocytes in the golden hamster. We chose this animal model since it offers several advantages in terms of studying finer details of sperm function, sperm-oocyte interaction and post-fertilization.
development. We show that hamster spermatozoa are capable of exogenous DNA uptake, the DNA-bound hamster spermatozoa undergo capacitation and acrosome reaction (AR) and are able to transfer the DNA to oocytes.

Materials and Methods

Preparation of spermatozoa—Golden Hamsters (Mesocricetus auratus; 3–4 months), used in the study, were from our institute colony. Procedures for handling and experimentation followed the guidelines on the Use of Laboratory Animals for Research (INSA, New Delhi). Animals were sacrificed by cervical dislocation. Cauda epididymal spermatozoa were obtained by puncturing distal tubules using a sterile 26.5 g needle. About 10 μl of caudal content was suspended into 35 mm petridishes (Greiner Labortechnik, Frickenhausen, Germany) containing 3 ml of pre-equilibrated, filter sterilized (Sartorius AG D-3400 Gottingen, Germany) TALP-PVA medium i.e., modified Tyrode's solution supplemented with 3 mg/ml bovine serum albumin, 10 mM lactate and 0.2 mM pyruvate and 1 mg/ml polyvinyl alcohol. The medium had an osmolarity of 285–300 mOsmols/Kg water and a pH of 7.4 after equilibration. About 1–2 million spermatozoa were placed in dishes containing 2 ml medium and incubated for 6 hr at humidified atmosphere of 5% CO₂ in air at 37°C. For preparation of human spermatozoa, semen samples, collected from healthy volunteers with consent were allowed to liquefy (20–30 min). 1 ml each of semen and TALP-PVA medium were mixed and centrifuged in a sterile tube at 700 rpm for 10 min. Supernatant was removed and the sperm pellet was washed three times. After this, the sperm pellet was overlaid with 0.5 ml of the medium and left undisturbed for 30 min. The count and motility of the swim-up sperm were determined and about 1–2 million spermatozoa were cultured as above.

Preparation of plasmid DNA—The plasmid DNA (pCMXoGH; 5.16 kb) used in the present study was an eukaryotic expression vector encoding the ovine growth hormone under the control of cytomegalovirus promoter. BamHI-linearized unlabeled or radiolabeled DNA was used. Radiolabeling, using random primers, was carried out by the method of Feinberg and Vogelstein. The specific activity of labeled DNA was ≈10² cpm/μg.

Kinetics of sperm DNA uptake—Different amounts of spermatozoa (4x10⁶, 1x10⁶ and 20,000) were cultured with the radiolabelled DNA (1.5x10⁵ cpm; ≈15 ng) and samples were recovered at various time points (0, 15, 30, 60, 120 and 240 min). Samples were washed thrice in Dulbecco's phosphate buffered saline (DPBS) and radioactivity measured using a scintillation counter. In all samples, negligible radioactivity remained in the supernatants after the 3rd wash while the sperm pellet retained substantial amount of radioactivity. DNA was extracted from sperm pellets, electrophoresed on 1% agarose gels and subjected to autoradiography. Extracted DNA was not subjected to any restriction enzyme digestion.

Preparation of human seminal fluid and inhibition of DNA uptake—Human seminal fluid (hSF) was prepared according to Lavitrano et al. by sequential centrifugation of freshly ejaculated semen. Sperm were sedimented at 700 g for 10 min. Supernatant was further centrifuged for 1 min at 12,000 g in a microfuge. About 1x10⁶ hamster spermatozoa were incubated for 30 min with increasing volumes of hSF: 1, 10, 20, or 40 μl. After this, radiolabeled DNA (1.5x10⁵) was added for an additional 30 min. Washing, counting and autoradiography were carried out as described above.

Slot blot and PCR analysis—Total DNA isolated from sperm was spotted on nitrocellulose filters for slot blot analysis using standard protocols. The cDNA encoding oGH was amplified from sperm DNA samples in a PCR reaction using the following primers:

5' AGCCATATGCTCGAGATGATGGCTGCAGGC CCC
3' and
5'ACGAA TTCGGATCCCT AGAAG GCGGCGCAGC
3'

The PCR products were visualized on 1.5% agarose gels after ethidium bromide staining.

Effect of DNA on sperm capacitation and acrosome reaction—Sperm capacitation was assessed by carrying out sperm motility assay. Briefly, 1–2 million spermatozoa were suspended in 3 ml TALP-PVA medium in 35-mm petridishes, without or with DNA (1 μg). At 2, 4 and 6 hr time points, the percentage motility and quality of motility were scored. Sperm motility index (SMI) was calculated by the formula: SMI: (% motility × quality). After 6 hr, sperm were washed twice with DPBS and DNA was extracted as described. The extracted DNA was used for slot blot and PCR analyses as detailed above.

