Lipsome-mediated uptake of exogenous DNA by Sahiwal cattle spermatozoa

Thomas V. Babu[1], M. D. Pratheesh[2], N. Anandlaxmi[1], K. Amarnath[2]

1. National Dairy Research Institute, Karnal, Hissar, Haryana, India.
2. Indian Veterinary Research Institute, Izatnagar, Dist. Bareilly, UP, India.
Corresponding author: M. D. Pratheesh, email: pratheeshmd@gmail.com
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Abstract

Aim: To investigate the influence of lipofection treatment and exogenous DNA uptake on the quality of sahiwal cattle spermatozoa.

Materials and Methods: Semen collected from sahiwal bulls (n=7) were evaluated separately for color, volume, mass activity, concentration, motility and viability using standard procedures. Pooled sperm samples from selected bulls (n=3) were transfected with a model gene construct enhanced green fluorescent protein (p-EGFP) via lipofection method and confirmed the genome integration by PCR technique. Furthermore the effect of transfection on spermatozoa was assessed based on apoptosis, viability and motility.

Results: In the current investigation sahiwal bulls were selected based on their breeding records and better semen characteristics. Although the transfected sperm samples failed to show florescence under fluorescence microscope, PCR studies confirmed the successful uptake of the p-EGFP gene in to the host sperm cell genome. Moreover transfected samples showed a significant reduction in the viability and motility without causing any DNA damage induced apoptosis as demonstrated by DNA Ladder assay.

Key words: EGFP, lipofection, Sahiwal cattle, spermatozoa, transfection

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Introduction

Liposome mediated DNA delivery method i.e. lipofection is a technique used for introducing nucleic acids into cells and embryos. Liposomes are small vesicles consisting of membrane like lipid layers that can actually protect foreign DNA from digestion of proteases and DNases. Cationic liposomes are capable of spontaneously interacting with DNA molecules, giving rise to lipid–DNA complexes [1].

Sperm cells constitute the male genetic material with haploid number of chromosomes, having capability of maintaining constancy of chromosome number in a species through fertilization. Sperm cells from echinoderm to man under certain conditions can take up foreign genes and integrate into its genome. The capacity of sperm cells to capture foreign DNA had been reported by Brackett *et al.* (1971) where, they observed that Simian virus 40 (SV40) adsorbs on surface of rabbit spermatozoa, but does not penetrate the cells [2]. This report provided the first evidence that a heterologous genome can be incorporated into mammalian spermatozoa and subsequently carried into an ovum during the process of fertilization. This finding and its implications were ignored for around twenty years and rediscovered after the report by Lavitrano et al, in 1989 that mouse sperm cell can act as a vector for transferring foreign gene to the next generation [3]. Similarly it has been reported in Sea urchin sperm cells. [4]. After this many success reports of *in-vitro* uptake of DNA constructs by animal sperm cells have been presented [3,5-10].

Green-fluorescent protein (GFP), is one of the hottest new biological tool responsible for the stunning bioluminescence of the Pacific Northwest Jellyfish, *Aequorea Victoria* [11]. In order to overcome the slow rate of flourescence acquisition in wild type GFP, a mutant variety was introduced known as Enhanced GFP (EGFP) and it is commonly used as a transfection reporter. EGFP permits the use of fluorescence-based technologies not only qualitative, but quantitative analysis of fluorescence intensity [12].

The sperm mediated gene transfer (SMGT) is a novel method for the generation of transgenic animals, where sperm is used as a vector for transferring the gene of interest to the zygote. Success is dependant on the sperm's ability to bind and internalize exogenous DNA and to transfer it into ovum at fertilization [3,7]. This method offers a powerful tool in the field of animal transgenesis and biotechnology. This technique has a widespread application to all species in which reproduction is mediated by gametes, but is refractory to microinjection technique, for example in fish, insects, amphibians, swine, and cattle [13,14].

Sahiwal cattle is one of the well established disease and heat resistant breed of tropical and sub tropical areas thereby serves as a model for determining the potential role of indigenous cattle in contributing to the economic and efficient milk and meat supply under the prevailing tropical conditions [15]. Despite of its utmost importance and unique characteristics to date there are no reports were presented regarding the genetic manipulation of sahiwal cattle spermatozoa.

In the current study sperm cells from the selected sahiwal bulls were lipofected with a model gene pEGFP and evaluated its efficacy with respect to viability, motility, apoptosis and gene expression studies. It is a preliminary work but definitely paves way for further studies to find widespread application in gene transfer for welfare of livestock species.

