Optimization of buffalo spermatozoa transfection with pbIFN-tau-EGFP gene by simple incubation technique

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ABSTRACT
The present study was conducted to optimize and analyze the uptake of pbIFN-tau-EGFP by spermatozoa of Murrah buffaloes (Bubalus bubalis) by simple incubation technique. Fresh semen samples from two Murrah buffaloes were selected based on mass activity (+3 and above) from the herd. Three different variables i.e. bovine Interferon tau gene conjugated to enhanced green fluorescent gene (pbIFN-tau-EGFP) gene concentration, incubation time and number of spermatozoa, were considered for optimization of spermatozoa transfection by simple incubation technique. Before and after transfection, the spermatozoa samples were evaluated for sperm functional parameters, motility and viability. Spermatozoa incubated with different concentrations (10 and 20µg/ml) of gene (pbIFN tau-EGFP) and without gene which served as control were subjected to *in vitro* fertilization (IVF). A significant decrease (P<0.05) in percentage of motile spermatozoa of fresh semen sample was observed. For further 1 h of incubation the decrease observed was significant (P<0.05) when compared with control group. Similarly a significant decrease (P<0.05) in percentage of viable spermatozoa was observed after 1h with a further 1h of incubation. Positive results were obtained for uptake of the gene (amplified 585bp product) on PCR analyses of DNA extracted from sperm samples incubated with either concentration of the gene. A 40- 47 percentage of cleaved embryos were obtained post IVF with spermatozoa of fresh semen samples. Either, concentration of the gene or spermatozoa used in the present study did not result in any significant difference in percentage of cleaved or morula stage embryos. The resulting embryos were also positive for uptake of pbIFN-tau-EGFP gene.

Key words- Gene transfer, IFN-tau, *In Vitro* fertilization, Spermatozoa, Transfection.

INTRODUCTION
Transfection is a molecular tool by which there can be a modification of genetic make up of an organism by transferring *in vitro* recombined gene construct through gametes. The sperm mediated gene transfer (SMGT) system is a technique that could provide the opportunity to carry out transgenesis on a mass scale (Spadafora, 1998). The first report showing that exogenous DNA could be introduced into sperm was provided in rabbits (Brackett et al., 1971). Since then, this technique has been used by multiple laboratories, but results are controversial (Smith and Spadafora, 2005). Although transgenic animals have been produced in various species, the efficiency of sperm-mediated DNA transfer is still questionable and the mode of transmission to the egg has not yet been well understood. In bulls the binding of exogenous DNA to spermatozoa has been reported by several authors (Schellander et al., 1995; Alderson et al., 2006; Anzar and Buhr, 2006), although the mechanism of binding and internalization of exogenous DNA is in question that has not been solved. Some reports are available with respect to development of bovine transgenic embryos with transfected spermatozoa (Alderson et al., 2006; Canovas et al., 2010) or calves (Shemesh et al., 2000) using SMGT technique. Although the efficiency with this technique was quite low. During the internalization, an endogenous nuclease activity is triggered upon interaction of spermatozoa with foreign molecules as one barrier against intrusion of exogenous molecules (Maione et al., 1998). However, no report is available with respect to SMGT by simple incubation in buffaloes for obtaining transfected spermatozoa and embryos. More information about bovine SMGT by simple incubation technique and the effect of exogenous DNA on sperm function would provide insight into the fertilizing capacity of sperm carrying exogenous DNA in conventional fertilization systems.

Sperm mediated gene transfer has the advantages of simplicity and cost-effectiveness, in contrast with more established methods of transgenesis (Zani et al., 1995; Maione et al., 1998).
et al., 1998). It becomes more effective if the procedure is by simple incubation of spermatozoa with desired exogenous DNA construct. So SMGT technique can be used to produce transgenic farm animals which can be used for production of improved economic traits and for the production of pharmaceuticals, medically or biologically important proteins.

MATERIALS AND METHODS

Semen collection: For the present study semen from Murrah bulls, with mass activity +3 or above were selected from the animal herd maintained at Artificial Breeding complex, National Dairy Research Institute, Karnal. Collection of semen was according to the routine practices followed at Artificial Breeding Complex. Preliminarily semen samples were evaluated as follows: diluting the semen samples, one drop of diluted sample was placed on a clean warm glass slide and covered with cover slip and observed under phase contrast microscope at 200X magnification, the samples were selected, based on type of motility and number of motile spermatozoa in at least 5-6 different fields.

