IN VITRO ELECTROPORATION OF CAPRINE SPERMATOZOA FOR INTRODUCTION OF FOREIGN DNA

Thesis

Submitted in partial fulfilment of the requirement for the degree of

MASTER OF VETERINARY SCIENCE

in

ANIMAL GENETICS AND BREEDING

By

Dr. Rakesh Kumar

Roll No. 4886

To

DEEMED UNIVERSITY

INDIAN VETERINARY RESEARCH INSTITUTE

Izatnagar – 243 122 (U.P.)

2012

Dedicated to....

My beloved Parents

&

Brother

भारतीय पशु चिकित्सा अनुसंधान संस्थान
(सम विश्वविद्यालय)
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Dated: 15th June, 2012

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Certified that the research work embodied in this thesis entitled **"In vitro electroporation of caprine spermatozoa for introduction of foreign DNA"** *submitted by* **Dr. Rakesh Kumar, Roll No. 4886,** *for the award of* **Master of Veterinary Science** *degree in* **Animal Genetics and Breeding** *at Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate himself under my supervision and guidance.*

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Abbreviations

A	Adenine
AI	Artificial Insemination
BSAFAF	Bovine Serum Albumin Fatty Acid Free
CFDA	Carboxyfluorescein Diacetate
CAT	Chloramphenicol acetyl transferase
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's Minimum Essential Media
ECMV	Encephalomyocarditis Virus
EDTA	Ethylene Diamine Tetra Acetic acid
EGFP	Enhanced Green Fluorescent Protein
EP	Electroporation
EF	Electric Field
E-N	Eosin-Nigrosin
EYC	Egg Yolk Citrate
FITC	Flourescein Isothiocyanate
GFP	Green Fluorescent Protein
GAA	Glacial Acetic Acid
HOST	Hypo-osmotic Swelling Test
ICSI	Intracytoplasmic Sperm Injection
Ifs	Inhibitory' factors
IRES	Internal Ribosome Entry Site
IVF	In vitro Fertilization
MCS	Multiple Cloning Site
MEM	Minimal Essential Medium
M	Mannitol
MT	Mannitol-TALP
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PSA	Pisum Sativum Agglutinin
REMI	Restriction Enzyme-Mediated Integration
RQ	Relative Quantization
RT	Reverse Transcriptase

RT	Room Temperature
RT-PCR	Reverse Transcriptions- Polymerase Chain Reaction
RT-qPCR	Quantitative Real Time PCR
SDS	Sodium Dodecyl Sulphate
SLB	Sperm Lysis Buffer
SMGT	Sperm Mediated Gene Transfer
SMRGT	Sperm-Mediated Reverse Gene Transfer
TAE	Tries acetate EDTA
TMGT	Testis Mediated Gene Transfer
TALP	Tyrodes Albumin Lactate Pyruvate

UNIT OF MEASUREMENT

%	Percent
°C	Degree Centigrade
bp	Base pair
cm	Centimeter
dl	Deci litre
g	Gram(s)
Hr	Hour
IU	International unit
Kb	Kilo base pair
kDa	Kilo Dalton
M	Molar
Ma	Milli ampere
mg	Milli gram
min	Minute
ml	Milli litre
mM	Millimolar
mm	Millimeter
MW	Molecular weight
ng	Nanogram
rpm	Revolution (s) per minute
rcf	Relative centrifugal force
S	Second
V	Volts
V/v	volume/volume
W/v	Weight/volume
µg	Microgram
µl	Micro liter
pg	Pico gram
nM	Nanomolar

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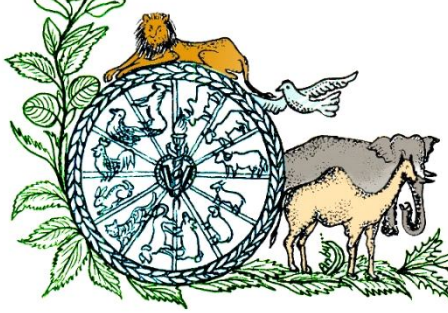
Introduction



1 Introduction

Transgenic animals, commonly referred as "transgenics", carry a gene-sequence of interest, often from a different species, which integrates into the genome of the host animal giving it a new characteristic (Gordon and Ruddle, 1980). These animals serve as a powerful tool in the study of gene regulation and developmental biology. Four techniques are commonly used in making transgenic animals, viz., DNA microinjection (Gordon *et al*., 1980), embryonic stem cell mediated gene transfer (Torres *et al.*, 1997), retrovirus mediated gene transfer (Robertson *et al*., 1986) and nuclear transfer (Wilmut *et al*., 1997). Each of these methods with its own advantages and disadvantages and have been successful in laboratory animal species particularly in mice. Attempts to introduce transgenes into large animal species, mainly livestock species using these methods have limited success (Houdebine *et al.*, 2002). Even DNA microinjection (Gordon *et al*., 1980) which is the most widely used method has a very limited success rate (<1%) in large animal species. Success of viral mediated gene transfer has been limited due to the difficulty in transferring large transgene and bio-safety issues. The success rates of embryonic stem cell mediated gene transfer and nuclear transfer methods are only 2% (Gossler *et al.,* 1986) and 1-3% (Solter, 2000), respectively. Further limitations include longer gestation and generation times, small litter sizes and requirement of large number of donor animals (Mullins and Mullin, 1996). Besides, these methods are often technically challenging, labour intensive, require costly equipment and highly specialized skill (Sato, 2005).

In the year 1971, the first evidence of mammalian spermatozoa being able to take up and transfer exogenous DNA was demonstrated by Bracket *et al*. In this pioneering report, rabbit sperm cells were exposed to [^{3}H]-thymidine-labelled SV40 DNA and subsequently

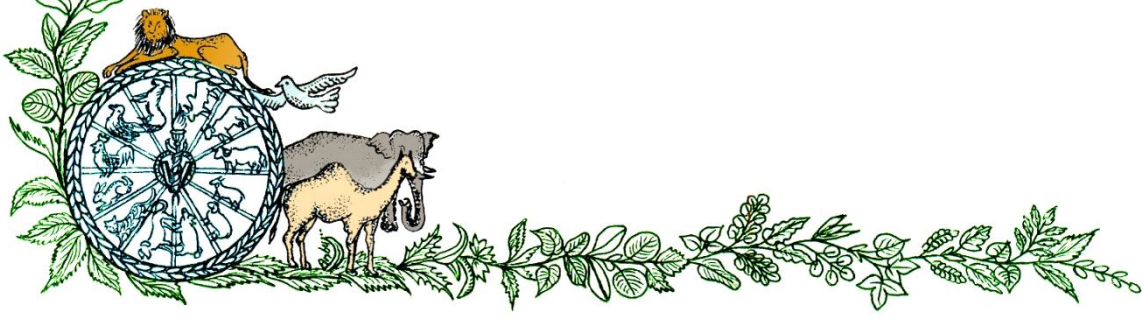
radioactive material could be detected in the heads of spermatozoa. Binding of exogenous DNA molecules to sperm cells is mediated by a specific class of DNA-binding proteins (Zani *et al*., 1995), and is modulated by a conserved glycoprotein abundant in the seminal fluid of mammals or associated to spermatozoa of lower organisms (Lavitrano *et al*., 1992; Zani *et al*., 1995). A portion of sperm-bound DNA is internalized within sperm nuclei (Francolini *et al*., 1993) in a process mediated by CD4 molecules (Lavitrano *et al*., 1997). The DNA associated with the sperm is then spontaneously incorporated (via protein inter actions) into the sperm nuclei (Zani *et al*., 1995). The sperm then carries the genetic material of interest to the egg, which in the process of fertilization incorporates the exogenous DNA (Celebi *et al*., 2003).

Successful gene transfer based on Sperm mediated gene transfer (SMGT) has been reported in crustacean (Chen *et al*., 2006), fishes (Khoo, 2000; Kurita *et al*., 2004), amphibians (Jonak, 2000), avies (Gruenbaum *et al*., 1991; Nakanishi and Iritani, 1993), and mammals (Lavitrano *et al*., 1989; Seperandio *et al*., 1996). However, due to the variable success rate and lack of repeatability, researchers have been finding ways to increase the capability of spermatozoa to capture exogenous DNA by chemical as well as physical methods. Chemical methods include utilization of liposome (Bachiller *et al*., 1991) and linkers (Epperly, 2007) while physical methods include freezing-thawing and electroporation (Gagne *et al*., 1991). Chemical mediated transgenesis has a variable success rate of approximately 4% for liposome (Bachiller *et al*., 1991) and 20% for linker based methods (Epperly, 2007). Anzar and Buhr (2006) reported a greater transfection efficiency of frozen-thawed bull spermatozoa than fresh spermatozoa from the same ejaculate. While comparing the efficiency and ability of fresh and frozen-thawed sperm to pick up exogenous DNA, Zhao *et al*. (2005) also demonstrated that frozen-thawed goat sperm were more efficient and reliable than fresh sperms. A recent report demonstrated a variable success of binding (3.08 -73.39%) and internalization (4.83–70.00%) of foreign DNA when buck semen was simply incubated with the DNA (Zhao *et al*. 2005). In contrast, the physical method which primarily includes electroporation has been used with better success rates. In a study to test the ability of the spermatozoa to carry foreign DNA into the bovine oocyte revealed that electroporation resulted in an increased absorption of DNA by the spermatozoa (Gagne *et al.,* 1991). Electroporation mediated *in vivo* transfection of sperm cells referred as Testis mediated gene transfer (TMGT) demonstrated in a success rate

of >94% in mice (Dhup and Majumdar, 2008). Available literatures although suggest the immense potential of electroporation to produce transgenic animals, it has not been used in large animals species. Keeping this in view, the present study was undertaken with the following objectives:

- **To study the effect of electroporation on semen parameters**
- **To standardize *in vitro* electroporation protocol for internalization of foreign DNA**

Review of Literature



2 Review of Literature

The term transgenic animal refers to an animal in which there has been a deliberate modification of the genome, in contrast to spontaneous mutation. Foreign DNA is introduced into the animal, using recombinant DNA technology, which gets transmitted through the germ line so that every cell, including germ cells, of the animal contains the same modified genetic material. If the germ cell line is altered, characters will be passed on to succeeding generations in normal reproduction. If the somatic cell line alone is altered, only the organism itself will be affected, not its offspring. Transgenesis may involve whole organisms, rather than individual cells, and there may be *in vivo* alteration of body function. Four main methods are used for the production of transgenic animals including pronuclear microinjection, embryonic stem cell transfer, retroviral vectors and nuclear transfer, all result in the insertion of new genetic material into an oocyte.

Transgenic technique has been successful in generating small animals and in particular, transgenic mice. In larger animals, these same methods have limited success (Chang *et al*., 2002., Houdebine, 2002), due to longer gestation period and generation times, reduced litter sizes and the large number of donor animals necessary (Mullins and Mullins, 1996). In the year 1971, the first evidence of mammalian spermatozoa being able to take up and transfer exogenous DNA was demonstrated by Bracket *et al*. (1971). Over time Sperm mediated gene transfer (SMGT), including its alternative approach testis mediated gene transfer (TMGT), becomes an established and reliable method for transgenesis. They have been extensively used for producing transgenic animals and offers exciting prospects for experimental and applied biology, agricultural and medical sciences. In the following review, this method will be reviewed under two broad headings, sperm medited gene transfer and testis mediated gene transfer.

2.1 SPERM MEDIATED GENE TRANSFER (SMGT)

The first evidence that mammalian spermatozoa are able to take up exogenous DNA molecules is now 25 years old (Brackett *et al*., 1971). This report was ahead of its time since both molecular biology and *in vitro* fertilization were at their very beginning. As a consequence, it was largely forgotten until 1989, when two reports (Arezzo, 1989; Lavitrano *et al*., 1989) described the binding of exogenous DNA in sea urchin and mouse, respectively. Since then, numerous strands of evidence have clearly indicated that spermatozoa have the peculiar property of allowing the internalization of molecules of different size and different nature, including DNA. Brackett *et al*. (1971) showed that [^{3}H]-thymidine labelled SV40 virus DNA was found in the post-acrosomal area of 30 -35% of exposed sperm cells. The capacity of spermatozoa exposed to SV40 DNA to infect cells even after their exposure to DNase suggest that at least part of the DNA penetrated into the spermatozoa. This hypothesis was confirmed by the autoradiography results. More recently, similar observations have been described by Kuznetsov and Kuznetsova (1995).This property is common to different species of sea urchins as described by Arezzo (1989) and it is not limited to DNA.

Sperm mediated gene transfer uses sperm as a vector to generate transgenic animals. Sperm cells are exposed to foreign DNA, which binds to the surface (of the sperm) through specific protein-protein interactions. The DNA associated with the sperm is then spontaneously incorporated (via protein inter actions) into the sperm nuclei (Zani *et al.*, 1995). The sperm then carries the genetic material of interest to the egg, which in the process of fertilization incorporates the exogenous DNA (Celebi *et al.*, 2003). The performance of SMGT, if improved could be one of the most rapid ways to produce transgenic animals.

Sperm mediated gene transfer has been a highly controversial issue since its first appearance (Lavitrano *et al*., 1989), because shortly after the earliest report several groups reported their failure to reproduce the original protocol (Brinster *et al*., 1989). There is currently a general consensus that only two steps in the SMGT process are well-established and fully reproducible: (i) the spontaneous interaction between sperm cells and foreign DNA molecules, and (ii) the delivery of sperm-bound DNA to oocyte at fertilization. The subsequent fate of sperm-bound DNA, after delivery in the oocyte, is still a contradictory issue; in particular, the question of whether foreign molecules of nucleic acids become integrated into the host genome or remain as extra chromosomal structures is still unsolved.

Lavitrano *et al*. (2003); Lavitrano *et al*. (2006), demonstrated that there are two important parameters that must be optimal for the SMGT technique to be effective in swine: selection of sperm donor and optimization of DNA uptake. Brinster *et al*. (1989) attempted to repeat Lavitrano's experiment, but they failed. Other research groups, such as Birnstiel and Busslinger (1989), held negative attitude because there is no selective advantage and will be severe chaos in evolution if sperm cells are able to take foreign or contaminated DNA. In spite of the controversy, many studies were performed to investigate the possibility of using spermatozoa as a vector for gene transfer.

Zhao *et al*. (2005) found that different goat sperm donors appeared to differ in ability to bind foreign genes. They selected two bucks, one Boer goat and another Yudong White goat, as sperm donors. They concluded that these two bucks could supply high quality semen and serve as good vectors for exogenous DNA. Frozen-thawed spermatozoa could also be used as a vector for introduction of exogenous DNA and produce transgenic goats. Anzar and Buhr (2006) found transfection efficiency of bull spermatozoa to be greater in frozen-thawed than in fresh spermatozoa, both from the same ejaculate. They have investigated the efficiency and the ability of fresh and frozen-thawed sperm to pick up exogenous DNA, and found that frozen-thawed goat sperm were more efficient and more reliable than fresh sperm cells.

For a long time, researchers have been finding ways to increase the capability of spermatozoa to capture exogenous DNA, such as utilization of liposome (Bachiller *et al*., 1991), electroporation (Gagne *et al*., 1991), combination of restriction enzyme-mediated integration (REMI) with SMGT (Kroll and Amaya, 1996), as well as combination of intracytoplasmic sperm injection (ICSI) with sperm/DNA interaction (Perry *et al*., 1999). Other alternative means of SMGT, including TMGT (Sato *et al*., 1994), have been developed at the end of last century. To a great extent, the efficiency of gene transfer by spermatozoa has been increased by all the new techniques and improvements. These experiments can be broadly classified into two distinct categories, such as 'Autouptake' and 'Augmented' uptake of naked DNA molecules.

2.1.1 'Autouptake' of Naked DNA Molecules

Seminal fluid reportedly contains an inhibitory factor (IF-1) that appears to actively block the binding of exogenous DNA to sperm. These IFs are envisaged to prevent exogenous

DNA uptake so as to protect the genetic integrity of the conceptus. The corollary of this notion is that successful instances of sperm cells taking up exogenous DNA may be attributed to the fortuitous removal or inhibition of IF(s) (Zani *et al*., 1995). Incubation of foreign DNA with spermatozoa, termed "DNA incubation", is the direct method reported by Brackett *et al*. (1971). This straightforward approach suspends the seminal plasma-free sperm cells in an appropriate medium containing foreign DNA molecules. Then the resultant sperms carrying foreign DNA are used to fertilize eggs by *in vitro* fertilization (IVF) or artificial insemination (AI).