Materials and Methods

Experimental design

Experiment 1: Two step procedure for the selection of Sahiwal bulls for the transfection experiment.

Experiment 2: Based on the results obtained from experiment 1, three animals were selected for semen collection and for further transfection studies. Transfected cells were evaluated for efficiency with respect to apoptosis, viability, motility and corresponding gene expression.

Selection and Management of Bulls: In the present investigation six sahiwal cattle bulls, with an average age of 22-30 months were selected based on their body weight and activity index from the animal herd maintained at Artificial breeding complex, National Dairy Research Institute, Karnal. All animal procedures were approved under the guidelines of the institutional animal ethics committee, NDRI, India. The selected bulls were of high activity score (above +2.5) [16] and body weight ranging from 400-500kg, in addition these bulls were screened against genital infections also. The selected sahiwal bulls were maintained as per the standard practices followed at the Artificial Breeding Complex, NDRI, Karnal. The animals were housed in bull pen and maintained on grass (hay) supplemented with concentrate, minerals and vitamins through out the experiment.

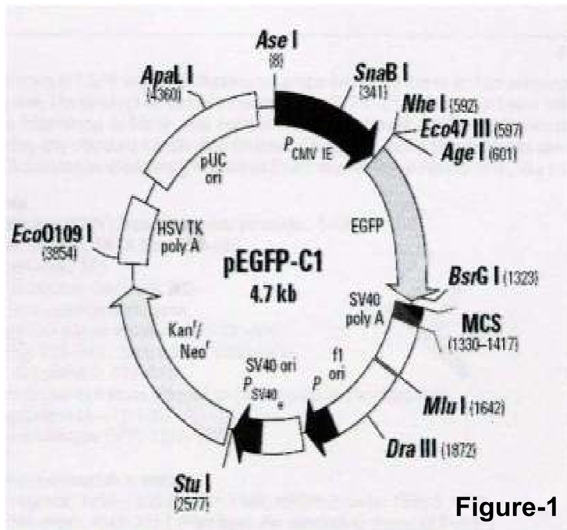
Collection of semen: Semen was collected from the selected sahiwal bulls twice a week for a month using sterile artificial vagina (CMV France) maintaining internal temperature of 42-44°C. Sterile and hygienic conditions were maintained during collection. Collection of semen was according to the routine practices followed at Artificial Breeding Complex, NDRI, Karnal.

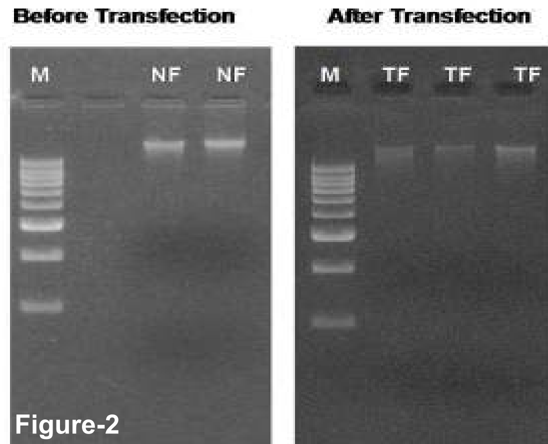
Semen evaluation: Semen collected from selected sahiwal bulls were evaluated separately for color, volume, mass activity, concentration, motility and viability using standard procedures.

Mass activity was assessed by visual analysis using an arbitrary scale ranging from + 1to +4 based on the whirling movement of the semen sample drops on a warm stage at 37 °C under light microscope at 10X magnification. Sperm concentration and viability were calculated by eosin–nigrosine staining procedure. Enumeration of diluted sample on haemocytometer ,which was determined by counting the sperm cells in the central chamber of Neubauer's haemocytometer under light microscope at 100x magnification and the number of total spermatozoa in four corner and one central square of RBC chamber were counted separately and concentration was calculated as per formula given below. Number of spermatozoa (millions /ml) = 50N x DF x 10^3, where N = no. of spermatozoa in five squares and DF = dilution factor. Number of viable sperms that did not take up stain were considered as live and those stained pink were considered as dead. Percentage of viable sperms= [Total no. of sperms – stained sperms (in 10 fields)] /total no. of sperms X 100.

Transfection of spermatozoa: Based on the evaluation of semen characterstics, three sahiwal bulls were selected and semen collected from these animals was pooled, diluted, processed and subjected for further transfection studies. Fresh semen samples were transfected with linearized pEGFP using cationic liposomes (DNAfectin) as mediator.