Quantitative AR was assessed by observing the presence or absence of the acrosomal cap on viable spermatozoa under bright-field microscope (40X).
Viable spermatozoa from a total number of sperm examined were counted by eosin-Y staining.

*Hamster egg penetration assay*—Oocytes, collected from oviducts of adult superovulated (PMSG/hCG) female hamsters, were treated with hyaluronidase (1 mg/ml) containing 0.01 mg/ml of soybean trypsin inhibitor in TL-HEPES-PVA medium to remove cumulus mass. They were made zona-free by treatment with acid Tyrode’s medium (pH 4). Hamster or human spermatozoa, after 3 hr culture, were incubated with radiolabeled DNA (5x10^5 cpm) in 100 µl coincubation drop overlaid with mineral oil. To each sperm sample, 20 zona-free oocytes were added and coincubated for additional 3 hr. After this, oocytes were washed, scored for sperm penetration status using an Olympus IMT-2 inverted microscope with Nomarski DIC (X10 & X20) objectives. DNA was extracted from oocytes and sperm and analyzed for the presence of pCMXoGH as before.

Statistical analysis—The data in Figs 2-5 represent mean±SEM from a minimum of 3-6 replicate experiments. Differences in mean values (Figs 4-5) were analyzed using Student’s t-test.

Results

The ability of hamster spermatozoa to take up foreign DNA (pCMXoGH) was demonstrated both by slot blot analysis as well as PCR. oGH DNA was detected only in DNA isolated from sperm treated with pCMXoGH (Fig. 1A, lanes 3, 4 and Fig. 1B lanes 3, 4) but not in DNA isolated from untreated sperm (Figs 1A, lanes 1, 2 and Fig. 1B, lanes 1, 2). The sperm pellet was washed three times with DPBS before DNA isolation. Trace amounts of oGH DNA could be detected only in the first wash (Fig. 1B, lanes 7, 8) but not in the subsequent washes (Fig. 1B, lanes 9-12) indicating that the added DNA detected by PCR in the sperm pellet was not a contamination of the DNA present in culture medium.

Hamster spermatozoa bound a substantial amount of exogenously added DNA within 30 min of encounter, regardless of their capacitated state (Fig. 2A). The kinetics of uptake of DNA increased over a period of time and the quantum of increase was maximum when a higher (4x10^6) concentration of spermatozoa was used, followed by lower concentrations (Fig. 2A). It was estimated that 1.4x10^3 DNA molecules were associated with a single

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**Fig. 1**—Slot blot (A) and PCR (B) analyses of pCMXoGH DNA uptake by hamster spermatozoa. About 4x10^7 hamster spermatozoa were incubated with 5 µg/ml (slot-blot) or 1 µg/ml (PCR) of Bam HI-linearised pCMXoGH at 37°C for 6 hr. Total DNA was extracted and analysed by slot blot analysis. Panel A, lanes 1 & 2: DNA-untrcatcd sperm (control); lanes 3 & 4: sperm treated with pCMXoGH. Panel B, lanes 1 & 2: DNA from control sperm pellets, lanes 3 & 4: DNA from sperm treated with pCMXoGH, lanes 5 & 6: supernatants of control sperm after 1st wash, lanes 7-12 supernatants of pCMXoGH treated-sperm after 1st wash (7-8), 2nd wash (9-10) and 3rd wash (11-12), lanes 13, 14, & 15: positive controls of 2, 0.2, 0.02 mg of oGHcDNA, respectively. The arrow indicates the PCR product of expected size. The arrow head indicates primers/carrier tRNA.
sperm. Under these conditions, the DNA-untreated samples failed to show any uptake. The intensity of bands obtained at different time points correlated with the amount of labeled-DNA bound to spermatozoa (Fig. 2B). Moreover, this interaction was not DNA sequence-specific since similar results were obtained when different plasmid DNAs, such as pCMX\(\beta\)gal, were used (data not shown). The sperm-DNA interaction was specific, as observed by the dose dependent inhibition of sperm DNA binding by the hSF with 20 \(\mu\)l of it, inhibiting DNA uptake by about 80\% (Fig. 3A). This was also evident during electrophoretic analysis of sperm DNA. The presence of increased amounts of hSF proportionately decreased sperm bound DNA (Fig. 3B).