The Lavitrano *et al*. (1989) experiments employed pSV2CAT plasmid, to which washed epididymal mouse sperm cells were exposed for 30 minutes at 37°C (at a concentration of 0.2-2.0 μg/10^6 sperm cells). Southern blot analysis of 250 progeny arising from IVF of eggs with pSV2CAT-treated sperm cells indicated the presence of the transgene in ~30% of the animals. Additionally, restriction mapping and sequence analysis confirmed the presence of transgene, as did the detection of CAT (chloramphenicol acetyl transferase) gene expression. Further, the transgene was detected in the F_1 progeny. To increase the efficiency of capturing exogenous DNA by spermatozoa, modification of sperm cells or media has shed light on the SMGT strategy. Horan *et al*., (1991) suggested that motile sperm were more efficient at capturing DNA molecules than non-motile sperm. Recently, the overall efficiency of SGMT was reported to be higher when ejaculated spermatozoa were used, compared to epididymal spermatozoa, while development of transgenic embryos was a DNA dose-dependent effect in which high DNA doses arrested the embryonic development (Sciamanna *et al*., 2003).

2.1.2 'Augmented' Uptake of Naked DNA Molecules

In augmentation techniques sperm cells integrate with exogenous DNA by Chemical and Physical methods.

2.1.2.1 Chemical method mediated transgenesis

The search for an optimized DNA carrier system for efficient gene delivery is one of the most important tasks of non-viral gene therapy. Receptor-mediated or cationic lipid-mediated systems are presently the most favored approaches. Structural requirements of the resulting complexes such as DNA condensation and complex surface charge, fusogenic action and minimum complex size have a very important influence on transfection.

It includes liposome mediated and linker based gene transfer methods.

2.1.2.1.1 Liposome's mediated technology

Liposome is small bodies consisting of membrane-like lipid layers surrounding hydrous compartments. Furthermore, liposome can be used to protect a foreign DNA from digestion of proteases or DNase I present in the cytoplasm of egg (Schaefer-Ridder *et al*., 1982). Commercial available cationic liposome can spontaneously interact with RNA/DNA and fuse with the plasma

membrane in a wide variety of cell types in culture to produce transfect ions (Schaefer-Ridder *et al*., 1982; Felgner *et al*., 1987; Francolini *et al*., 1993). Cationic liposome was used to increase the transfection efficiency of sperm cells. Association of the cationic liposome/ DNA complexes with sperm cells may allow DNA to be carried into oocytes at fertilization (Bachiller *et al*., 1991). However, sperm motility and fertilizing capability of spermatozoa was lower at the higher concentration of liposome as assessed by microscopic observation.

To determine the dose concentration of DNA and cationic liposome required for optimal transfection of sperm cells. Recently reported that BSA, a major serum protein, could prevent the cellular uptake of liposome/DNA complexes in cells. Rabbit spermatozoa were incubated with liposome/DNA mixture before *in vitro* fertilization, and 66% spermatozoa were detected carrying foreign DNA. The high transgenic rates were reported more recently in mouse F_1 (41%) and F_2 (37%) offspring via TMGT using liposome (DOTAP) treated plasmid DNA (He *et al*., 2006). However, Gorlova and Torchilin, (1991) reported negative results following successful uptake of DNA by sperms of mouse. Furthermore, the existence of several different types of liposome makes it difficult to make general predictions as to the likelihood of success, in the absence of specific empirical studies.

2.1.2.1.2 Linker (receptor) based method

The process of linking the exogenous DNA to the head of the sperm was reported by (Chang *et al*., 2002) using the monoclonal antibody mAbC. The antibody (mAbC) is a positively charged basic linker protein; it binds to negatively charged DNA via ionic interactions. These interactions specifically bind exogenous DNA to sperm in a precise way. DNA can bind to polycations in a strong but no covalent manner forming soluble complexes. DNA coupled with

antibodies or antibody-fragments offers the ability to internalize the complexes via receptor-mediated endocytosis (Varga *et al*., 2000).

2.1.2.2 Physical method mediated transgenesis

It primarily includes electroporation technique for gene transfer

2.1.2.2.1 Electroporation

2.1.2.2.2 Electrode Assemblies Used for Electroporation of Cultured Cells

Electroporation was initially developed for the introduction of DNA into cells which grow in suspension and was performed in a cuvette with two flat electrodes on opposite sides. Different configurations were subsequently developed for the electroporation of adherent cells in situ, while the cells were growing on nonconductive surfaces or a gold-coated, conductive support. They developed an assembly where the cells grow and are electroporated on optically transparent, electrically conductive indium-tin oxide (ITO). This material promotes excellent cell adhesion, growth, is inert, durable, as well as it does not display spontaneous fluorescence, making the examination of the electroporated cells by fluorescence microscopy possible. The molecules to be electroporated are added to the cells and introduced through an electrical pulse delivered by an electrode placed on top of the cells. several electrode and slide configurations which allow the electroporation of large numbers of cells for large-scale biochemical experiments or for the detection of changes in cell morphology and biochemical properties in situ, with control, nonelectroporated cells growing on the same type of ITO-coated surface, side by side with the electroporated ones.

2.1.2.2.3 *In vitro* Electroporation

Electroporation is particularly useful in cells that are difficult to transfect by other means (Jordan *et al*., 2008). A very useful development that has allowed researchers to avoid lengthy optimisation steps is a commercial product called nucleofection developed by Amaxa laboratories (now owned by Lonza Group Ltd). Nucleofection is a variant of electroporation that uses pulse settings and solutions optimized for specific cells. A number of papers report the use of this technology to efficiently transfect otherwise difficult to transfect cells, for example in cardiovascular research (Iversen *et al*., 2005; Thiel and Nix, 2006). Electroporation can be used to transfer a range of genetic materials into cells including DNA, RNA and oligonucleotides.

2.1.2.2.4 *In vivo* Electroporation

The most substantial change in the efficiency of plasmid-based gene transfer has been seen when delivery of plasmid is followed by the application of a series of electrical pulses as a form of *in vivo* electroporation. *In vivo* electroporation of plasmid DNA was first used for skin and liver but skeletal muscle has recently attracted a lot of attention, as expression of the episomal plasmid can be long lived in this tissue. Indeed, a wide range of tissues have been studied including skin, kidney, lung, liver, skeletal, cardiac muscle, joints, spinal cord, brain, retina, cornea and the vasculature. In most studies, electroporation increased gene expression by 100 to 1000 fold compared to injection of naked plasmid DNA.

The exact mechanism by which delivery of plasmid into cells is enhanced is not certain, although it is clear that membranes become effectively permeable once a critical voltage has been achieved (in the order of 200 V/cm *in vivo*). This is thought to occur by the formation of hydrophilic pores and subsequent movement of plasmid through these pores as a local electrophoretic effect. Golzio *et al*. (2005) used fluorescently labeled plasmid to visualize the interaction with single cells *in vitro*. In their system, plasmid was seen to accumulate at the cell membrane via an electrophoretic effect but did not immediately move into the cytosol. Movement into the cytoplasm was relatively slow and continued after the application of the electrical field ended. This is consistent with the DNA active uptake mechanism proposed by Budker *et al*. (2000), but may represent a novel physical interaction with the cell membrane as, with time, the plasmid that had not yet entered the cytoplasm became inaccessible to a DNA dye. However, this plasmid accumulation has yet to be observed *in vivo* and the complex organization of tissues such as muscle may significantly modify this process. In general, the plate electrodes appear to give a more uniform electrical field and are more commonly used for small animal's studies but they may not be suitable for electrotransfer in large animals due to the large electrical fields that would need to be applied to larger tissues. The pattern of electrical pulses also varies considerably between studies ranging from moderate voltage (e.g. 200 V/cm) pulses of tens of milliseconds to high-voltage microsecond pulses.

Electroporation has been extensively tested for gene transfer into tumors. Even the transfer of empty plasmid vector is sufficient to cause significant tumor regression in some models. Electroporation of plasmids containing interleukin 12 (IL-12) and interleukin 18 (IL-

18) has been shown to produce a synergistic effect in inhibiting tumor growth, both for the treated tumor and also for the untreated contra lateral tumor. Electrotransfer can also be used for genetic vaccination and a number of laboratories have demonstrated substantial improvements in responses to a variety of antigens. Finally, integration of plasmid DNA has not been previously reported following direct intramuscular injection despite a number of careful studies from several laboratories.

2.1.2.3 Mechanism of integration of exogenous nucleic acid in sperm chromosomes

Binding of exogenous DNA molecules to sperm cells is mediated by a specific class of DNA-binding proteins (Zani *et al*., 1995), and is modulated by a conserved glycoprotein abundant in the seminal fluid of mammals or associated to spermatozoa of lower organisms (Lavitrano *et al*., 1992; Zani *et al*., 1995). A portion of sperm-bound DNA is internalized within sperm nuclei (Francolini *et al*., 1993) in a process mediated by CD4 molecules (Lavitrano *et al*., 1997). The internalized foreign DNA reaches the nuclear scaffold, in close contact with the chromosomal DNA, becomes heavily rearranged and eventually undergoes recombination events that cause the integration of exogenous sequences in the sperm genome (Zoraqi and Spadafora, 1997). Consistent with those observations is the finding that the interaction of exogenous DNA with sperm cells activates endogenous nucleases in a DNA dose-dependent manner (Maione *et al*., 1997). Sperm nucleases heavily degrade the foreign DNA and can also locally degrade sperm

Chromosomal DNA, suggesting that discrete sites of hypersensitivity exist within the tightly packed chromatin of mature sperm cells which are triggered upon interaction with foreign DNA molecules.

More recently, they have suggested that the DNA is also integrated into the sperm chromatin (Zoraqi and Spadafora, 1997). Several other laboratories have reported that live spermatozoa both from the mouse (Huguet and Esponda, 1998) and other species (Lavitrano *et al*., 1989; Gagne *et al*., 1991; Camaioni *et al*., 1992; Horan *et al*., 1992) have the ability to bind to exogenous DNA. Mammalian sperm DNA is organized into loop domains of about 30–50 kb in length. Mouse spermatozoa can interact with exogenous pSV2CAT plasmid DNA. In this work, they explored this interaction and examined the sub cellular localization of

the exogenous DNA. They found a repeatable association of exogenous DNA with a specific region of the sperm nuclear matrix. This region of the nucleus correlates with the equatorial segment of the sperm head.

2.1.2.4 Sperm-Mediated Reverse Gene Transfer (SMRGT)

Reverse transcriptase (RT) activity plays a central role in SMGT. 'Retro-genes' are generated either through reverse transcription of exogenous RNA internalized in spermatozoa, or through sequential transcription, splicing or reverse transcription of exogenous DNA. The resulting retro-genes are delivered to oocytes and transmitted to embryos and born animals as low-copy, transcription ally competent, extra chromosomal structures capable of determining new phenotypic traits. Retro-genes can be further transmitted through sexual reproduction from founders to their F1 progeny (Giordano *et al*., 2000; Sciamanna *et al*., 2003). New genetic and phenotypic features, unlinked to chromosomes, can thus be generated and inherited in a non-Mendelian ratio. They have called this phenomenon as a sperm-mediated 'reverse' gene transfer (SMRGT). Thus, RT-mediated machinery operates in sperm cells and is responsible for the genesis and non-Mendelian propagation of new genetic information. The features of RT-generated traits elicited in SMRGT resemble those characterized in recent studies of RNA-mediated inheritance of extra-genomic information. Therefore, spermatozoa can reverse-transcribes foreign RNA and subsequent resultant cDNAs can be transmitted to offspring.

2.1.2.5 Restriction Enzyme-Mediated Integration (REMI)

A new method for introducing foreign DNA into spermatozoa is restriction enzyme-mediated integration (REMI). In brief, plasmids were linearized with a restriction enzyme to generate single-stranded cohesive ends and then introduced *in vitro* into decondensed sperm nuclei using REMI. Shemesh *et al*. (2000) produced transgenic bovine sperms by combining REMI with liposome, and demonstrated that these transgenic sperms could be used to produce transgenic embryos and live offspring by IVF or AI. Sperm mediated gene transfer has also been successfully combined with intra-cytoplasmic sperm injection (ICSI) to produce transgenic animals (Chan *et al*., 1998; Lai *et al*., 2001). Before incubating with foreign DNA, however, spermatozoa must be pretreated with Triton-X or undergone repeated freeze-thaw cycles (Perry *et al*., 1999).

The important experiments of sperm mediated gene transfer can be summarized in following table 1.

Species	Approach	Sperm Uptake[1]	Fertilization[2]	Fertilization Method	Latest Development Stage	Reference
Rabbit	DNA incubation	Yes	Yes	AI	two cell ova	Brackett *et al*., 1971
Sea urchin	DNA incubation	Yes	Yes	IVF	Embryos	Arezzo, 1989
Mouse	DNA incubation	–	N	IVF		Brinster *et al*., 1989
Pigs	DNA incubation	Yes	Yes	IVF	Adults	Gandolfi *et al*., 1989
Chicken	DNA incubation	–	Yes	IVF	adults & progeny	Fainsold *et al*., 1990
Mouse	Liposomes	Yes	N	IVF	Progeny	Bachiller *et al*., 1991
Bull	Electroporation	Yes	Yes	IVF	Blastocysts	Gange *et al*., 1991
Mouse	Liposomes	Yes	–			Gorlova and Torchilin, 1991
Pig	Electroporation	Yes	-			Horan *et al*., 1992
Rooster	Liposomes	–	Yes	AI	12-day fetuses	Rottmann *et al*., 1992
Salmon	Electroporation	–	Yes	IVF	Fry	Sine *et al*., 1993
Salmon	Electroporation	Yes	-			Symonds *et al*., 1994
Rabbit, bull chicken	Liposomes	Yes	Yes	IVF	fetuses & adults	Sperandio *et al*., 1996
Bull and pig	DNA incubation	–	Yes	AI	Adults	Cabrera *et al*., 1997
Human	DNA incubation	Yes	Yes	IVF	Blastocysts	Chen & Tsai, 1997
Pig	DNA incubation	_	Yes	AI	Piglets	Lavitrano *et al*., 1999

[1]direct evidence (e.g. from autoradiography) presented that spermatozoa have taken up exogenous DNA.
[2]evidence presented that exogenous DNA-bearing sperm have upon fertilisation transferred the transgene
IVF = *in vitro* fertilization, AI = artificial insemination, N = Negative result

2.1.2.6 DETECTION OF THE TRANSGENE

For the detection of the transgene following technique can be used.

2.1.2.6.1 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is a technique widely used in molecular biology. It derives its name from one of its key components, a DNA polymerase used to amplify a piece of DNA by an *in vitro* enzymatic replication. PCR entails enzymatic amplification of specific DNA sequences using two oligonucleotide primers that flank the DNA segment to be amplified. The rapid production of large quantities of a specific DNA sequence took a leap forward with the development of the PCR. Polymerase chain reaction, now a common and

indispensable technique was developed by Mullis *et al.* (1986), is used in medical and biological research laboratories for a variety of applications.

Innis and Gelfand, (1990) pointed out that "time of temperature" was the main reason for loss of activity of *Taq* DNA polymerase. They suggested that reducing the denaturation time to 30 s for short template sequences and increasing the denaturation temperature up to 96 ^{0}C might prolong the activity of the enzyme. Yap and Mc Gee, (1991) suggested the possibility of reducing the denaturation temperature after about 10 rounds of amplification as the mean length of target DNA was decreased. For templates of 300 bp or less, denaturation temperatures may be reduced to as low as 88^{o}C. For templates with 50 per cent (G+C) content as many as 40 cycles could be carried out without much decrease in enzymes efficiency. Hongbao, (2005) used a peltier heat pump to quickly heat and cool the DNA and used *Taq* polymerase for the synthesis of DNA.