Transgene construct: pEGFP-C1 (Fig:1) encodes a red-shifted variant of wild-type GFP, which has been optimized for brighter fluorescence and higher expression in mammalian cells (Excitation maximum=488nm; emission maximum=507nm.). The vector contains an SV-40 origin for replication in mammalian cells expressing the SV-40 T antigen. A





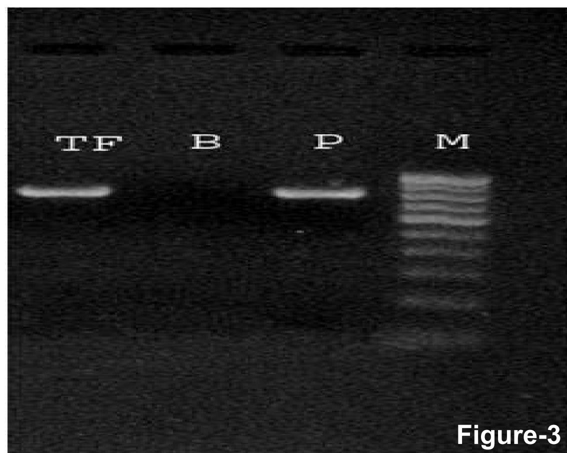


Figure-1. pEGFP-C1 transgene construct used as model gene for transfection studies in sperm samples.

Figure-2. Apoptosis assay of sperm samples before and after transfection M- 100bp DNA ladder, NF- Non Transfected samples and TF- Transfected samples.

Figure-3. PCR product showing expression of pEGFP (885bp) in transfected sample. TF- Transfected sample, B- Control sample, P- positive control for pEGFP and M- 100bp marker.

SV-40 early promoter for neomycin resistance cassette and bacterial promoter for kanamycin resistance cassette are present in back bones of this vector. Vector DNA was linearized by digesting with restriction enzyme:*Mlu* I. This linearized vector was purchased from Chromus Co. Bangalore.(Gene bank accession no.# U55763).

Preparation of sperm samples: The transfection of spermatozoa with pEGFP-DNAfectin complex with respect to their corresponding concentrations and incubation time were already optimized in our lab. Out of different combinations, 4 million spermatozoa, 2hrs of incubation time and 1.5µg of pEGFP-DNAfectin complex were found to be optimum for liposome mediated transfection in bull spermatozoa (Data not shown).

The neat semen sample was diluted with egg yolk citrate diluent (EYC) at a ratio of 1:10. The diluted semen was again diluted with Ca^{2+}, Mg^{2+} free PBS, so that the sperm numbers in the samples were adjusted to four millions. The samples were washed twice with Ca^{2+}, Mg^{2+} free PBS by centrifugation at 1000 rpm for 3 min. The supernatant was discarded and the pellet obtained was resuspended in 500µl of Opti-MEM-I medium, a commercial medium that is specially used for lipid mediated transfections. Opti-MEM-I is a modification of eagle's minimal essential medium, buffered with HEPES and sodium bicarbonate and supplemented with hypoxanthine, thymidine, sodium pyruvate, L-glutamine, trace minerals and growth factors.

Preparation of plasmid (pEGFP) – DNAfectin

complex: DNAfectin (0.50µg) and pEGFP (1.5µg) were mixed in 125 µl of Opti-MEM-I medium taken in a cryovial, by gentle movement and incubated at room temperature for 30 min to allow the formation of lipid DNA complexes.

Transfection of spermatozoa with pEGFP-DNA fectin complex : A six well plate (sterile TC grade, Orange scientific, USA) was used for transfection trials. Trials were performed for both fresh and cryopreserved semen samples separately. The wells were seeded with sperm suspension in Opti-MEM-I medium at a rate of $4x10^6$ cells in duplicates . The pEGFP –DNAfectin complex prepared in Opti-MEM-I medium was added to each well. Controls were also similarly processed without pEGFP-DNA fectin complex. The plates were incubated for 45 minute at 37 ^{0}C in a CO_2 incubator under controlled conditions. The transfected samples at the end of incubation period were treated with DNase (Promega.USA) for 30 min at 37^{0}C and terminated its action by co-incubating with stopping buffer at 65^{0}C for further 10 min. After the end of the specific incubation period the above samples were observed for fluorescence under fluorescent microscope using FITC filter.