Spermatozoa concentration in the sample was determined by counting the number of spermatozoa cells in the five squares of central chamber of haemocytometer under light microscope at 100X magnification. Spermatozoa cell concentration was expressed as millions per ml.

Viability of sperm cells was assessed by one step Eosin- Nigrosin staining technique. The stain solution comprised 0.67% Eosin and 10% Nigrosin dissolved in 0.9% sodium chloride. Sperms that did not take up stain were considered as live and those stained pink were considered as dead (Mortimer 1994).

Transgene construct: The promoter region of IFN-t was PCR amplified using sequence specific primers designed based on cattle genome sequence data. The promoter region was amplified, the amplicon was gel purified and cloned within a cloning vector (pUC18). A few clones were sequenced to get a clone with no mutations. The coding region of the IFN-tau was PCR amplified using sequence specific primers designed based on cattle genome sequence data. The primers had suitable restriction sites for assembling the gene into the final construct. The gene was subcloned into pUC18 for sequence confirmation. A few clones were sequenced to get one with no mutations. The coding region of the IFN-tau was PCR amplified using sequence specific primers designed based on cattle genome sequence data. The primers had suitable restriction sites for assembling the gene into the final construct. The gene was subcloned into pUC18 for sequence confirmation. A few clones were sequenced to get one with no mutations. The gene was PCR amplified using primers designed in such a way that the gene would be in-frame after it’s cloned within the vector backbone (Fig 1).

Preparation of spermatozoa samples: The neat semen sample was diluted with egg yolk citrate diluents (EYC) at a ratio of 1:10. The diluted semen was diluted with washing bracket and Oliphant (BO) medium (BO medium containing 10 µg/ml heparin, 137.0 µg/ml sodium pyruvate and 1.942 mg/ml caffeine sodium benzoate), so that the sperm numbers in the samples were adjusted to forty and eighty millions/ml respectively. The samples were washed with washing Bracket and Oliphant (BO) medium twice by centrifugation at 1000 rpm for 5 min. The supernatant was discarded and the pellet obtained was resuspended in 500 µl of BO medium.

Incubation of spermatozoa with phiIFN-tau gene: A six well plate was used for transfection trials. Different combinations as given in Table 2 were subjected to transfection experiments. The wells were seeded with sperm suspension in BO medium at the rate of 40*10^6 and 80*10^6 cells/ml in duplicate into four wells respectively, so that two of these wells contained same number of spermatozoa (40 million/ml in 2 wells and 80 million/ml in other 2 wells). The phiIFN-tau-EGFP transgene was added to these wells in such a way that wells containing 40*10^6 cells/ml and 80*10^6 sperm cells, received either concentration of phiIFN-tau- EGFP transgene-(10µg/ml and 20µg/ml). Care was taken to see that the different concentrations of phiIFN-tau- EGFP transgene

![FIG 1: bIFN-tau gene cloned in to pEGFP-C1 plasmid used for transfection of spermatozoa.](image-url)

**TABLE 1:** Reaction Mix for PCR

<table>
<thead>
<tr>
<th>INGREDIENTS</th>
<th>VOLUME (µl)</th>
<th>CONCENTRATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water, Nuclease-free</td>
<td>Variable</td>
<td>10x</td>
</tr>
<tr>
<td>10X Taq buffer(with KCl and 25mM MgCl₂)</td>
<td>0.5</td>
<td>10mM</td>
</tr>
<tr>
<td>DNTPs</td>
<td>2</td>
<td>100ng</td>
</tr>
<tr>
<td>Forward primer (10 µM)</td>
<td>1</td>
<td>100ng</td>
</tr>
<tr>
<td>Reverse primer (10 µM)</td>
<td>1</td>
<td>100ng</td>
</tr>
<tr>
<td>Taq DNA polymerase (native, without BSA)</td>
<td>1</td>
<td>3units/µl</td>
</tr>
<tr>
<td>Template DNA</td>
<td>1-2</td>
<td>100ng-1µg</td>
</tr>
<tr>
<td>Total volume</td>
<td>20 µl</td>
<td></td>
</tr>
</tbody>
</table>
was available for transfection with samples containing different numbers of spermatozoa. Similarly controls were also taken with respect to 40×10^6 cells/ml and 80×10^6 sperm cells/ml (without pbIFN-tau-EGFP transgene) in BO medium in the remaining two wells of the six well plate. The different combinations used for transfection trials are given in Table 2. The six well plate was covered with a lid and samples were incubated for 1h and 2h at 37°C in CO₂ incubator under controlled conditions. Before and after the end of the incubation, motility (percentage of motile sperms) and viability (percentage of viable sperms) were estimated.