2.1.2.6.2 Reverse Transcription -Polymerase Chain Reaction (RT- PCR)

Reverse transcription polymerase chain reaction (RT-PCR) is a variant of polymerase chain reaction (PCR), a laboratory technique commonly used in molecular biology to generate many copies of a DNA sequence, a process termed "amplification". In RT-PCR, however, an RNA strand is first reverse transcribed into its DNA complement (*complementary DNA*, or *cDNA*) using the enzyme reverse transcriptase and the resulting cDNA is amplified using traditional PCR or real-time PCR. Reverse transcription PCR is not to be confused with real-time polymerase chain reaction (Q-PCR/qRT-PCR), which is also sometimes abbreviated as RT-PCR.

RT-PCR includes three major steps. The first step is reverse transcription (RT), in which RNA is reverse transcribed to cDNA using reverse transcriptase. This step is very important in order to perform PCR, since DNA polymerase can act only on DNA templates. The RT step can be performed either in the same tube with PCR (one-step PCR) or in a separate one (two-step PCR) using a temperature between 40°C and 50°C, depending on the properties of the reverse transcriptase used (Bustin, 2000).

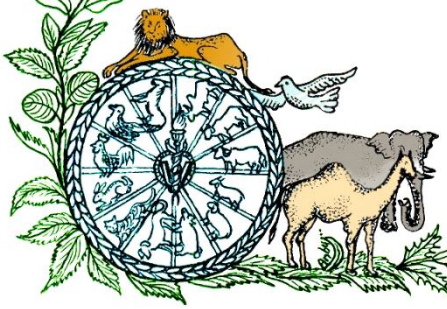
The next step involves the denaturation of the dsDNA at 95°C, so that the two strands separate and the primers can bind again at lower temperatures and begin a new chain reaction.

Then, the temperature is decreased until it reaches the annealing temperature which can vary depending on the set of primers used, their concentration, the probe and its concentration (if used), and the cations concentration. The main consideration, of course, when choosing the optimal annealing temperature is the melting temperature (Tm) of the primers and probes (if used). The annealing temperature chosen for a PCR depends directly on length and composition of the primers. This is the result of the difference of hydrogen bonds between A-T (2 bonds) and G-C (3 bonds). An annealing temperature about 5 degrees below the lowest Tm of the pair of primers is usually used (Innis and Gelfand, 1990).

The final step of PCR amplification is DNA extension from the primers. This is done with thermostable *Taq* DNA polymerase, usually at 72°C, the temperature at which the enzyme works optimally. The length of the incubation at each temperature, the temperature alterations, and the number of cycles are controlled by a programmable thermal cycler. The analysis of the PCR products depends on the type of PCR applied. If a conventional PCR is used, the PCR product is detected using agarose gel electrophoresis and ethidium bromide (or other nucleic acid staining). Conventional RT-PCR is a time-consuming technique with important limitations when compared to real-time PCR techniques (Mackay *et al*., 2002). This, combined with the fact that ethidium bromide has low sensitivity, yields results that are not always reliable. Moreover, there is an increased cross-contamination risk of the samples since detection of the PCR product requires the post-amplification processing of the samples. Furthermore, the specificity of the assay is mainly determined by the primers, which can give false-positive results. However, the most important issue concerning conventional RT-PCR is the fact that it is a semi- or even a low-quantitative technique, whereas the amplicon can be visualized only after the amplification ends. Real-time RT-PCR provides a method in which the amplicons can be visualized as the amplification progresses using a fluorescent reporter molecule. There are three major kinds of fluorescent reporters used in real time RT-PCR, which are general non-specific DNA Binding Dyes such as SYBR Green I, TaqMan Probes and Molecular Beacons (including Scorpions). The real-time PCR thermal cycler has a fluorescence detection threshold, below which it cannot discriminate the difference between an amplification generated signal and background noise. On the other hand, the fluorescence increases as the amplification progresses and the instrument performs data acquisition during the annealing step of each

cycle. The number of amplicons will reach the detection baseline after a specific cycle, which depends on the initial concentration of the target DNA sequence. The cycle at which the instrument can discriminate the amplification generated fluorescence from the background noise is called the threshold cycle (Ct). The higher the initial DNA concentration, C_t .were low.

Materials and Methods



3 Materials and Methods

The present study was conducted at AG Division, IVRI, Izatnagar, Bareilly (UP), India, during 2011-2012. The institute is located at an altitude of 564 feet above the sea level and at latitude of 28º north and a longitude of 79º east. The climate touches both the extremes of cold and hot weather experienced in the country and the relative humidity ranges between 15 and 85%

3.1 MANAGEMENT OF BREEDING BUCKS

Adult bucks (n=2) of 2-3 years age, maintained under identical managemental condition at the experimental herd under Genome Analysis Lab were included for this study. Deworming and vaccination against major diseases at regular interval was done for prevention of major disease. Green leaves and concentrate feeds were given on the daily basis

3.2 STERILIZATION OF ARTICLES

All the glassware's used during the course of study were thoroughly washed with soap water and rinsed with double distilled water. Buffer solutions, rubber articles, micropipettes, micro tips and artificial vagina with its accessories were autoclaved at 15 psi pressure, 121 º C temperatures for 20 min

3.3 PREPARATION OF ARTIFICIAL VAGINA

Semen from Bucks were collected using artificial vagina which was made of heavy rubber with 20 cm length and 5 cm diameter. A rubber lining (latex liner) of 30 cm length and 5 cm diameter was inserted in the artificial vagina. The space between the rubber lining and AV were filled three-fourths with warm water (42-45^{0}C) and the air was blown in through the

valve to adjust the pressure. The quantity of water filled inside was 150-175 ml, the temperature of AV ready for use was around 42^{0}C. A graduated glass semen collection cup was held at one end (nearer to valve) of AV and the other end was lubricated with the soft paraffin to avoid injury to buck penis due to friction.

3.4 SEMEN COLLECTION

Collections were done in the morning hours before feeding following standard practice. When flehming reaction and mounting behavior were seen then bucks were allowed to mount on the dummy goat and ejaculated semen samples were collected in artificial vagina. Immediately after collection, the tubes containing semen were placed in the water bath maintained at 37 ºC and brought in the laboratory for further processing. The effect of two seasons i.e. winter (November to Feburary) and summer (March to May) were also assessed on seminal parameters. Average temperature varied from a maximum of 42^{0}C to a minium of 4^{0}C. Relative humidity varied in the range of 71.5 to 47.6% respectively.

3.5 ASSESSMENT OF SEMEN PARAMETERS

Various semen parameters viz., progressive motility, live-dead spermatozoa, membrane integrity, acrosome integrity and chromatin integrity were assessed in winter (November to February) and summer season (March to May) respectively.

3.5.1 Progressive Motility

The estimate of initial gross motility was not very precise. Some percentage of spermatozoa which were weakly motile, might be exaggerated under the influence of actively motile spermatozoa. Superiority of semen depends on the concentration of progressively motile spermatozoa present in it. The neat semen was diluted at 37ºCwith sodium citrate glucose buffer. Diluted film of semen was examined under high power phase contrast microscope (40X) for the presence of total and non-motile spermatozoa. Different fields were observed and the percentage of motile spermatozoa was ascertained. Hence, sperm motility was evaluated in semen by subjective assessment.

3.5.2 Percent Live Spermatozoa

The percentages of live spermatozoa were estimated by differential staining techniques using Eosin- Nigrosin stain (Campbell *et al.,* 1953) in fresh sample. The smears were prepared in duplicate, after gently mixing a small drop of neat semen with 4 drops of stain on a clean grease-free microscopic slide. At least 200 spermatozoa were counted under the oil-immersion objective (100X) of a phase contrast microscope for estimating the percentage of live (unstained) spermatozoa. The pinkish spermatozoa (eosinophilic) were classified as dead whereas unstained colorless spermatozoa against background of Nigrosin were scored as live. Partially stained spermatozoa were also considered as dead.

3.5.3 Hypo-Osmotic Swelling (HOST) Test

Hypo-osmotic swelling test was performed after slight modification of the method described by Jeyendran *et al.,* (1984) in fresh samples to assess the functional integrity of the sperm tail membrane which gives an idea of spermatozoal membrane integrity.

Procedure:

i) 0.1 ml of semen with 1.0 ml of hypo-osmotic solution were taken in test tube and mixed properly

ii) Mixture were incubated at 37 ºC for 60 min

iii) One drop of mixture was taken on grease free glass slide covered with cover slip

iv) For good visibility a drop of eosin solution was added and

v) Slides were examined under 40X of phase contrast microscope

vi) A total of 200 spermatozoa were counted in each slide and classified in four classes based on tail swelling pattern such as, Pattern A: No swelling, no membrane reaction, Pattern B: Swelling of the tip of the tail, Pattern C: Different types of hair-pin like swelling pattern or swelling of the mid piece, Pattern D: Complete tail swelling, Spermatozoa displaying either pattern B, C or D were considered positive for HOST.

3.5.4 Acrosome Integrity

The acrosome integrity of mammalian spermatozoa is pre-requisite for capacitation, normal acrosome reaction and successful fertilization *in vivo.* The acrosome contains a number

of hydrolytic enzymes and several acids hydrolyse. The enzymes localized in the acrosome determine the sperm penetrating and fertilizing capacity. Acrosome can be detached from the sperm head under the influence of different physical and chemical factors. Optimum fertility depends on the acrosome being structurally and functionally intact. The most commonly used staining method to detect acrosomal changes, based on staining intensity and contrast of background is Giemsa stain (Watson, 1975).

Procedure:

Acrosomal integrity of spermatozoa was assessed using Giemsa stain (Watson, 1975).

i) A smear of diluted semen was prepared on a clean, grease free glass slide and air-dried

ii) The smear was fixed in Hancock's fixative for 15 minutes

iii) Fixed smears were washed in slow running water for 15 minutes

iv) The smears were then rinsed with distilled water and air-dried

v) The smears were stained in Giemsa working solution for 90-120 min.

vi) Smears were then removed from the stain solution and rinsed quickly in distilled water, air dried and mounted in DPX

vii) The smears were examined under oil immersion objective of the microscope to assess acrosome integrity.

viii) At least 200 spermatozoa were counted for each slide for estimation of intact acrosome percentage. The acrosomal abnormalities were classified as swollen acrosome, ruffled acrosome, lost acrosome and others (including knobbed etc.).

3.5.5 Acrosome integrity by FITC-PSA Staining

The acrosome status of sperm samples was assessed with fluorescent-labeled lectin from the peanut plant, *Arachis hypogea* (FITC-PSA) using a slightly modified version of the procedure described by Sukardi *et al.* (1997). Modifications included use of PBS instead of HEPES buffer and removal of excess Propidium iodide by diluting the contents several fold and centrifugation instead of filtration.

Procedure:

i) 100 µl of semen sample was taken in a micro centrifuge tube and volume was made up to 1000µl with PBS.

ii) The sample was washed twice by centrifugation at 170g for 10 min, supernatant removed and the final volume made up to 100µl with PBS.

iii) 2 µl of PI solution (500µg/ml) was added to get a final concentration of 10µg/ml PI in the sperm suspension.

iv) The spermatozoa were allowed to interact with PI exactly for two minutes.

v) The excess PI was removed by adding 10 fold volumes (1ml) PBS, centrifuged gently for 5 min and supernatant removed. The final volume was again adjusted to 100µl.

vi) 20µl of diluted semen suspension was smeared on clean grease free glass slide in duplicate and dried.

vii) Spermatozoa were permeabilized by flooding the slide with 100% methanol for 5 min.

viii) Excess methanol was removed by washing the slides with PBS.

ix) Permeabilized slides were then flooded with FITC-PSA working solution (40µg/ml) in PBS.

x) The slides were kept in dark chamber at 37^{o}C for half an hour.

xi) Excess FITC-PSA was removed by rinsing the slides with PBS.

xii) A drop of anti fade solution of 0.22M 1, 4-diazo-bicyclo (2, 2, and 2) octane was placed on the stained smears in order to preserve fluorescence.

xiii) A cover slip was placed, pressed and edges sealed with nail varnish.

xiv) Slides were examined within two hours under the fluorescence with FITC filter set at 40X magnifications.

xv) A total of 200 spermatozoa were counted. PSA positive sperm showed green to yellowish fluorescence. PI positive spermatozoa showed red colored nuclear material,

indicating damaged membrane, as intact membranes are impermeable to PI. Cells, which retained staining of the equatorial segment, were classified fully acrosome reacted as these cells were considered totally devoid of PSA staining

3.5.6 Carboxy Fluorescein Diacetate/Propidium Iodide (CFDA/PI) Test

This test was performed for viability and membrane stability of spermatozoa. CFDA crossed cell membrane and were de-esterified by esterase within the cell. They reacted with the intact cell and produced green fluorescence whereas, PI entered only in broken cell and produced red fluorescence.

Procedure:

i) Semen samples were mixed with a 0.5% skim milk powder in PBS to make a final concentration 50 million sperm/ml

ii) Aliquots of 20µl of CFDA stock solution consisting of 0.46 µg CFDA in 1.0 ml of DMSO and 10µl of PI stock solution (0.5 mg PI in 1 ml of 0.9% NaCl solution) were mixed with 950µl of above semen sample and,

iii) Incubated for 8 min

iv) A 5 µl drop of incubated sample was placed on a slide and overlaid with cover slip

v) Fluorescent cells were counted from fluorescent microscope at 40X

vi) A total 200 spermatozoa were counted.

3.5.7 Chromomycin A_3 Assay

Chromomycin A_3 is a guanine cytosine specific fluorochrome that reveals chromatin status in spermatozoa and was used to assess the packaging quality of the chromatin in spermatozoa which allow indirect visualization of Protamine deficiency. High CMA_3 fluorescence is a strong indicator of the low protamination state of spermatozoa

Procedure:

i) Semen smears were fixed in methanol-glacial acetic acid (3:1) at 4ºC for 20 min

ii) Then smears were air dried at room temperature for 20 min.

iii) Air- dried slides were treated for 20 min with 100µl CMA_3 solution.

iv) The slides were then rinsed in PBS buffer and mounted with 1:1 v/v PBS-glycerol

v) Finally, Slides were kept at 4ºC overnight and next day observed in fluorescent microscope

vi) A total 200 spermatozoa were counted. CMA_3 positive sperm showed bright yellow fluorescence whereas, CMA_3 negative spermatozoa showed dull yellow fluorescence.

3.6 PREPARATION OF ELECTROPORATION MEDIA

In order to assess the effect of media, two different media viz., TALP and Mannitol were used

3.6.1 PREPARATION OF TALP MEDIA

Fertilization TALP media were prepared by different type of constituents which has mentioned in Annexure. Sperm TALP was always prepared fresh by addition of heparin, sodium pyruvate, BSA (fraction V) with fertilization TALP media. Final pH of media was maintained around 7.6 to 7.8

3.6.2 PREPARATION OF MANNITOL MEDIA

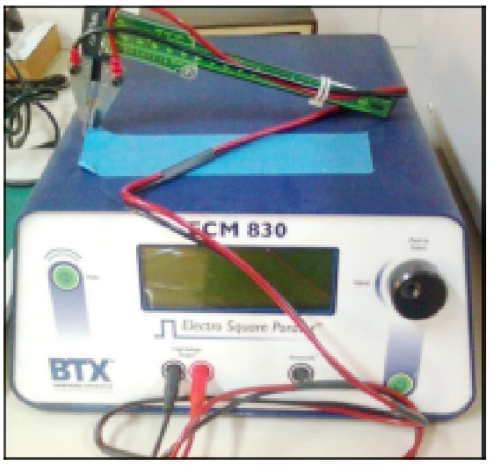
Mannitol media were prepared by using 0.3 M Mannitol, 0.1mM $MgCl_2$, 0.05 $CaCl_2$ in distilled water

3.7 ELECTROPORATION

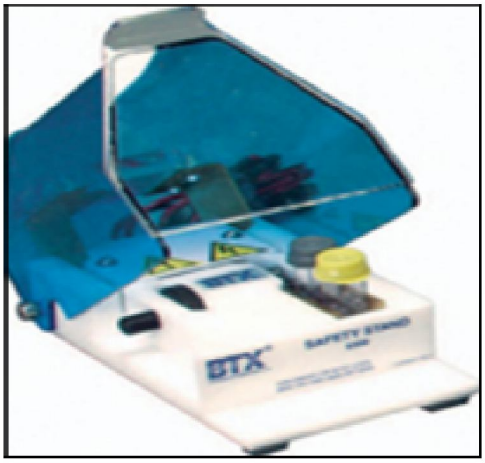
Electroporation was carried out in a cuvette, placed in safety stand using a square wave electroporator (ECM830, BTX Fig. 3.1). Different voltages 100 V, 300 V, 500 V and 1000 V were used. Different electroporation parameters combination has been depicted in table 3.1.