Evaluating the effect of transfection on sperm cells: Transfected sperm cells of either group were evaluated in terms of viability, number of motile spermatozoa, apoptosis, and pEGFP gene uptake parameters. After the specific incubation period the viability and motility of the transfected samples were evaluated under visible microscope (200x) and by eosin–nigrosine staining procedure as described in the previous section. The DNA laddering technique was used to visualize the endonuclease cleavage products of apoptosis as described by Wyllie,1980 [17]. In brief genomic DNA was extracted from approximately 10 million spermatozoa by standard salting out protocol using DTT and Proteinase K [18]. Purity of the genomic DNA was checked by measuring the absorbances at 260 nm and 280 nm respectively. 1µg of extracted DNA was subjected to electrophoresis on 0.8% agarose gel, using 1kb ladder as marker.

Genomic DNA extraction and PCR for evaluating uptake of p EGFP: Genomic DNA was extracted from control and transfected sperm samples after lysis [16] using DNA extraction kit according to manufacturer's instructions. The extracted genomic DNA was subjected to quality check by taking the ratio of the absorbances at 260 and 280 nm. Genomic DNA (4 µl. for each sample) was amplified by polymerase chain reaction (PCR) using the specific primer sequences for EGFP;
(F-primer:5'ATGGTGAGCAAGGGCGAGGAG CT
R-primer:5' GTACCGTCGACTGCAGAATTCGAA GCT)

The thermal cycling conditions in this study were initially at 94^{0}C for 4 min denaturation, followed by 30 cycles of 94^{0}C for 30 sec, 56^{0}C for 30 sec, 72^{0}C for 1min respectively. Final extension step was performed at 72^{0}C for 15 min. The amplified products were resolved on 1.8% agarose gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet illumination (Alpha Imagetech, USA).

Results

From the herd maintained in the NDRI cattle farm, based on the average activity index (2-3) and body weight (300-400kg), six donor Sahiwal bulls were shortlisted (Table-1). This was also confirmed from farm breeding records. In the present study the semen was evaluated from these selected animals for different parameter like color, volume, mass activity, motility, viability, concentration and semen samples from three bulls were utilized for further transfection studies (SW- 1719, SW- 1681, SW- 1772). Data of various semen parameters of the selected Sahiwal bulls were displayed in Table-2. As spermatozoa are very sensitive to thermal shocks, all evaluation steps were done at 37°C on a thermostat stage.

Table-1. Mean activity index and bodyweight of selected sahiwal bulls.

Bull No.	Mean activity index	Average Body weight (Kg)
SW-1719	2.8	454
SW-1681	2.5	417
SW-1730	2.5	434
SW-1731	2.5	436
SW-1682	2.8	502
SW-1772	2.8	390

The semen samples incubated with p EGFP gene for a period of 2 h were checked for motility and viability parameters along with respective controls. The percentage of motile and viable sperms significantly ($P<0.05$) decreased after subjeced to transfection with p EGFP gene-DNA fectin complex, (Table: 3). In addition the transfected sperm samples when observed under fluorescence microscope found negative for green fluorescence. This could be due to the less active transcription and translation machinery in sperm cells. The purity of extracted DNA samples as analyzed by taking the ratio was observed to range between 1.8-1.9. Apoptosis in sperm samples due to DNA fragmentation was evaluated by DNA ladder

Table-2. Evaluation of semen parameters of the selected sahiwal bulls.

Bull no.	Color& volume	Mass activity	Motility %	Viability%	Concentration (millions per ml)
SW-1719*	Milky,6ml	+2.5	90%	89%	940
SW-1681*	Milky,5ml	+2.5	80%	70%	970
SW-1730	Milky,5ml	+2.5	80%	64%	560
SW-1731	Milky,4ml	+2.5	80%	79%	690
SW-1682	Milky,7ml	+3.0	90%	86%	830
SW-1772*	Milky,7ml	+3.5	>90%	92%	1160

*- Selected animals for further transfection experiments.

Table-3. Evaluation motility and viability of the transfected sperm samples.

Stages	Bull Number SW-1719	SW-1681	SW-1772
Motility%			
Before transfection	70%^{a}	78%^{a}	67%^{a}
After transfection	29%^{b}	34%^{b}	30%^{b}
Control	33%^{b}	36%^{b}	32%^{b}
Viability%			
Before transfection	89%^{x}	85%^{x}	90%^{x}
After transfection	50%^{y}	53%^{y}	59%^{y}
Control	57%^{y}	45%^{y}	61%^{y}

a,b Values with different superscripts in the same column are significantly different (P<0·05)
x,y, Values with different superscripts in the same column are significantly different (P<0·05)

assay on 0.8% agarose gel, Apoptosis was not observed in any of the samples before and after transfection (Fig.2). Furthermore the gene uptake via lipofection was evaluated by PCR amplification of the pEGFP gene in the transfected sperm samples. An 885bp product was observed on 1.8% agarose gel, demonstrating the successful uptake of the pEGFP gene to the host sperm by lipofection technique (Fig-3).