Influence of addition of foreign DNA on sperm function was evaluated. Upon addition of DNA, there was no change in either capacitation (Fig. 4) or AR (Fig. 5) of hamster spermatozoa. The SMI followed the normal pattern of increase with time during 2 to 6 hr period for both control and DNA-treated samples (Fig. 4, panel C). There was no significant difference in the SMI values between the control and DNA-treated samples at these time points; also in the quality of motility (panel A). However, we observed differences in the % motility at 15 min and 2 hr \((P < 0.1)\); this was not observed at the 4th and 6th hr time points (panel B). Exogenous addition of DNA did not also affect the percentage of spermatozoa undergoing AR compared to untreated controls (Fig. 5) both at 3 hr \((34.5\pm2.3\% \text{ vs } 35\pm1.5\%)\) and 6 hr \((38\pm3.1\% \text{ vs } 40\pm2.3\%)\).

The results so far indicated that hamster spermatozoa have an inherent ability to take up foreign DNA without being compromised in capacitation and AR processes. We then examined the ability of hamster and human spermatozoa to transfer foreign DNA into zona-free hamster oocytes. The results were presented in Table 1. As expected the mean numbers of spermatozoa per penetrated oocytes for hamster and human spermatozoa were 23.45 and 1.41.3, respectively. All the penetrated oocytes contained the radiolabeled DNA, though the amount varied i.e., 30.2 cpm/oocyte and 19.2 cpm/oocyte for

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**Fig. 2**—(A) Kinetics of uptake of exogenous DNA by hamster spermatozoa. Radiolabeled pCMXoGH \((1.5\times10^5 \text{ cpm})\) was incubated with varying amounts of spermatozoa (i.e., \(2\times10^4, 1\times10^5\text{ and }4\times10^5\)) at 37°C as described in the text. Samples were withdrawn at different time points i.e., 0, 15, 30, 60, 120 and 240 min, washed thoroughly (3 times) and supernatants and pellets were measured for radioactivity in a scintillation counter. (B) Electrophoretic analysis of DNA from hamster spermatozoa incubated with radiolabeled DNA. Undigested sperm DNA was loaded on 1% agarose gels, electrophoresed, dried and subjected to autoradiography. Lane 1: positive control DNA, lanes 2 to 7: DNA from sperm \((4\times10^5)\) incubated with \([^3P]-pCMXoGH \(1.5\times10^6 \text{ cpm}\) for 0, 15, 30, 60, 120 & 240 min, respectively. The arrow head indicates linearized pCMXoGH DNA.
hamster and human spermatozoa, respectively. Under these conditions, the uptake of DNA by oocytes (without sperm) was very minimal (7.2; Table 1).

**Discussion**

Our results demonstrate that hamster spermatozoa spontaneously bind exogenous DNA and are able to deliver the DNA to oocytes without compromising their functional competence in terms of their ability to undergo capacitation and AR. Hamster spermatozoa exhibit two important features for transgenesis i.e., to capture DNA and to transfer it into eggs. The DNA uptake by sperm is not a sequence-specific phenomenon since, any DNA added is taken up by hamster spermatozoa. This indicates that the use of sperm as a gene transfer vehicle could be a promising approach for transgenesis in mammals. The observations made in our study, for the first time in hamsters, on the sperm-mediated DNA delivery to oocytes are consistent with those reported for other mammalian species such as mouse, rabbit, pig, and cattle.

The kinetics of DNA uptake by hamster spermatozoa (Fig. 2) that we observed has been quite interesting. Approximately, $1.4 \times 10^5$ DNA (pCMXoGH) molecules are associated with each sperm cell. This is quite significant in view of the

<table>
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<th>Oocyte treatment</th>
<th>No. of oocytes inseminated (penetrated)</th>
<th>No. of sperm/penetrated oocyte</th>
<th>$[^3]P$-DNA (cpm) in penetrated oocytes</th>
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<tr>
<td>Nil</td>
<td>50</td>
<td>18 (penetrated)</td>
<td>362</td>
</tr>
<tr>
<td>Hamster sperm</td>
<td>50</td>
<td>23±4.5</td>
<td>1510</td>
</tr>
<tr>
<td>Human sperm</td>
<td>40</td>
<td>1.4±1.3</td>
<td>768</td>
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*Zona-free hamster oocytes were coincubated with either hamster or human spermatozoa (or no sperm: nil). At the end of 3 hr, oocytes were washed, scored for sperm penetration and radioactivity measured. Results are from 3 replicate experiments.