3.8 ASSESSMENT OF THE EFFECTS OF ELECTROPORATION ON VITAL SEMINAL PARAMETERS

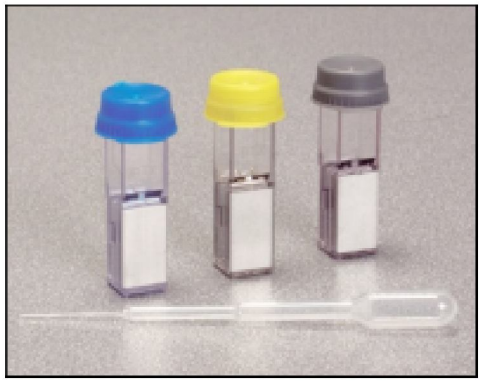
In order to assess the adverse effect of electroporation on vital semen parameters, electroporation was carried out at different voltages with variable time constant. Further, for each voltage and time constant two different media were used. The objective of this experiment



A. ELECTROPORATOR ECM 830, BTX



B. SAFETY STAND



C. CUVETTE PLUS

Fig. 3.1 : Electroporator assembly

Table.3.1: Different electroporation parameters combinations

SI No.	Media	Voltage A	Voltage B	Voltage C	Voltage D
1	TALP	100 V	300 V	500 V	1000 V
2	Mannitol	100 V	300 V	500 V	1000 V

was to identify a combination of media and electroporator condition that have least possible adverse effect on spermatozoa.

3.9 PREPARATION OF PLASMID VECTOR

3.9.1 Gene Construct for Electroporation

In the present investigation *pIRES2-EGFP* (Clontech, USA, a kind gift by Dr. Majumdar, Core Scientist NII, New Delhi) was used for electroporation. The map of the vector has been shown in fig. 3.2. *pIRES2-EGFP* contains an internal ribosome entry site (IRES; 1, 2) of the encephalomyocarditis virus (ECMV) between the multiple cloning site (MCS) and the enhanced green fluorescent protein (EGFP) coding region permitting both the gene of interest (cloned into the MCS) and the EGFP gene to be translated from a single bicistronic mRNA

3.9.2 Transformation

The transformation of *pIRES2-EGFP* in *E. coli* DH5á strain was carried out by using Transform Aid Bacterial Transformation Kit (Fermentas, USA) according to manufacturer's protocol as described below:

i) Frozen culture of *E. coli* strain DH5á was revived by inoculating LB broth (100 µl/ 10ml) and kept in shaker incubator at 37ºC, 180 rpm for overnight (12 -14 hrs).

ii) Next day, 75 µl of above culture was inoculated in 750 µl of pre-warmed Cmedium in 1.5 ml microfuge tube. The cells were incubated by constant shaking (180 rpm) at 37ºC for 2 hrs.

iii) Cells were pelleted by centrifugation at 12000 rpm for 2 min. at 4ºC and discarded the supernatant.

iv) Then 125µl each of (T) solution A and (T) solution B was mixed in a fresh 1.5 ml microfuge tube and kept on ice for 5 min.

v) The cell pellet was reconstituted by adding 150 µl of above mixture and kept in ice for 5 min. Centrifugation was done at 12000 rpm for 1 min at 4ºC and supernatant was discarded. Pellet was again reconstituted by adding 60 µl of T mixture and kept in ice for 5 min.

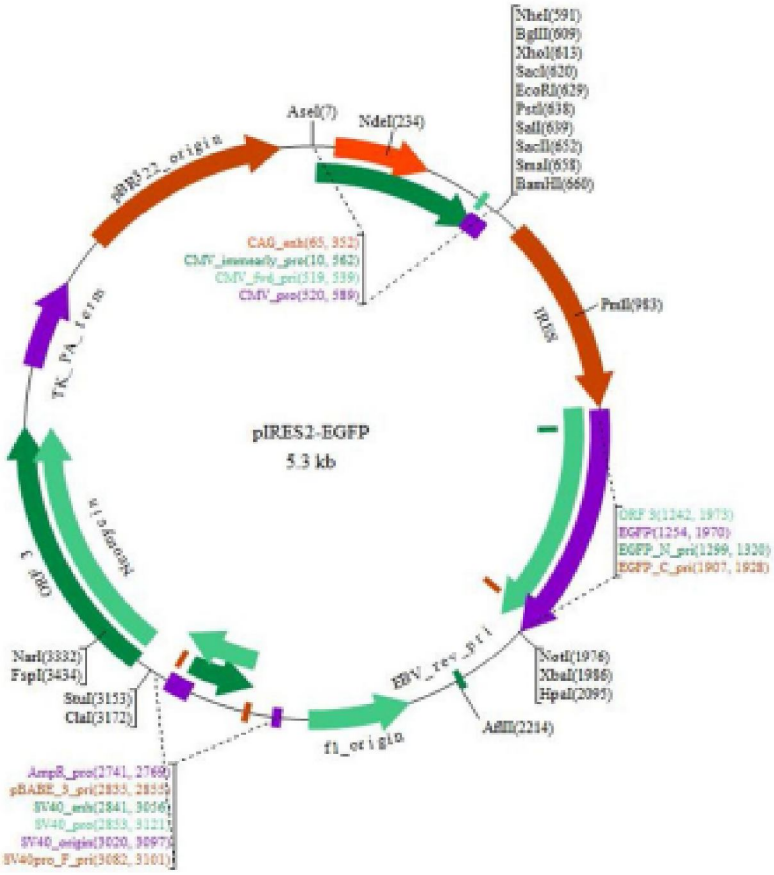


Fig. 3.2 : Map of the vector *pIRES2-EGFP*

vi) Then 8 µl of plasmid (30 ng/µl) was added to this mixture kept on ice and mixed very gently.

vii) Finally, 50 µl of above mixture was spreaded onto prewarmed LB agar plate (37ºC) containing antibiotic kanamycin (30 µg/ml). The plates were incubated at 37ºC for 14 -16 hrs.

viii) After 14 -16 hrs, single colonies were picked and added to 10ml LB broth containing antibiotic Kanamycin (30 µg/ml) and incubated at 37ºC, 180 rpm overnight. Next day glycerol stocks were prepared by taking 850 µl culture and 150 µl autoclaved glycerol in 1.5 ml tube. The tubes were vortexed and kept at -80 ºC.

3.9.3 Isolation of Plasmid

Glycerol stock prepared above was revived in LB broth (100 µl/10ml) containing antibiotic Kanamycin (30 mg/ml @ 1µl/ml of broth) and incubated at 37ºC for 8 hrs in a shaker incubator (180 rpm). After 8 hrs of incubation, two cultures were set up by inoculating 1ml of incubated culture in 1 liter flasks (2) each containing 250 ml of LB broth and Kanamycin (@ 1µl/ml). Cultures were kept in shaker incubator at 37ºC, 180 rpm for overnight. Remaining culture was used to make glycerol stock (850 µl culture +150 µl autoclaved glycerol, vortex and store at -70ºC). Plasmid was isolated from transformed cells using Pure Link™ HiPure Plasmid Endotoxin Free Quanta Maxi Kit (MDI, India) according to manufacturer's protocol as described below:

3.9.4 Plasmid Isolation Using MDI Kit with Some Minor Modifications

i) The overnight bacterial culture (300ml) was pelleted by centrifuging at 8000rpm for 15 min at 4 ºC.

ii) The pellet was resuspended by adding 16 ml of M1 buffer followed by addition of 16 ml of M2 buffer and incubation was done at room temperature for 3 minutes.

iii) Then 16 ml of cooled M3 buffer was added and tube was mixed gently by inverting 4-6 times until white fluffy material had formed.

iv) The above lysate was poured immediately into barrel of 50 ml syringe with mdi filter device attached to it and then incubation was done at room temperature for 20-25

min. The red stopper from the mdi filter device outlet nozzle was removed and the plunger was gently inserted into barrel and filtration of lysate done in 50 ml falcon tube.

v) Then 16 ml of MB buffer was added followed by incubation at room temperature for 5 min.

vi) Now Quanta maxi spin column with tube extender was attached to 250 ml bottle with help of green adapter and the lysate was passed through spin column by applying vacuum (approx. 300 mbar).

vii) 1.6 ml of EF buffer was added and filtered by applying vacuum followed by washing with 16 ml of MPW buffer. The tube extender was removed and spin column was washed with 750 µl of MW buffer by applying vacuum.

viii) The spin column was placed in 2ml collection tube and centrifugation was done at 12000rpm for 2 min.

ix) Now the spin column was placed in fresh 1.5 ml microfuge tube and elution was done by adding 300 µl of ME buffer by centrifuging at 12000 rpm for 2 min.

x) The eluted DNA was checked for its quality and quantity and then stored at -20 ºC till further use.

3.9.5 Evaluation of Quality, Purity and Concentration of Plasmid DNA

The Plasmid DNA isolated from transformed cells was checked for its quality, purity and concentration. Only the Plasmid samples of good quality, purity and concentration were used for further analysis.

3.9.5.1 Quality of plasmid DNA

Horizontal submarine agarose gel electrophoresis was used to check the quality of plasmid using 0.8% (w/v) agarose (Low EEO). For loading the samples, 6 µl autoclaved triple distilled water along with 4 µl diluted DNA was taken and after mixing it with 2 µl of 6 X gel loading dye (Xylene cyanol, Bromophenol blue and Orange G), it was loaded into the wells with the help of micro tips. Electrophoresis was performed at 10V/cm (90-100 V) for 45min. Once the electrophoresis was over, the gel was visualized under UV transilluminator and documented by photography under Gel Documentation System (Syngene, USA).

3.9.5.2 Purity and concentration of plasmid

The purity and concentration of plasmid was checked by using nanodrop 1000 (company). A total of 2 µl of diluted plasmid was used for taking the reading. The OD ratios (260:280) of plasmid samples lied in the range of 1.8 to 2.0 and were considered as free from protein or any other contamination. To know the concentration of genomic DNA in all the samples the spectrophotometer reading was taken at OD_{260}. For estimating the concentration of genomic DNA following formula was employed:

DNA concentration (µg/ml) = OD260 x dilution factor x 50

(1 OD value at 260nm is equivalent to 50 ng dsDNA/ml)

3.10 LINEARIZATION OF *pIRES2-EGFP* PLASMID

Circular plasmid was linearized by using *StuI* restriction digestion. After digestion the plasmid was checked through agarose gel electrophoresis which showed single band around 5.3 kb (Fig. 3.3)

3.11 ADDITION OF PLASMID CONTAINING REPORTER GENE

For electroporation, 0.8 ml of media containing one 1µg of reporter gene (*pIRES2-EGFP*) construct was added into an electroporation cuvette. Concentration of reporter gene was 0.54µg/µl in stock solution. The $OD_{260/280}$ ratio of reporter gene was 1.84 and was considered free from protein or any other contamination. An appropriate volume, 10µl of fresh semen was added into electro-cuvette so that final total concentration of spermatozoa remains approximately 30 million

3.12 ELECTROPORATION

Electroporation was carried out in 800 µl cuvette, placed in safety stand using a square wave electroporator (ECM830, BTX). Electroporation was done at 300 V and 200 ms

3.13 ASSESSMENT OF SEMEN PARAMETERS

After electroporation, semen parameters were assessed. The adverse effect of the electroporation was assessed by comparing the vital semen parameters before and after the electroporation

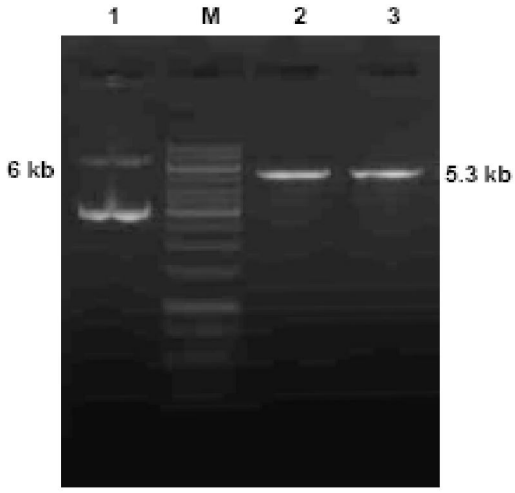


Fig. 3.3 : **Linearized plasmid of *pIRES2-EGFP***

Lane M : 1 kb ladder
Lane 1 : Undigested plasmid
Lane 2-3 : Linearized plasmid

3.14 DETECTION OF INTERNALIZATION OF REPORTER GENE

As per the optimized condition of media, voltage and time constant that lead least possible adverse effect on seminal parameters was used further. Electroporation was carried out as described in section 3.7. Additionally controls were also set up as follows

3.14.1 CONTROL

Optimized semen sample and media was taken into cuvette. But neither it contained the plasmid nor it was electroporated

3.14.2 CONTROL ELECTROPORATED

This control was set as a negative control. It contained the plasmid of reporter gene but was not electroporated

3.15 SEPARATION OF SPERM PELLET AND MEDIA

Both control (naïve and non-electroporated) and Sperm pellet were subjected for centrifugation at 500 g for 10 min. After centrifugation, supernatant containing the media was collected in a separate microfuge tube. Separated sperm pellet and supernatant were washed six times with PBS and supernatant were collected from each centrifugation. Supernatant 1, 4, 7 and sperm pellet processed immediately and remaining supernatant were stored at -20°C

3.16 ISOLATION OF DNA FROM SPERM PELLET AND SUPERNATANT

Genomic DNA were isolated using the standard phenol-chloroform extraction method (Sambrook and Russell, 2001) as well as by GeneipureID™ isolation kit

3.16.1 Isolation of DNA by Phenol-Chloroform Extraction Method

Procedure:

i. Sperm pellet was dissolved in 800µl sperm lysis buffer (SLB) and mixed very gently by multiple pipetting

ii. Placed the tube in incubator for 1.5 hrs at 50°C

iii. Added proteinase K (20mg/ml) 15µl to each tube and mix gently by rotating

iv. Kept the tubes in water bath (56°C) over night

v. Next day, equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) were added and mixed properly with gently inversion (5-7 min.)

vi. The tube was centrifuged @ 12000 rpm for 15 min. at room temp.

vii. Transfered the upper aqueous phase carefully in a separate 2 ml tube

viii. Added equal volume of chloroform: isoamyl alcohol (24:1) in the tube and mixed gently by inversion (10min.)

ix. Centrifuged the tube as before and transfered the aqueous phase in a new 2 ml tube

x. Added 2M sodium acetate (1:10 vol.) to the aqueous phase and added 2 volume of chilled ethanol to precipitate the DNA and tube was kept the tubes in -20^{o}C for 1 hr. for total precipitation

xi. Spooled the DNA and washed twice with 70% ethanol

xii. Dried the pellet and dissolved in TE

xiii. Finally kept overnight at room temp for proper dissolution and proceeded for further heat treatment and OD measurement

xiv. Stored the stock DNA in -20^{o}C.

3.16.2 Isolation of DNA by GeneipureID™ Isolation Kit

Procedure:

i. 350 µl of lysis buffer (LB) were added to the sperm pellet and resuspended the pellet by votexing at full speed for 1 min.

ii. Added 150 µl of 1M Di-thiotheritol (DTT) and tube was kept at 55^{o}C for 3-4 hrs. and vortexed at full speed for 5 seconds after every 60 min in a dry bath or water bath

iii. Added 400 µl of Binding Buffer (BB), 200 µl isopropanol to the sample and mixed thoroughly for 5 sec.

iv. GeneipureID™ column was placed in 2 ml collection tube. Loaded the supernatant, 600 µl each time and spin at 10000 rpm for 1 min. Discarded the flow-through, before proceeding to the next step

v. Washed GeneipureID™ column with 500 µl wash buffer I (WBI) and centrifuged at 10000 rpm for 1 min. discarded the flow through and placed the GeneipureID™ column in same collection tube

vi. Diluted one volume of Wash Buffer II (WBII) with one volume of absolute ethanol e.g. for I prep. diluted 350 µl of WBII with 350 µl of absolute ethanol and mixed thoroughly

vii. Washed column with 700 µl WB II and centrifuged at 10000 rpm for 3 min. Discarded flow-through and placed the GeneipureID™ column in same collection tube before proceeding with the next spin.

viii. Final spin for 2 min. was given at 10000 rpm to remove traces of WBII. Discarded the collection tube

ix. Placed the GeneipureID™ in new 1.5 ml vial (sterile) and eluted DNA with 50-100µl elution buffer (EB).

x. Incubated at room temperature for 2 min. and centrifuged at 10000 rpm for 1 min.

xi. Stored the eluted DNA at -20°C

3.17 Amplification of Reporter Gene Construct with Real Time PCR (RT-qPCR)

3.17.1 Designing of primers for PCR/RTqPCR:

Specific primers for Enhanced Green Fluorescent Protein (EGFP) were designed using Primer Express 3.0 software.