Discussion

Transfection is a tool for the introduction of foreign DNA into eukaryotic or prokaryotic cells and it is one of the important steps in transgenesis. Transgenic technology is of particular relevance in the rapid genetic modification of farm animal species as it facilitates augmentation of production characteristics like growth, development, disease resistance, reproduction, lactation performance, feed efficiency, immune response and fibre production [19]. For example, transgenic cattle expressing a mammary specific transgene encoding lysostaphin are resistant to mastitis due to *Staphylococcus aureus*, and this finding leads the way for targeting specific infections through the use of transgenic technology [20]. For the present study sahiwal donor bulls were selected based on their semen qualities and thereby demon-strated that good quality sperm cells can be successfully transfected with pEGFP via lipofection without causing any apoptosis induced DNA fragmentation.

Liposomes have been used to introduce a variety of molecules into living cells [8,9,10], but their utility as a carrier for exogenous DNA in transgenic experiment was not explored until a later stage. Lipofection is one of the most sought methods for transfection of DNA molecules into spermatozoa . The efficiency of sperm-mediated gene transfer (SMGT) via lipofection appears to be much higher than that obtained with other transfection methods and rates as high as 88% have been obtained in some liposome-mediated uptake of exogenous DNA by spermatozoa and applications in SMGT studies [21], compared to only 1–8% of transgenic offspring after microinjection techniques [22]. The major benefits of this technique are the high efficiency, low cost, and ease of use compared with that of other transfection methods [23]. The use of transgenic sperm has the advantage over somatic cell nuclear transfer for producing transgenic animals as it is a simple procedure and there is no trauma to the oocytes [22]. Additionally, sperm mediated gene transfer has resulted in germ-line transmission of transgenes by incorporation into F1 and later progeny [24]. Therefore, SMGT offers potential advantages over other methods for inducing transgenesis in domestic livestock as been successfully reported [6,25].

DNA uptake is directly correlated with semen quality. Lavitrano et al., (2003) demonstrated that quality of semen sample, must be optimum for the

SMGT technique to be effective, quality of the semen sample is primarily dependent on viability and motility of spermatozoa in the samples [24,26]. The semen samples should pass the evaluation tests for selection as a good vector for exogenous DNA, for example volume, concentration, presence of abnormal sperm, motility etc. The ability of the sperm cells to internalize exogenous DNA is directly correlated with the progressive motility of spermatozoa in the samples [24].

In adult males, during normal spermatogenesis germ cell apoptosis plays an important role in sperm production. Approximately 25–75% of potential spermatozoa degenerate and die in mammalian testis itself [27,28]. Fresh ejaculated semen also contains significant numbers of apoptotic cells [29,30]. These abnormal and immature spermatozoa display DNA damage, defective protamination and chromatin packaging [31,32]. DNA fragmentation in ejaculated spermatozoa is one of the most important characteristics of programmed cell death; however, the extent of DNA fragmentation does not correlate with the percentage of motile spermatozoa or other markers of apoptosis. So DNA integrity should be considered as an independent measure of sperm quality apart from other sperm parameters. In the present study we found that transfection of sperm cells by lipofection method does not cause any DNA damage induced apoptosis as demonstrated by the DNA ladder assay.

Conclusion

To conclude in the present study, we demonstrated that Sahiwal cattle sperm cells can be successfully transfected with pEGFP gene of interest via lipofection without causing DNA damage. In this study, the motility and viability of sperm cells had significantly reduced post transfection. It will be an inexpensive technique for transfer of genes. Keeping in view of enhancing the socio-economic conditions of farming community and to bridge the gap of supply and demand of animal protein in the developing countries, traits of commercial value such as milk yield and feed efficiency needs genetic improvement in indigenous breeds like Sahiwal cattle. Considering this research outcome along with the extensive availability of livestock semen, sperm mediated gene transfer can be considered as an alternative method for the production of genetically modified superior quality farm animals.

Author's contribution

All authors contributed equally. All authors read and approved the final manuscript.

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Competing interest

Authors declares that they have no competing interest.

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