Sperm samples at the end of incubation period were treated with 0.1 mg DNase I for 30 min at 37°C. After 30 minutes DNase activity was stopped with stopping buffer and followed by washing step twice in PBS, to separate pbIFN-tau gene which was not incorporated in sperms and was used for in vitro fertilization experiments or lysed for extraction of genomic DNA and frozen for later analysis of pbIFN-tau gene uptake by PCR and agarose gel electrophoresis technique.

**In vitro maturation and fertilization of oocytes with spermatozoa incubated with or without pbIFN-tau gene:**
Buffalo ovaries were collected from Derabassi slaughter house, New Delhi, immediately after slaughter. These were washed 3-4 times with isotonic saline (32-37°C) containing 100 mg/ml streptomycin. The washed ovaries were then put in a thermos flask containing warm saline and antibiotics. The collected ovaries were transported to the laboratory within 6 hrs of slaughter. In the laboratory, the ovaries were rinsed twice, trimmed to remove the extra tissue and washed properly with warm saline containing antibiotics. Oocytes were collected by aspiration of surface follicles (2-8 mm diameter) with a 18 gauge needle attached to a 10 ml syringe containing the aspiration medium (TCM -199 + 0.3% BSA + 0.8% L-glutamine + 50 mg/ml gentamicin sulfate). The contents of the syringe, which included the aspirated oocytes, were collected by aspiration of surface follicles (2-8 mm diameter) with a 18 gauge needle attached to a 10 ml syringe containing the aspiration medium.

The collected ovaries were transported to the laboratory in a thermos flask containing warm saline and antibiotics. The washed ovaries were then put in a thermos flask containing warm saline and antibiotics. The collected ovaries were transported to the laboratory within 6 hrs of slaughter. In the laboratory, the ovaries were rinsed twice, trimmed to remove the extra tissue and washed properly with warm saline containing antibiotics. Oocytes were collected by aspiration of surface follicles (2-8 mm diameter) with a 18 gauge needle attached to a 10 ml syringe containing the aspiration medium (TCM -199 + 0.3% BSA + 0.8% L-glutamine + 50 mg/ml gentamicin sulfate). The contents of the syringe, which included the aspirated oocytes, were collected by aspiration of surface follicles (2-8 mm diameter) with a 18 gauge needle attached to a 10 ml syringe containing the aspiration medium (TCM -199 + 0.3% BSA + 0.8% L-glutamine + 50 mg/ml gentamicin sulfate). The contents of the syringe, which included the aspirated oocytes, were collected by aspiration of surface follicles (2-8 mm diameter) with a 18 gauge needle attached to a 10 ml syringe containing the aspiration medium (TCM -199 + 0.3% BSA + 0.8% L-glutamine + 50 mg/ml gentamicin sulfate).

**TABLE 2:** Effect of different parameters (Time, concentration of sperms and gene) on sperm functional parameters on incubation with IFN-tau gene

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Duration of incubation</th>
<th>Group I 40 million</th>
<th>Group IA 40 million and 20µg/ml</th>
<th>Group IB 80 million</th>
<th>Group IIA 80 million and 10µg/ml</th>
<th>Group IIB 80 million and 20µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motility (%)</td>
<td>0h</td>
<td>82.63±0.80</td>
<td>82.63±0.80</td>
<td>82.63±0.80</td>
<td>82.63±0.80</td>
<td>82.63±0.80</td>
</tr>
<tr>
<td></td>
<td>1h</td>
<td>75.75±0.59</td>
<td>75.88±0.77</td>
<td>75.38±0.84</td>
<td>75.88±0.44</td>
<td>76.50±0.73</td>
</tr>
<tr>
<td></td>
<td>2h</td>
<td>70.75±0.45</td>
<td>70.75±0.82</td>
<td>70.38±0.63</td>
<td>71.88±0.69</td>
<td>71.25±0.65</td>
</tr>
<tr>
<td>Viability (%)</td>
<td>0h</td>
<td>91.25±0.45</td>
<td>91.25±0.45</td>
<td>91.25±0.45</td>
<td>91.25±0.45</td>
<td>91.25±0.45</td>
</tr>
<tr>
<td></td>
<td>1h</td>
<td>84.13±0.74</td>
<td>84.00±0.73</td>
<td>83.75±0.53</td>
<td>85.00±0.71</td>
<td>84.88±0.79</td>
</tr>
<tr>
<td></td>
<td>2h</td>
<td>81.88±0.67</td>
<td>80.13±0.55</td>
<td>80.13±0.52</td>
<td>82.15±0.84</td>
<td>81.75±0.70</td>
</tr>
</tbody>
</table>

a, b, c- values with different superscript differ significantly (P<0.05). Values are expressed as Mean±SE
Each trial was conducted in triplicate. Six trials were conducted. 0h- at the initiation of experiment
Group I: Control with 40 and 80 million sperms.
followed for IVF was according to protocol followed by Chauhan et al., 1998).