3. 17.2 Real-Time quantitative PCR:

Amount of plasmid DNA was quantified using Real-Time PCR (Mode 7500 Applied Biosystems Real-Time PCR System) (Fig. 3.4). Beta actin (Fig. 3.5) was used as a house keeping gene (endogenous control) for the analysis of data.

The reaction mixture: All PCR reactions were performed in triplicate. The amplification was carried out in 20 µl volume reaction containing 1X SYBR Green PCR master mix (Applied Biosystems, Cat no.# 436765), gene-specific primer in respective primer combinations and 4.0 µl, containing 40ng of plasmid DNA were used. PCR cycling conditions were as described Table 3.2. No template control was also set up. in which plasmid DNA was not added using absolute quantification method a standard curve was used . A serial dilution of the template was run with each primer pair and the log of the dilution was plotted against the C_t value of each dilution. The slope of the resulting regression equation was used to calculate the efficiency of the PCR reaction using E= -1+10(-1/slope) equation. For each sample, a dissociation curve was also generated after completion of amplification to determine the specificity of PCR reaction.

3.18 AGAROSE GEL ELECTROPHORESIS

The confirmation of amplification of specific RT-PCR amplicon was done by agarose gel electrophoresis (appendix) (Fig.3.6). 2% agarose was mixed with 40 ml 1X TBE buffer and melted in a microwave oven. When the molten gel had cooled to about 60ºC, ethidium bromide (1µl) was added to final concentration of 0.5ìg/ml. The gel was mixed thoroughly by gentle swirling and then poured into the gel casting tray fitted with the comb. The gel was allowed to solidify and the comb was removed. The PCR product were mixed with 1X gel loading dye (final concentration) and loaded into the wells. For the comparison, a 50 bp molecular weight marker was gel electrophoresed in parallel to the RT-PCR amplicons. The gel was run at a voltage of 5 V/cm till the running dye crossed at least two third of the gel. The bands were visualized under UV light and recorded on a gel documentation system (GELDOC, USA).

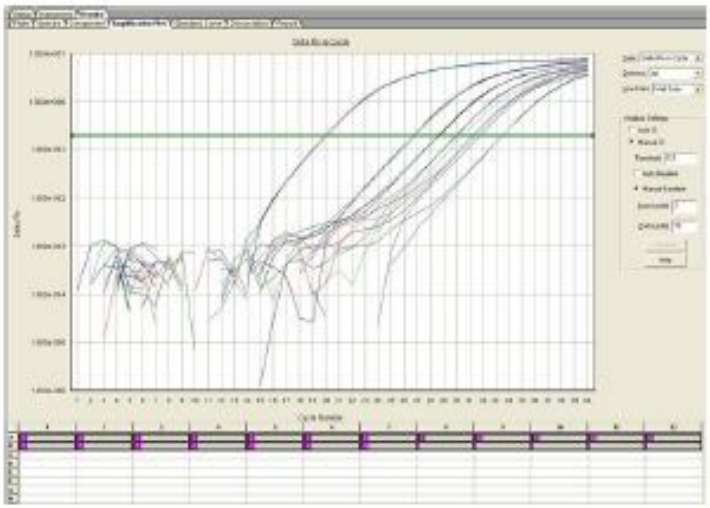


Fig. 3.4 : Amplification plot of EGFP gene

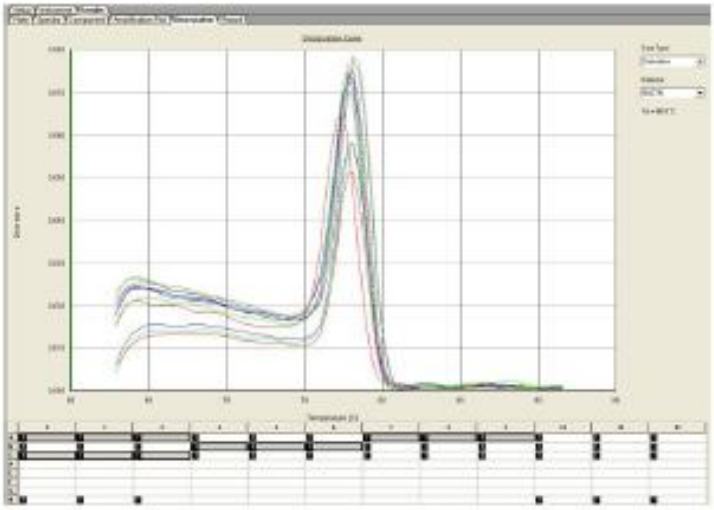


Fig. 3.5 : Dissociation curve of β-actin gene

Table 3.2 Cycling conditions for RT-qPCR

a) Thermocycling condition

Stage	Repetion	Temperature	Time
1.	1 Cycle	50°C	2 min.
2.	1 Cycle	95°C	10 min.
3.	40 Cycle	95°C	15 sec
4.	40 Cycle	60°C	1 min.

b) Denaturing condition

Stage	Repetion	Temperature	Time
1	1 Cycle	95°C	15 sec
		60°C	20 sec
		95°C	15 sec
		60°C	15 sec

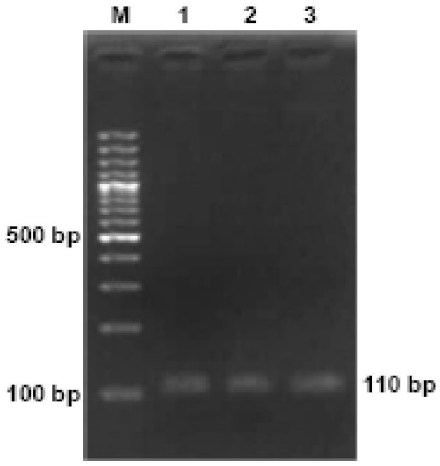


Fig. 3.6 **Agarose gel electrophoresis showing amplification of product using Real time PCR**

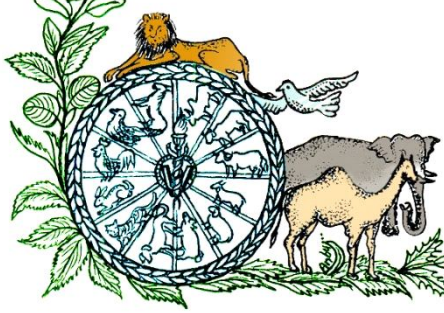
Lane M : 100 bp DNA ladder

Lane1-3 : PCR amplified product (110 bp)

3. 19 STATISTICALANALYSIS

Results were expressed as mean± S.D of three individual animals at designated time points. Three replicates were done. The results (pooled data of three experiments) were analyzed by one-way ANOVA test and comparisons with control data were made with two media mannitol and TALP by Dunnett's or Tukey's post test where appropriate. All of the analyses were done using Graph Pad Prism (version 3.03) software. The upper level of significance was chosen as $P < 0.001$.

Results and Discussion



4 Results and Discussion

The present study is comprised of two objectives. The first objective was aimed to study the effect of electroporation on semen parameters and to optimize an electroporation condition having least possible adverse effect. In the second objective, efficiency of the optimized electroporation condition for internalization of a foreign DNA into the spermatozoa was determined.

4.1 ASSESSMENT OF NORMAL SEMEN PARAMETERS

Semen from the selected bucks (n=2) were collected using artificial vagina (AV). Immediately after collection, the tubes containing the semen were placed in the water bath maintained at 37 ºC and brought to the laboratory for further processing. Various semen parameters viz., progressive motility, live-dead spermatozoa, membrane integrity, acrosome integrity and chromatin integrity were assessed in winter (November to February) and summer (March to May) season have been summarized in Table 4.1.

4.1.1 Progressive Motility

The range of progressive motility varied between 60 to 80 per cent. Progressive motility during winter (65±1.17) and summer (70.55±1.3%) season differed significantly ($P< 0.01$).

4.1.2 Live and Dead Spermatozoa Count

The live and dead spermatozoa were identified using Eosin-Nigrosin (Fig.4.1) and CFDA/PI (Fig.4.2) staining. There was a significant difference ($P<0.05$) in the mean percentage of live spermatozoa estimated on the basis of these two methods. Estimate of mean percentage of live spermatozoa in winter season was higher (61.11±0.60 vs. 49.44±1.23) as compare to

Table 4.1 Various seminal parameters in Black Bengal bucks during winter and summer season

Parameters	Season		Significance
	Winter	Summer	
Progressive motility (%)	65±1.17	70.55±1.30	**
Live and dead (%), Eosin-Nigrosin method	61.11±0.60	64.44±1.33	*
Live and dead (%), CFDA/PI method	49.44±1.23	49.44±1.75	NS
Acrosome Integrity (%), Giemsa stain	84.72±0.27	83.33±0.33	NS
Acrosome Integrity (%), FITC-PSA	88.05±0.55	86.11±1.38	NS
Hypo-osmotic swelling (HOST) test	82.77±0.27	76.66±2.35*	*
Chromomycin A_3 test (CMA_3)	70.00±0.00	62.77±2.22**	**

NS= non significant, * = p<0.05, ** = p<0.01

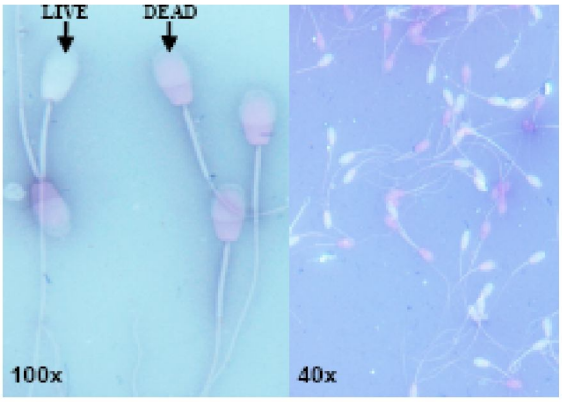


Fig. 4.1 : Live and dead caprine spermatozoa after staining with Eosin-Nigrosin

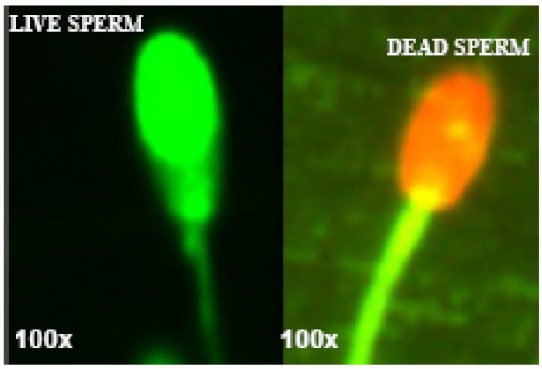


Fig. 4.2 : Live and dead caprine spermatozoa after staining with CFDA/PI

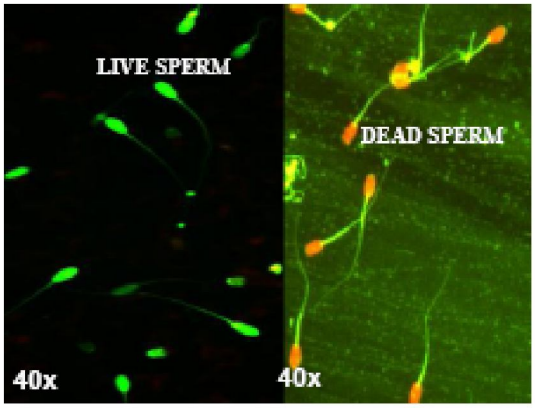


Fig. 4.2a : Live and dead caprine spermatozoa after staining with CFDA/PI (40x)

CFDA/PI estimation. While using Eosin-Nigrosin staining, mean percentage of live sperm was significantly higher ($P<0.05$) during summer season than that in winter (64.44±01.33 vs. 49.44±1.75). However, season differences could not be ascertained while using CFDA/PI staining.

4.1.3 HOST Positive Spermatozoa:

Hypo-osmotic swelling (HOS) test indicates the membrane integrity of the spermatozoa (Fig.4.3).The mean per cent HOST positive spermatozoa recorded in winter and summer seasons were 82.77±0.27 and 76.66±2.35 respectively. In winter season significantly higher ($p<0.01$) HOST positive sperm was observed in comparison to summer season.

4.1.4 Acrosome integrity

Intact acrosome bound to Giemsa stain gave violet color where as reacted acrosome looked colorless (Fig.4.4). In Fluorescein isothiocynate *Pisum sativum* agglutinin (FITC-PSA) staining, intact acrosome produced fluorescence where as it was absent in reacted acrosome (Fig.4.5).The mean value of per cent intact acrosome using Giemsa staining was estimated as 84.72±0.27 and 83.33±0.33 during winter and summer season, respectively. While using FITC-PSA staining, similar mean values (88.05±0.55 and 86.11±1.38) were also observed. However, season had no significant effect on the acrosome integrity.

4.1.5 Chromatin Integrity

Chromomycin A_3 stains assess the protamine content which is directly proportional to fertility status of the sperms (Fig.4.6).The mean value of percent intact chromatin (70.00±0.00 and 62.77±2.22) differed significantly ($p<0.01$) between summer and winter season, respectively. The result showed less number of protamine deficient sperms during winter indicating good fertility status of sperms.