Fig. 3—(A) Influence of human seminal fluid on the uptake of exogenously added DNA by hamster spermatozoa. Hamster spermatozoa ($1 \times 10^7$/ml) were incubated with increasing volumes (0, 10, 20 and 40 μl) of human seminal fluid for 30 min at 37°C in 5% CO₂ in air. Radiolabeled DNA ($1.5 \times 10^5$ cpm) was then added for additional 30 min. Spermatozoa were then washed, total DNA extracted and measured for radioactivity. (B) Electrophoretic analysis of undigested hamster sperm DNA showing inhibition of bound DNA by human seminal fluid. About one million spermatozoa were treated with $[^3]P$-pCMXoGH (1.5×10⁵ cpm). Lane 1: control sperm DNA incubated with labeled DNA pCMXoGH, lanes 2, 3 & 4: samples treated with 10, 20 and 40 μl of human seminal fluid. The arrow head indicates linearized pCMXoGH DNA.
very low (negligible) signal to noise ratio observed while using radiolabeled DNA, similar to that observed in other species. We observed that the hamster sperm-DNA interaction can be inhibited by the hSF in a dose dependent manner (Fig. 3), similar to that reported in the mouse.

It would be quite interesting to study the mechanism of DNA uptake by hamster spermatozoon. Recently, Zami et al. provided information on the molecular mechanisms underlying the uptake and the internalization of DNA. Specific DNA binding proteins (30-35 KDa) on the sperm head responsible for this interaction have been demonstrated and also the inhibition of sperm-DNA interaction by IF-1.

In this regard, our preliminary results, in the hamster, are quite encouraging. The human SF-derived factor responsible for the inhibition DNA binding is a protein of approximate Mr, 36,000. We believe that the reason for DNA-sperm (via the putative protein) interaction is the negative charge of the DNA molecules binding to presumptive (acidic) protein in the sperm head. These observations eliminate the possibility that the hSF-induced inhibition of DNA uptake in sperm is not an artifact as a consequence of degradation of the sperm-bound DNA. Studies are underway to identify the specific sperm DNA binding proteins responsible for this DNA-protein interaction by South-Western analysis and also to localize these proteins on the sperm head using fluorescent-labeled DNA.

It is believed that non-viable spermatozoa do not take up DNA. We observed that, within 30 min, a substantial amount of added DNA was associated with spermatozoa which gradually increased in course of time (Fig. 2A) indicating that the uptake of DNA is authentic during the functional maturation (capacitation) of spermatozoa. Moreover, there was no adverse effect on either the morphology or physiology of sperm as a consequence of addition of...
foreign DNA (Figs. 4, 5). No effect was seen of the added DNA on the capacitation as reflected by the absence of any significant change in the profiles of quality of motility as well as SMI (Fig. 4). Interestingly, the proportion of spermatozoa undergoing AR was also not affected at the two time points tested (Fig. 5). Values obtained for the two parameters were similar to those reported earlier for hamster spermatozoa.

One of the significant observations made in the present study is the oocyte uptake of foreign DNA, delivered by hamster or human spermatozoa. Data of Table I clearly illustrates that the DNA-carrying spermatozoa are able to retain the fertilizing potential. It also shows that there is no (negligible) uptake of DNA by hamster oocytes when incubated with pCMXoGH. A similar observation was also made earlier in the mouse using pSV2CAT DNA. Expectedly, the number of penetrated sperm/oocyte was higher for hamster spermatozoa than those of the human. This is attributed to the fact that the former is a homologous system unlike the latter. It is however, envisaged, that when in vitro fertilization (IVF) is carried out in the species of interest, it would be possible to produce transgenic embryos/offspring. Nevertheless, our studies confirm that hamster sperm can act as a vector to transfer DNA into oocytes and that the added DNA does not in any way affect sperm's fertilizing potential. Use of DNA-treated sperm for fertilization and post-fertilization development would be quite promising in developing germine transformatants.

The use of hamsters, in particular, would be of great advantage since sperm biology, and sperm-oocyte interaction in the context of molecular mechanism involved in DNA uptake can be examined in detail in this species, including the consequences of post-fertilization development. Of all the mammalian spermatozoa studied, hamster sperm is one of the largest, with a large sperm-head and an acrosomal cap which make intranspermatosal DNA localization studies easier. Besides, zona-free hamster oocytes are unique in that they permit penetration of spermatozoa from other mammals, including humans. Thus, the ability of foreign DNA uptake by human sperm as well as its penetration into oocytes can be conveniently examined in the hamster model. Moreover, peri-implantation hamster development can now be routinely achieved using optimized culture systems.

The challenge of reproductive biologists and animal bio-technologists is to find a simple and inexpensive method for generating transgenic animals with useful genes for commercial purpose. Viewed from this angle, the data in hamster (present study) and in other mammals on sperm mediated gene transfer is proving to be an easy, inexpensive and reliable method. Moreover, it is now possible to introduce genes in vivo into the male genital tract (vas deference) which is gained by spermatozoa, thereby providing an easier in vivo approach for germ-line transmission of foreign DNA. These offer potential opportunities to improve efficiency of transgenic technology.

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