**Extraction of genomic DNA from spermatozoa and embryos**: DNA was isolated from semen samples using phenol-chloroform extraction method (Sambrook and Russell, 2001). Collected embryos were treated with pronase (10% in modified PBS/BSA) to remove zona pellucida, rinsed twice in PBS and lysed by heating at 95°C for 10 min and freezing. The lysis step was repeated twice, and then DNA was extracted and further used for PCR later given in Table 1.

The purity of genomic DNA was determined by measuring the ratio of O.D. at 260 and 280 nm with a UV spectrophotometer (Nano Drop ND-1000-USA). The concentration of genomic DNA was estimated using the following formula. The amount of DNA (ng/µl) = (OD260 x 50 x dilution factor). (1 OD value at 260 nm is equivalent to 50 ng DNA/µl). The isolated genomic DNA samples were diluted to get 10 ng of DNA/µl for all samples.

**Polymerase chain reaction (PCR)**: PCR technique was adopted for analyzing the uptake of pbIFN-tau-EGFP gene. Oligonucleotide primers were specifically targeted against specific base pair regions of IFN-tau. The sequences of forward and reverse primers are given below (designed by Chromus Co. Bengaluru).

- CAGTGA CAT ATG GCC TTC GTG CTC TCT CTA CTG ATG- F Primer
- CAG TGA CTC GAG AAG TGA GTT CAG A TC TCC ACC CAT CT-R Primer

PCR based detection of IFN-tau sequences involved different steps like Template DNA was denatured at 94°C. Temperature and time required for denaturation, annealing and extension in the study were standardized as 94°C for 30 sec, 65.7°C for 30 sec and 72°C for 45 sec respectively. Final extension was performed at 72°C for 15 min., after 34 cycles of PCR are given in Table 1.

### Table 3: Percentage of Cleaved embryos obtained in vitro with spermatozoa subjected to transfection with IFN-tau-EGFP gene for different time intervals.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Time interval for incubation of gene with sperms</th>
<th>Concentration of spermatozoa and bIFN-tau gene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control 40million and devoid of exogene</td>
<td>Control 80million and devoid of exogene</td>
</tr>
<tr>
<td></td>
<td>40million and 10µg/ml exogene</td>
<td>40million and 20µg/ml exogene</td>
</tr>
<tr>
<td></td>
<td>80million and 10µg/ml exogene</td>
<td>80million and 20µg/ml exogene</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cleaved embryos</th>
<th>0h</th>
<th>47.60±3.63</th>
<th>47.85±2.45</th>
<th>47.97±2.17</th>
<th>48.47±3.09</th>
<th>48.12±2.73</th>
<th>48.09±2.63</th>
</tr>
</thead>
<tbody>
<tr>
<td>1h</td>
<td>45.66±1.90</td>
<td>45.58±2.23</td>
<td>45.40±1.90</td>
<td>44.42±2.04</td>
<td>44.32±2.16</td>
<td>44.54±2.55</td>
<td></td>
</tr>
<tr>
<td>2h</td>
<td>40.17±3.47</td>
<td>40.12±1.39</td>
<td>43.38±2.61</td>
<td>40.25±2.89</td>
<td>41.28±2.78</td>
<td>40.37±2.36</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as Mean±SE. Each trial was conducted in triplicate. Three trials were conducted. Values with superscript b are significantly different from values for different groups for 0h, but not for 1h interval groups. 0h- at the initiation of experiment.

**Statistical analysis**: Data is expressed as Mean ± SE and analyzed by ANOVA, considering the concentration of exogenous DNA, concentration of spermatozoa and time of incubation, as the main variables. The variables expressed in percentage was subjected to arc sine transformation and further subjected to ANOVA. Results revealing a significant effect were compared by the least significant difference pairwise multiple comparison test. Differences were considered statistically significant at P<0.05.