4.2 EFFECTS OF ELECTROPORATION ON SEMEN PARAMETERS

For electroporation two media (viz., TALP and Mannitol) were used. In a gene pulsar cuvette 800 µl of media (e.g., Mannitol and TALP) was taken to which neat semen containing 30 million spermatozoa was added and mixed thoroughly. This mixture was subjected to electroporation with the help of Square Wave Electroporator using following conditions i.e. 100 V at 200 ms, 300 V at 200 ms, 500 V at 600 µs and 1000 V at 300µs. Following

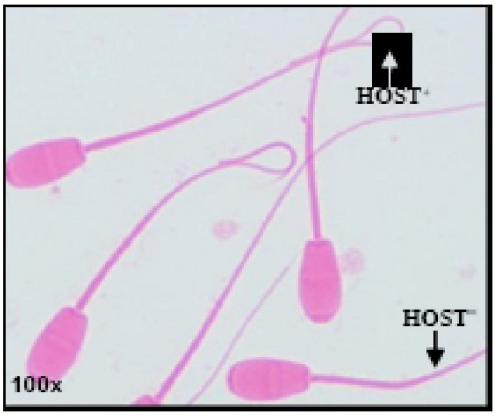


Fig 4.3 : Eosin stained caprine spermatozoa showing HOS positive and negative response

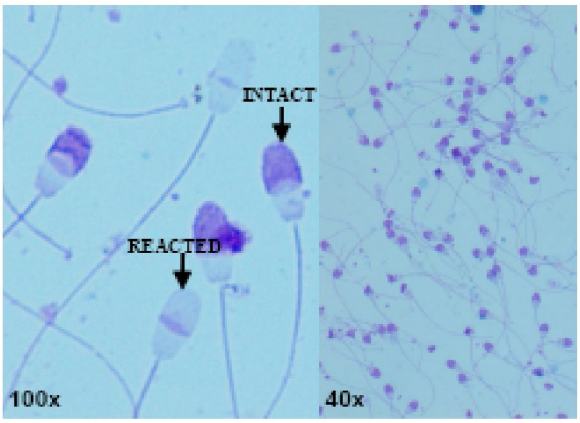


Fig. 4.4 : Giemsa stained caprine spermatozoa showing intact and reacted acrosome

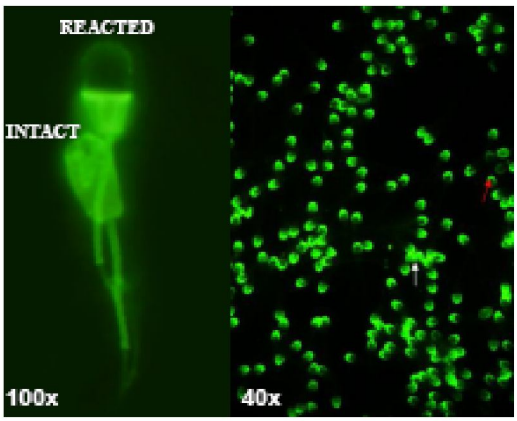
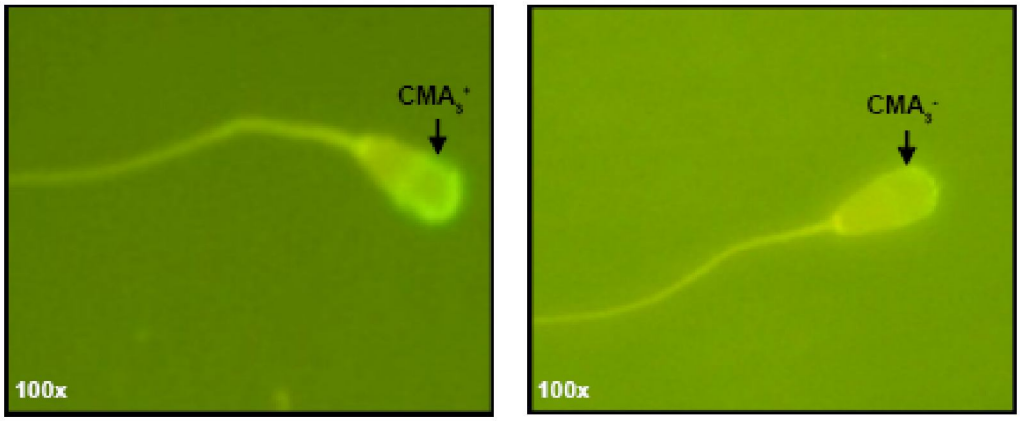


Fig. 4.5 : FITC-PSA stained caprine sperm showing intact and reacted acrosome



CMA_3^+ spermatozoa showing fluroscence

CMA_3^- spermatozoa showing no fluroscence

Fig. 4.6 : Chromomycin A_3 stained caprine spermatozoa

electroporation, semen parameters were assessed and the mean values of different parameters of electroporated as well as control samples are presented in Table 4.2 and Fig. 4.7. Objective of this experiment was to identify the best media and electroporation condition.

4.2.1 Progressive Motility

While using Mannitol media progressive motility was adversely affected for all the voltages (e.g.100 to 1000V) used. On the other hand, in TALP media, progressive motility was not adversely affected up to 300V, however, it was reduced significantly ($p<0.01$) from 500 V to 1000 V ($p<0.001$) (Fig.4.7.A)

4.2.2 Effect on Livability of Spermatozoa

Following electroporation, the per cent live spermatozoa was assessed by two methods i.e. Eosin-Nigrosin (Fig.4.7.B) and CFDA/PI staining (Fig.4.7.C). In Eosin-Nigrosin method, significant differences ($p<0.001$) were found for all the voltages in Mannitol media. Whereas, in TALP media significant differences ($p<0.001$) were found from 300 V up to 1000 V. However in CFDA/PI staining there was significant differences were found in both media from 500 V up to1000.

4.2.3 Acrosome integrity

The intactness of the acrosome was assessed using two methods i.e., Giemsa (Fig.4.7.D) and FITC-PSA staining (Fig.4.7.E). The per cent intactness of the acrosome as determined by Giemsa staining did not vary significantly for any voltage in both the media. In Mannitol media, voltages up to 500 V did not have any adverse effect on the acrosome integrity as a determined by using FITC-PSA staining. At 1000 V, however acrosome integrity was reduced significantly compared to naïve ($p<0.01$) and 100V ($P<0.05$). In TALP media, effect of the voltage becomes evident from 300 V to 1000 V. After exposure to 300 V in TALP media acrosome integrity was reduced to 85 percentage as a compared to 90 percentage in naïve control. However, acrosome integrity at 300 V did not differ significantly due to media.

4.2.4 Effect on membrane integrity of Spermatozoa using HOST

In Mannitol media, voltage did not have any adverse effect on the membrane integrity of spermatozoa, while using HOS test (Fig.4.7.F). In TALP at 1000 V, however membrane integrity is reduced significantly compared to naïve ($p<0.01$) and 100 V ($p<0.05$).

Table 4.2: Effect of voltages within media on various seminal parameters

Descriptive	TALP MEDIA					MANNITOL MEDIA				
	Naive	100 V	300 V	500 V	1000 V	Naive	100 V	300 V	500 V	1000 V
Progressive motility (%)	65.3 ±0.1	65.58 ±0.01	61.7 ±0.01	53.34 ±0.2	44.37 ±0.05	57.81 ±0.02	57.8 ± 0.02	40.97 ±0.08	30.89 ±0.08	22.68 ±0.15
Live and dead (%), Eosin-Nigrosin method	62.25 ±0.01	62.25 ±0.01	52.22 ±0.07	50.55 ±0.02	45.53 ±0.02	56.12 ±0.01	56.12 ±0.01	43.31 ± 0.01	37.18 ±0.01	30.86 ±0.08
Live and dead (%), CFDA/PI method	47.75 ±0.01	47.75 ±0.04	39.39 ±0.02	36.06 ±0.02	28.70 ±0.05	41.64 ±0.02	41.64 ±0.02	34.11 ±0.11	26.97 ±0.07	21.62 ±0.12
Acrosome Integrity (%), Giemsa stain	85.07 ±0.01	85.07 ±0.01	83.93 ±0.009	81.29 ±0.03	78.52 ±0.04	84.55 ±0.01	84.55 ±0.019	81.91 ±0.048	81.29 ±0.039	78.04 ±0.06
Acrosome Integrity (%), FITC-PSA	90.0 ±0.03	90 ±0.01	85 ±0.02	82.84 ±0.013	82.29 ±0.013	85.67 ±0.02	85.67 ±0.02	83.21 ±0.01	81.78 ±0.02	80.10 ±0.02
Hypo-osmotic swelling (HOS) test	82.89 ±0.01	82.84 ±0.013	79.15 ±0.05	76.82 ±0.03	73.08 ±0.08	82.84 ±0.01	82.84 ±0.01	81.78 ±0.02	76.82 ±0.03	76.82 ±0.03
Chromomycin A_3 test (CMA_3)	70.07 ±0.02	70.07 ±0.02	64.45 ±0.003	64.45 ±0.003	60.56 ±0.003	67.34 ±0.03	67.34 ±0.03	60.56 ±0.003	56.70 ±0.02	56.13 ±0.01

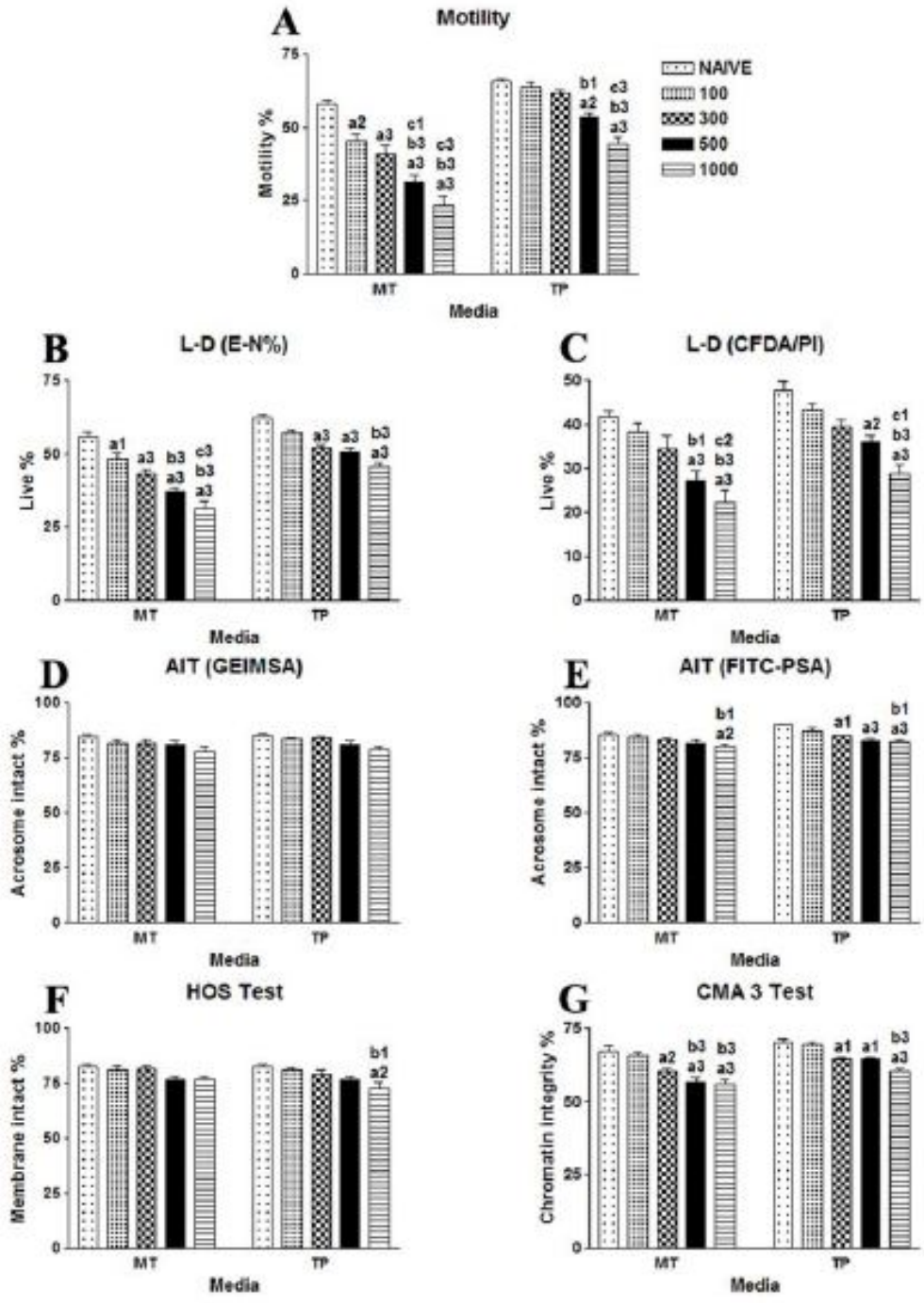


Fig. 4.7 : Effect of voltages within media on various semen parameters

4.2.5 Chromatin Integrity

Chromomycin A_3 (Fig.4.7.G) test showed a significant difference in both media from 300 to 1000V. In both Mannitol and TALP media, significant differences were observed at 300V to 1000V .

In summary, it was observed that, for all parameters, except acrosome integrity using Giemsa staining , and membrane integrity by HOS test, voltage above 300 V had an adverse effect. With respect to media (Table 4.3), for any given voltage Mannitol had an significantly adverse effect on motility and live-dead percentage of spermatozoa. Therfore, amongst all the above mentioned combinations, 300 V at 200 ms in TALP media was the best which is having the least adverse effect on semen parameters.

4.3 INCORPORATION OF REPORTER GENE INTO THE SPERM

Following electroporation, sperm pellet was separated from the media by centrifugation and supernatant containing media was collected in a separate microfuge tube. Separated sperm pellet was further subjected to six serial washings with PBS. Supernatants from each washing was collected. DNA was isolated from the final sperm pellet obtained after six washings and the supernatant numbers 1, 4, and 7.

4.3.1 ASSESSMENT OF SENSITIVITY AND SPECIFICITY OF REAL TIME PCR

Standard curve were plotted using ten-fold dilutions of the *pIRES2-EGFP* plasmid gene with their respective C_t values (Fig. 4.8). The R^2 value and linear equation for the standard curve was 0.984 and $Y = -3.1077 X + 12.145$, respectively.The log of the concentration and respective C_t values are given in Table 4.4. With respect to Sensitivity of the Real time a minimum copy no. 18-20 of plasmid was possible to detect by RTq-PCR.

The C_t values of the samples were compared with the standard curve and respective concentrations were determined to find out the copy number of the plasmid as given in (Table 4.5).The relative abundance of the plasmid in the sperm pellet and supernatant nos. 1 and 4 was determined using $\Delta\Delta C_t$ method (Fig.4.9).

Table : 4.3. Effect of media within voltage on various seminal parameters

Voltage	Motility			Live (%) spermpatozoa						Acrosome integrity (%)						HOS			CMA_3		
	MT	TP	Sig	E-N stain			CFDA/PI stain			Giemsa stain			FITC-PSA stain			MT	TP	Sig	MT	TP	Sig.
				MT	TP	Sig	MT	TP	Sig	MT	TP	Sig	MT	TP	Sig						
Naive	57.81 ±.02	65.3 ±.01	NS	56.12 ±.01	62.25 ±.01	NS	41.64 ±.02	47.75 ±.04	*	84.55 ±.01	85.07 ±.01	NS	85.67 ±.02	90 ±.03	*	82.84 ±.01	82.84 ±.01	NS	67.34 ±.03	70.07 ±.02	NS
100 V	57.8 ±0.02	65.58 ±0.01	***	56.12 ±0.01	62.25 ±0.01	**	41.64 ±0.02	47.75 ±0.04	*	84.55 ±0.01	85.07 ±0.01	NS	85.67 ±.02	90 ±.01	NS	82.84 ±.01	82.84 ±.01	NS	67.34 ±.03	70.07 ±.02	NS
300 V	40.97 ±0.08	61.7 ±0.01	***	43.31 ±0.01	52.22 ±0.07	**	34.11 ±0.11	39.39 ±0.02	*	81.91 ±0.04	83.39 ±0.009	NS	83.21 ±0.01	85 ±.02	NS	81.78 ±.02	79.15 ±.05	NS	60.56 ±.003	64.45 ±.003	NS
500 V	30.89 ±0.08	53.34 ±0.2	***	37.18 ±0.01	50.55 ±0.02	***	26.97 ±0.07	36.06 ±0.02	*	81.29 ±0.03	81.29 ±0.03	NS	81.78 ±.02	82.84 ±0.01	NS	76.82 ±.03	76.82 ±.03	NS	56.70 ±.02	64.45 ±.003	***
1000 V	22.68 ±0.15	44.37 ±0.05	***	30.86 ±0.08	45.53 ±0.02	***	21.62 ±0.12	28.70 ±0.05	*	78.04 ±0.06	78.52 ±.04	NS	80.10 ±.02	82.29 ±.01	NS	76.82 ±.03	73.08 ±.08.08	NS	56.13 ±.01	60.56 ±.003	NS

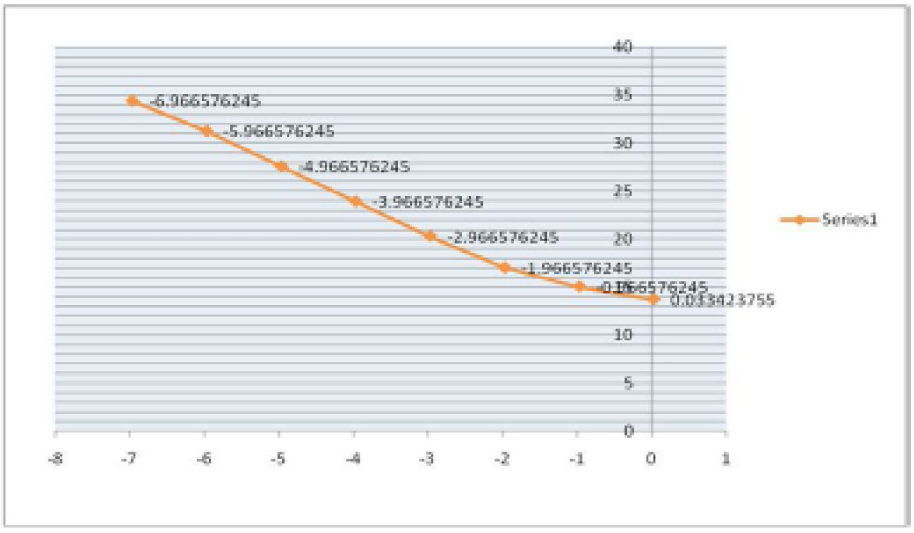


Fig. 4.8 : Standard curve of log (concentration) to C_t value

Table 4.4 : Standard curve parameters

Sr. No.	Concentration (ng/µl)	Log(concentration)	C_t value
1	1.08	0.033423755	13.736
2	0.108	-0.966576245	15.06903
3	0.0108	-1.966576245	17.01437
4	0.00108	-2.966576245	20.32157
5	0.000108	-3.966576245	23.94445
6	0.0000108	-4.966576245	27.5886
7	0.00000108	-5.966576245	31.25873
8	0.000000108	-6.966576245	34.41507

Table 4.5 : Various parameters of electroporated control and electroporated sample

Sample	Buck no. 1			Buck no. 2	
	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2
EC-pellet	17096235.34± 291948	11158490.62 ± 628114	75839440.85 ± 2806640	160898676.4 ± 5163580	124268578.9 ± 622215
EC- sup 1	8224279.389 ± 65224.3	9678350.704 ± 544634	14620349.05 ± 1117510	6084005.297± 18350.5	2450964.527 ± 551822
EC- sup 4	708661.4022 ± 6145	2370.179274 ± 485.216	8698094.822 ± 15974.6	120400.1883 ± 9377.99	885854.5382 ± 830.808
EC- sup 7	3253.27978 ± 1256.64	731.1227203 ± 162.064	33.54141242 ± 1.65996	122519.8044 ± 40988.6	1985663.398 ± 34448.4
E- pellet	24215349.86 ± 814557	42505043.79 ± 1433860	362358524.1 ± 0	362358524.1 ± 0	286008952.6 ± 34144600
E- sup 1	3401550.244 ± 116989	14187781.68 ± 54292.4	26843565.79 ± 772227	10672113.08 ± 497984	2887153.071 ± 20810.5
E- sup 4	10216.76388 ± 1162.42	56.36381918 ± 1.01652	524752.4043 ± 23872.2	1340.84452 ± 368.694	526791.1992 ± 7301.32
E- sup 7	119397.7252 ± 1326.42	5056.048735 ± 1971.17	131534.8811 ± 6621.55	2811.909555 ± 399.277	6177893.352 ± 410308

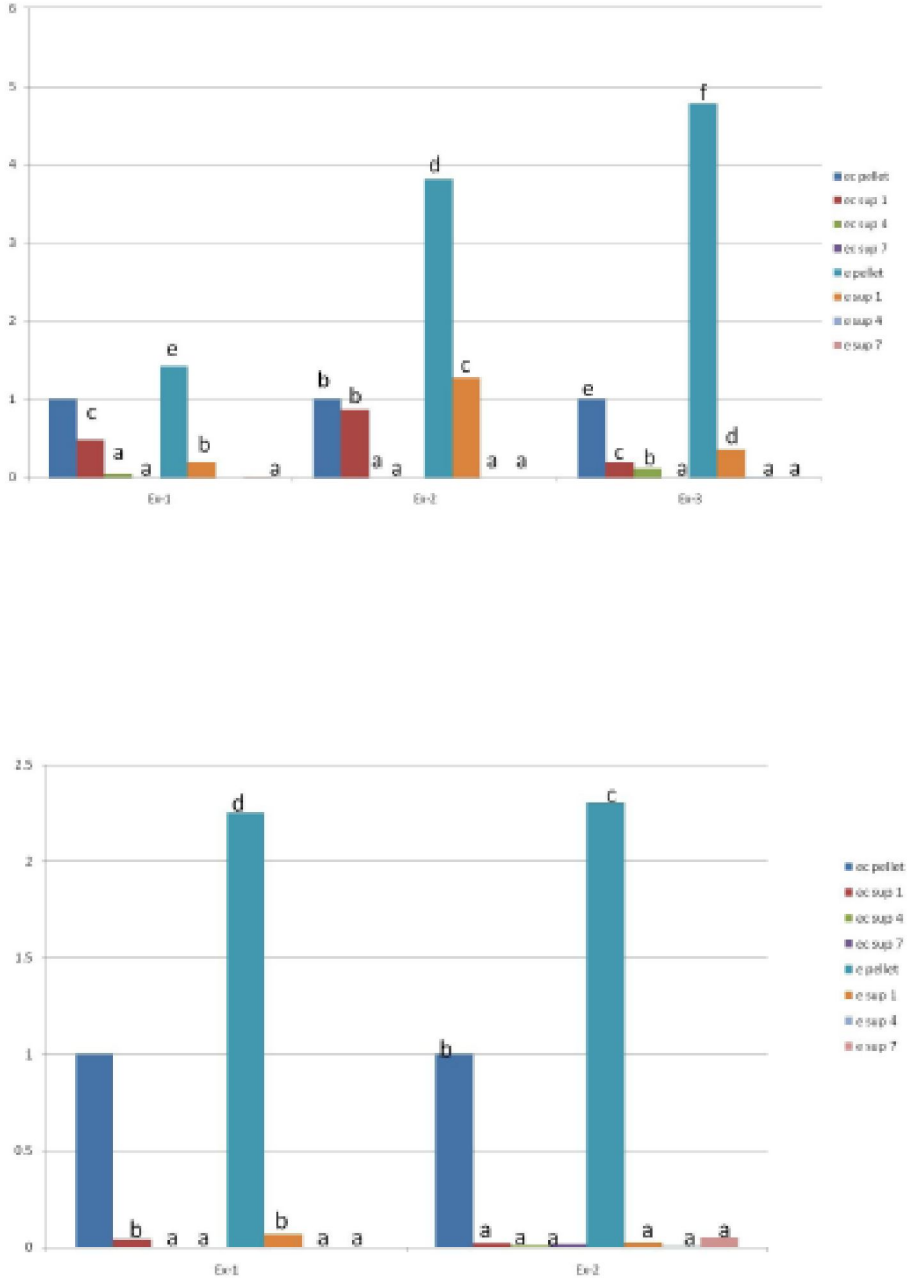


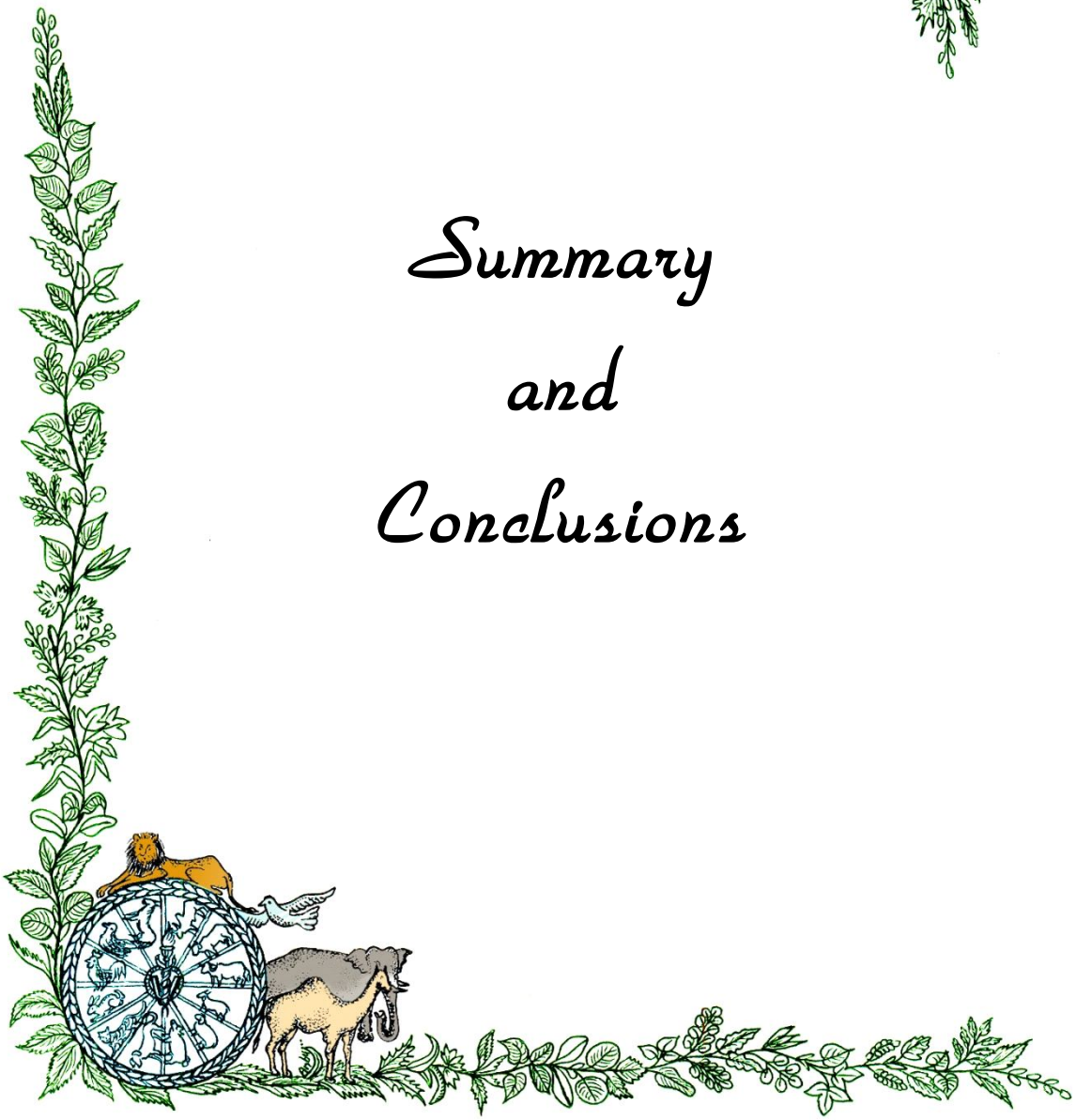
Fig. 4.9 : Fold increment of electroporated and electroporated control in different experiment of two bucks

4.3.2 The Efficiency of electroporation in incorporation of plasmid DNA

As depicted in (Fig. 4.9), in electroporated sperm pellet, after six serial washings, a 1 to 4 fold more ($p<0.01$) abundance of the plasmid was observed as compared to the control pellet. The presence of 0.5 to 1.5 fold more copies of plasmid in the control-supernatant 1 than that in electroporated also indirectly suggested greater internalization of the plasmid gene due to electroporation. Interestingly, there was a gradual decline in abundance of plasmid in Sup. 1 and 4 in electroporated sample. However, copy no. in the respective supernatants from control, a sudden reduction was observed. These observations are suggestive of the fact that in absence of electroporation, though large numbers of plasmid DNA might have been adhered to the surface of spermatozoa but could not be internalized. And these adhered plasmid might have simply got removed by centrifugation and appeared in the first washing i.e. sup 1.

It is evident from the above results that electroporation was far more efficient in internalization of foreign DNA than only incubation. The incubation of spermatozoa with DNA constructs seems to be sufficient to make sperm cells carriers of plasmids as already mentioned by Lavitrano *et al.* (1989) and Azerro. (1989), but success of incubation spermatozoa in making transgenic animals is yet to be validated. A recent report demonstrated a variable success of binding (3.08 -73.39%) and internalization (4.83–70.00%) of foreign DNA when buck semen was simply incubated with the DNA.

In summary, the electroporation of caprine sperm cells at 300 V in TALP media was the best combination showing least adverse effect on spermatozoa. Thus, from this study, it can be concluded that the above combination of voltage and time can be used for the subsequent transgenic animal production without producing much damage to the sperm cells.



Summary and Conclusions

5 Summary and Conclusions

Microinjection has been used successfully in generating transgenic laboratory animals, particularly in mice. In larger animals however, success rate have been limited (Chang *et al*., 2002; Houdebine, 2002), due to longer gestation period, generation time, less litter sizes and requirement of large numbers of donor animals (Mullins and Mullins, 1996). Out of the physical, viral and chemical methods of transgenesis, physical method primarily electroporation showed a better success rate and seems to be simple and efficient one. Sperm mediated gene transfer has been used successfully in mice (Gordon and Ruddle, 1980) such study has yet to be reported in livestock species including goat. Therefore, the present study was proposed with the following objectives;

- To study the effect of electroporation on semen parameters
- To standardize *in vitro* electroporation protocol for internalization of foreign DNA

Adult bucks (n=2) of 2 years age, maintained under identical managemental conditions at experimental herd were utilized for this study. Semen from these Bucks were collected using artificial vagina. Various semen parameters viz., progressive motility, live-dead spermatozoa, membrane integrity, acrosome integrity and chromatin integrity were assessed in winter (November to February) and summer season (March to May) respectively. The seasonal difference in percentage of live and dead spermatozoa could not be observed with CFDA/PI staining. Membrane integrity of spermatozoa were assessed through HOST method observed a significantly decrease from winter to summer. Acrosome integrity of spermatozoa were assessed through Giemsa and FITC-PSA staining technique. There was no seasonal effect were found in acrosome integrity of spermatozoa.Chromomycin A_3 test (CMA_3) determine

low protamination state of spermatozoa which is directly correlated to fertility status of the spermatozoa. Chromatin integrity were found higher in summer season than winter season.In order to assess the adverse effect of electroporation on vital semen parameters, electroporation was carried out with different voltages 100 V, 300 V, 500 V and 1000 V were used. Out of the all combinations, 300 V in TALP media was found to be the best parameter showing least adverse effect on spermatozoa. *pIRES2-EGFP* (EGFP gene cloned downstream of CMV promoter) construct (Clontech, USA) was used. The transformation of *pIRES2-EGFP* in *E. coli* DH5α strain was carried out by using Transform aid Bacterial Transformation Kit. Plasmid was isolated from Endotoxin Free Quanta Maxi Kit (mdi, India) according to manufacturer's protocol. The Plasmid DNA isolated from transformed cells was checked for its quality, purity and concentration. The plasmid was linearized using suitable restriction enzyme. Linearized plasmid was purified after gel electrophoresis and used for electroporation. In electroporation, 0.8 ml of media containing one 1µg of plasmid gene (*pIRES2-EGFP)* with, 10 µl of neat fresh semen was added into electro-cuvette so that final total concentration of spermatozoa remains approximately 30 million. For detection of internalization of plasmid DNA into the sperm, electroporated sperm pellet were separated from the media by centrifugation and supernatant containing media was collected in a separate microfuge tube. Separated sperm pellet was further subjected to six serial washings with PBS. Supernatants from each washing was collected. Supernatant 1, 4, 7 and sperm pellet processed immediately and remaining supernatant were stored at -20^{0}C. For assessment of sensitivity and specificity of Real time PCR for the detection of plasmid gene, standard curve were plotted using ten-fold dilutions of the *pIRES2-EGFP* plasmid gene with their respective C_t values. The R^2 value and linear equation for the standard curve was 0.984 and Y= -3.1077 X + 12.145, respectively. Sensitivity of the Real time PCR is the minimum copy no. of plasmid which can be detected by RTq-PCR was found to be 18 to 20. Finally, copy numbers of pellet, supernatant nos. 1, 4 and 7 from the control as well as electroporated samples were determined using supernatant 7 of control as a calibrator. To determine efficiency of electroporation in internalization of plasmid DNA in electroporated sperm pellet, after six serial washings, a 1 to 4 fold more ($p<0.01$) abundance of the plasmid was observed as compared to the control pellet. The presence of 0.5 to 1.5 fold more copies of plasmid in the control-supernatant 1 than that in electroporated also indirectly suggested

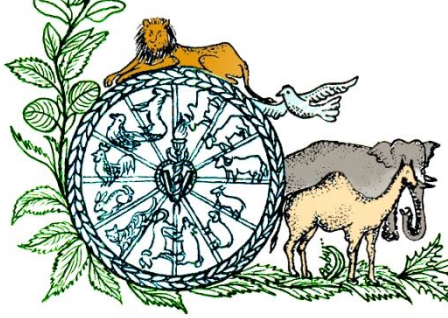
greater internalization of the plasmid gene due to electroporation. Interestingly, there was a gradual decline of copy no. in sup. 1, 4 and 7 in electroporated sample. However, copy no. in the respective supernatants from control a sudden reduction was observed. This observation is suggestive of the fact that in absence of electroporation, though large numbers of plasmid DNA might have been adhered to the surface of spermatozoa but could not be internalized. These adhered plasmid might have simply got removed by centrifugation and appeared in the first washing i.e. sup 1.