**RESULTS AND DISCUSSION**

**Effect of time of incubation on sperm functional parameters**: Mean ± SE of percentage of motile sperms before and after incubation with pbIFN-tau-EGFP is presented in Table 2. A significant decrease (P<0.05) in percentage of motile spermatozoa was observed at different time intervals (1h and 2h) in the subgroups when compared with the 0h period. The percentage of motile spermatozoa at 1h of incubation when compared with 0h time of incubation in Group I (Control, 40million/ml) and groups with 10ug, 20ug gene (Group IA,IB) was observed to be 91.67, 91.83 and 91.22 respectively. Similarly percentage of motile spermatozoa was 91.83%, 92.58%, 92.28% was observed for Group II (Control, 80million/ml) or with 10ug, 20ug gene (Group IIA,IB) respectively. Percentage of motile spermatozoa observed after 2h of co-incubation when compared with 0h time of incubation was 85.62%, 85.62%, 85.17%, for Group I with IA and B subgroups and 86.99%, 86.22%, 86.38% for Group II, IIA and B subgroups respectively as mentioned earlier. The values at 0h were adjusted to 100%.

Mean ± SE of percentage of viable sperms before and after incubation is presented in Table 2. A significant decrease (P<0.05) in percentage of viable spermatozoa was observed when different time intervals were considered for all the groups. The decrease in percentage of viable spermatozoa in Group I (40million/ml) after 1h of co-incubation was observed to be 7.12%, (Control) 7.25%.
(IA), 7.50% (IB) and also in Group II (80 million/ml) a decrease of 6.25% (Control), 6.37% (IIA), 7.12% (IIB) was observed. A significant decrease in viability was also observed after 2h of co-incubation i.e. 9.37%, 11.12%, 11.12% for Group I with different subgroups and 9.10%, 9.50%, 10.75% in Group II, with different subgroups respectively as mentioned earlier. The percentage in the decrease of viable sperms was positively related with the increase in the time of incubation. When the time period was kept constant, difference in the percentage of motile or viable sperms was not significant between the groups or subgroups. The different concentrations of gene also did not affect both the parameters.

Spermatozoa incubated with different concentrations of gene (10 µg/ml and 20 µg/ml) or without (control) gene when subjected to IVF, resulted in successful fertilization of oocytes and cleaved embryos. Mean ± SE of resulting cleaved embryos (%) is presented in Table 3 and Fig. 1. There was no significant difference in the percentage of cleaved embryos obtained post fertilization of oocytes with sperm samples incubated with gene for 1h or without pBlIFN-tau-EGFP. It was observed that 2h time interval for incubation with either concentration of sperm or gene resulted in significantly (P<0.05) less number of cleaved embryos. (Table 3).

**PCR analyses of DNA extracted from transfected sperms and embryos obtained post fertilization:** The extracted DNA was subjected to amplification by PCR for IFN-tau gene. The PCR amplified products when electrophoresed on 1.8% agarose gel, a 585 bp product for IFN-tau gene was obtained, revealing transfer of the gene to the embryos through transfected spermatozoa (Fig. 3).

Embryos were observed for EGFP fluorescence under fluorescent microscope, green fluorescence could not be observed in cleaved or morula stage embryos. After fertilization process may be the expression of gene became silent or the expression was weak to be captured under fluorescence microscope, further research is required in this direction to improvise the quality of expression of transfected gene even though results were positive with respect to uptake of gene.

Simple incubation, transfection technique proved to be superior to lipofection (Camaioni et al., 1992) and electroporation (Gagne et al., 1991) techniques where sperm motility and viability decreased more than what has been reported in the present study.

From the present study, it can be suggested that Murrah buffalo spermatozoa can be subjected to simple incubation with pBlIFN-tau-EGFP gene for 1h/2h for yielding transfected spermatozoa which could be utilized further for IVF yielding cleaved embryos. Decline in motility and viability did not affect fertilizing potential as observed with respect to
percentage of cleaved /morula stage embryos obtained in the present study and also as reported by Canovas et al. (2010) in cattle. The media for sperm processing and transfection procedures were optimum for yielding cleaved embryos (41-48%) when subjected to IVF. These results are comparable with others where the results reported are similar without incubation with the gene (Bacci et al., 2009). During transfection process by simple incubation negligible toxic effect was observed. SMGT is a simple technique and becomes even more simpler and less cytotoxic when procedure for transfection by simple incubation is followed when compared with electroporation and lipofection techniques.

REFERENCES