On the basis of results observed in the present study, the following conclusions can be drawn:

1. Effect of voltage for all parameters except acrosome integrity by Giemsa staining and membrane integrity by HOS test, voltage above 300 V had an adverse effect.
2. For any given voltage, Mannitol had an significantly adverse effect on motility and live-dead percentage of spermatozoa
3. The electroporation of caprine sperm cells at 300 V in TALP media was the best combination having least adverse effect on spermatozoa and causing significant internalization of the plasmid

Thus from this study, it can be concluded that the above combination of voltage and time constant can be used for the subsequent transgenic animal production without producing much damage to the sperm cells. However, results of this study should be validated by in vitro fertilization using electroporated semen. The expression of the plasmid carrying Enhanced Green Fluorescent Protein (EGFP) or any other reporter gene in the embryo (at morula or blastocyst) stage will confirm both the fertilizibility of the electroporated spermatozoa as well as the efficiency of electroporation for internalization of the plasmid.

Mini Abstract



6 Mini Abstract

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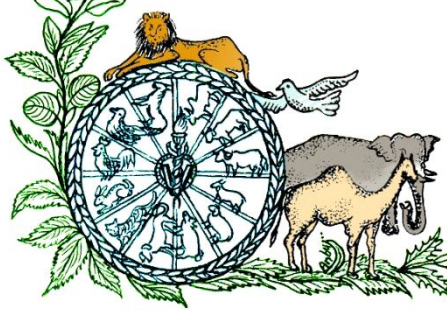
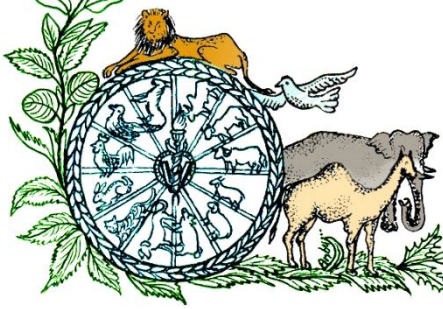
लघु सारांश



7 लघु सारांश

शुक्राणु मध्यस्थता जीन अंतरण के प्रयोग से बाहरी डी.एन.ए. को अंडजनाणु में प्रवेश कराकर ट्रांसजेनिक पशु का उत्पादन किया जा सकता है। शुक्राणु मध्यस्थता द्वारा डी.एन.ए. का अंतरण एक बहुत सरल तथा शीघ्र होने वाली तकनीक है। इस प्रणाली से बकरों के आनुवंशिकी में बदलाव लाकर इसे बायोरेक्टर या अन्य रूप में विभिन्न क्षेत्रों में प्रयोग कर सकते हैं। इस अध्ययन का प्रमुख उद्देश्य इलेक्ट्रोपोरेशन का शुक्राणु पर प्रभाव और इन विट्रो इलेक्ट्रोपोरेशन संलेख का मानकीकरण करके बाह्री डी.एन.ए. का शुक्राणु में समावेशन करना। तीन स्वस्थ बकरों से वीर्य को कृत्रिम वेजाइना के द्वारा लिया गया और उसका सामान्य मूल्यांकन किया गया इसके बाद विभिन्न वोल्टेज और समय नियतांक पर इलेक्ट्रोपोरेशन किया गया। वर्तमान अध्ययन के लिए pIRES2- EGFP वेक्टर जिसमें कि EGFP प्रोटीन के जीन था का प्रयोग किया गया। प्लाज्मिड को E.coli की DH5α स्ट्रेन में प्रबर्धित किया गया तथा इसे व्यवसायिक रूप से उपलब्ध किट से अलग किय गया। इलेक्ट्रोपोरेशन के लिए वीर्य एक्सटेंडर के रूप में मैनिटॉल और टॉल्प मीडिया लिया गया। ट्रांसफेक्शन के बाद पेलेट और प्रत्येक अधित्त्यवी द्रव्य से DNA को अलग किया गया। रीयल टाइम PCR का सेन्सटीवीटी 18–20 कॉपी प्लाज्मिड का सेन्सटीभीटी रीयल टाइम PCR से पता लगाने पर 18–20 कॉपी पाया गया। सुपरनेटेन्ट सात को कैलिबेरेटर के रूप में लिया गया। इलेक्ट्रोपोरेटेड पेलेट को कन्ट्रोल पेलेट से कम्परिजन करने पर 1 से 4 गुणा ज्यादा पाया गया। कन्ट्रोल सुपरनेटेन्ट प्रथम में 0.5 से 1.5 गुणा ज्यादा प्लाज्मिड पाया गया, जो अप्रत्यक्ष रूप से इन्टरलाईजेशन को सूचित करता है। रिर्पोटर जीन को RTq-PCR से पता लगाया गया। अन्ततः इन विट्रो इलेक्ट्रोपोरेषन संलेख द्वारा टॉल्प मीडिया में छः वॉशिंग के बाद प्रचूरता में रिर्पोटर जीन पाया गया। सबसे अच्छा पैरॉमीटर टॉल्प मीडिया में 300 वोल्टेज पर पाया गया। जिसको इन विट्रो निशेचन द्वारा भ्रूण में पक्का किया जायेगा।

References



8 References

Anzar, M. and Buhr, M.M. (2006). Spontaneous uptake of exogenous DNA by bull spermatozoa. *Theriogenology*, **65**: 683 -690.

Arezzo, F. (1989). Sea urchin sperm as vectors for foreign genetic transformation. *Cell Biol. Int. Rep*., **13**: 391 -404.

Bachiller, D., Schellander, K., Peli, J. and Ruther, U. (1991). Liposome-mediated DNA uptake by sperm cells. *Mol. Reprod. Devel*., **30**: 194 -200.

Brackett, B.G., Baranska, W., Sawicki, W. and Koprowski, H. (1971). Uptake of heterologous genome by mammalian spermatozoa and it transfer to ova through fertilization. *Proc. Natl. Acad. Sci. USA*., **68 (2)**: 353 -357.

Brinster, R.L., Sandgren, E.P., Behringer, R.R. and Palmiter, R.D. (1989). No simple solution for making transgenic mice. *Letters to the editor. Cell,* **59**: 239 -241.

Brinstiel, M.L. and Busslinger, M. (1989). Dangerous liaisons: Spermatozoa as natural vectors for foreign DNA. *Cell,* **57**: 701 -702.

Bustin, S.A. (2000). Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J. Mol. Endocrinol.*, **25**: 169 -193.

Cabrera, M., Chan, P.J., Kalugdan, T.H. and King, A. (1997). Transfection of the inner cell mass and lack of a unique DNA sequence affecting the uptake of exogenous DNA by sperm as shown by dideoxy sequencing analogues. *J. Assisted Reprod. & Genet.*, **14**: 120 -124.

Camaioni, A., Russo, M.A., Odarisio, T., Gandolfi, F., Fazio, V. M. and Siracusa, G. (1992). Uptake of exogenous DNA by mammalian spermatozoa - specific localization of DNA on sperm heads. *J. Reprod. Fertil.*, **96**: 203 -212.

Campbell, R.G., Hancock, J.C. and Rothschild, L. (1953). Counting live and dead bull spermatozoa. *J. Exp. Biol.,* **30**: 44.

Celebi, C., Guillaudeux, T., Auvray, P., Vallet-Erdtmann, V. and Jegou, B. (2003). The making of "transgenic spermatozoa". *Biol. Reprod.,* **68:**1477 -1483.

Chan, A.W.S., Homan, E.J., Ballou, L.U., Burns, J.C. and Bremel, R.D. (1998). Transgenic cattle produced by reverse-transcribed gene transfer in oocytes. *Prod. Natl. Acad. Sci. USA.*, **95:** 14028 -14033.

Chang, K., Qian, J., Jiang, M.S., Liu, Y.H., Wu, M.C., Chen, C.D., Lai, C.K., Lo, H.L., Hsiao, C.T., Brown, L., Jr, J.B., Huang, H.I., Ho, P.Y., Shih, P.Y., Yao, C.W., Lin, W.J., Chen, C.H., Wu, F.Y., Lin, Y.J., Xu, J. and Wang, K. (2002). Effective generation of transgenic pigs and mice by linker based sperm-mediated gene transfer. *BMC Biotechnol.*, **2:** 5 -17.

Chen, H.L., Yang, H.S., Huang, R. and Tsai, H.J**.** (2006). Transfer of a foreign gene to Japanese abalone (*Haliotis diversicolor supertexta*) by direct testis-injection. *Aquaculture,* **253:** 249 -258.

Chen, Y.L. and Tsai, H.J. (1997). Effect of electroporation conditions on loach sperm for successful gene transfer and early development. *Fisheries Science*, **63:** 527 -532.

Dhup, S. and Majumdar, S.S. (2008). Transgenesis via permanent integration of genes in repopulating spermatogonial cells *in vivo*. *Nat. Methods*, **5**: 601 -603.

Epperly, J.M. (2007). Linker -based sperm mediated gene transfer method for the production of transgenic rat. A thesis presented to the Graduate Faculty of the University of Akron. Ohio State, USA., *pp.* 57.

Fainsold, A., Frumkin, A., Rangini, Z., Revel, E., Yarus, S., Benyehuda, A. and Gruenbaum, Y. (1990). Chicken homeogenes expressed during gastrulation and the generation of transgenic chicken. *EMBO-EMBL Symp. The molecular biology of vertebrate development pp.* 31.

Felgner, P. L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, M. and Danielsen, M. (1987). Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. USA.,* **84:** 7413 -7417.

Francoline, M., Lavitrano, M., Lamia, C. L., French, D., Frati, L., Cotelli, F. and Spadofora, C. (1993). Evidence for nuclear internalization of exogenous DNA into mammalian sperm cells. *Mol. Reprod. Dev.,* **34:** 133 -139.

Gagne, M.B., Pothier, F. and Sirard, M.A. (1991). Electroporation of bovine spermatozoa to carry foreign DNA in oocytes. *Mol. Reprod. Devel.*, **29**: 6 -15.

Gandolfi, F., Lavitrano, M., Camaioni, A., Spadafora, C., Siracusa, G. and Lauria, A. (1989). The use of sperm-mediated gene transfer for the generation of transgenic pigs. *J. Reprod. Fertil. Abstr. Ser.*, **4**: 10.

Giordano, R., Magnano, A.R., Zaccagnini, G., Pittoggi, C., Moscufo, N., Lorenzini, R. and Spadafora, C. (2000). Reverse transcriptase activity in mature spermatozoa of mouse. *J. Cell Biol.,* **148**: 1107 -1113.

Gordon, J.W., Scangos, G.A. and Plotkin, D.J., Barbosa, J.A. and Ruddle, F.H. (1980). Genetic transformation of mouse embryos by microinjection of purified DNA. *Proc. Natl. Acad. Sci. USA*., **77**: 7380 -7384.

Gorlova, A.V. and Torchilin, V.P. (1991). Use of liposomes to associate foreign genetic material with spermatozoa. *Bulletin Exp. Biol. & Med.*, **112**: 1309 -1311.

Gossler, A., Doetschman, T., Korn, R., Serflingt, E. and Kemler, R. (1986). Transgenesis by means of blastocyst-derived embryonic stem cell lines. *Proc. Natl. Acad. Sci. USA.,* **83:** 9065 -9069.

Gruenbaum, Y., Revel, E., Yarus, S. and Fainsod, A. (1991). Sperm cells as vectors for the generation of transgenic chickens. *J. Cell Biochem*., **15**: 194.

He, X., Qi, B., Liu, G., Yu, W. and Chen, Q. (2006). A novel method to transfer gene *in vivo* system. *Prog. Biochem. Biophys.,* **33**: 685 -690.

Hongbao, M. (2005). Development application of polymerase chain reaction (PCR). *J. Am. Sci.,* **1(3)**: 1-47.

Horan, R., Powell, R., Bird, J.M., Gannon, F. and Houghton, J.A. (1992). Effects of electropermeabilization on the association of foreign DNA with pig sperm. *Arch. Androl*., **28**: 105 -114.

Horan, R., Powell, R., McQuaid, S., Gannon, F. and Houghton, J.A. (1991). Association of foreign DNA with porcine spermatozoa. *Arch. Androl.,* **26**: 83 -92.

Houdebine, L.M. (2002): Animal transgenesis: recent data and perspectives, *Biochimie,* **84**: 1137 -1141.

Huguet, E. and Esponda, P. (1998). Foreign DNA introduced into the vas deferens is gained by mammalian spermatozoa. *Mol. Reprod. Dev.*, **51**: 42 -52.

Innis, M.A. and Gelfand, D.H. (1990). Optimization of PCRs. *PCR protocols*. (eds. Innis, M.A., Gelfand ,D.H., Snisky, J.J. and White, T.J). Academic press, New York, pp.3-12.

Iversen, N., Birkenes, B., Torsdalen, K. and Djurovic, S. (2005). Electroporation by nucleofactor is the best nonviral transfection technique in human endothelial and smooth muscle cells. *Genet. Vaccines. Ther.*, **3(1)**: 2.

Jeyendran, R. S., Perez-Pelaez, M., Crabo, BG. and Zaneveld, L.J.D. (**1984**). Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *J. Reprod. Fertil.,* 70: 219-228.

Jonak, J. (2000). Sperm-mediated preparation of transgenic Xenopus laevis and transgenic DNA to the next generation. *Mol. Reprod. Dev.*, **56**: 298 -300.

Jordan, E.T., Collins, M., Terefe, J., Ugozzoli, L. and Rubio, T. (2008). Optimizing electroporation conditions in primary and other difficult-to transfect cells. *J. Biomol. Tech.*, **19**: 328 -334.

Khoo, H.W. (2000). Sperm-mediated gene transfer studies on zebrafish in Singapore. *Mol. Reprod. Dev.*, **56**: 278 -280.

Kroll, K.L. and Amaya, E. (1996). Transgenic Xenopus embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. *Development*, **122**: 3173 -3183.

Kurita, K., Burgess, S.M. and Sakai, N. (2004). Transgenic zebra fish produced by retroviral infection of *in vitro*-cultured sperm. *Proc. Natl. Acad. Sci. USA.*, **101**: 1263 -1267.

Kuznetsov, A.V. and Kuznetsova, I.V. (1995). Binding of exogenous DNA pRK3lacZ by the rabbit spermatozoa, its transfer in the oocytes and expression in the preimplantation embryos. *Ontogenez,* **26:** 300 -309.

Lai, L., Sun, Q., Wu, G., Murphy, C.N., Kuhholzer, B., Park, K.W., Bonk, A.J., Day, B.N. and Prather, R.S. (2001). Development of porcine embryos and offspring after intracytoplasmic sperm injection with liposome transfected or non-transfected sperm into *in vivo* matured oocytes. Zygote, **9:** 339-346.

Lavitrano, M., Busnelli, M., Cerrito, M.G., Giovannoni, R., Manzini, S. and Vargiolu, A. (2006). Sperm-mediated gene transfer. *Reprod. Fertil. Dev.,* **18:** 19 -23.

Lavitrano, M., Camaioni, A., Fazio, V.M., Dolci, S., Farace, M.G. and Spadafora, C. (1989). Sperm cells as vectors for introducing foreign DNA into eggs: genetic transformation of mice. *Cell*, **57**: 717 -723.

Lavitrano, M., Forni, M., Bacci, M.L., Di-Stephano, C., Varzi, V., Wang, H. and Seren, E. (2003). Sperm mediated gene transfer in pigs: selection of donor boars and optimization of DNA uptake. *Mol. Reprod. Dev.,* **64:** 284 -291.

Lavitrano, M., French, D., Zani, M., Frati, L. and Spadafora, C. (1992). The interaction between exogenous DNA and sperm cells. *Mol. Reprod. Dev.*, **31**: 161 -169.

Lavitrano, M., Maione, B., Forte, E., Francolini, M., Sperandio, S., Testi, R. and Spadafora, C. (1997). The interaction of sperm cells with exogenous DNA: a role of CD4 and major histocompatibility complex class II molecules. *Exp. Cell Res.,* **233**: 56 -62.

Lavitrano, M., Stoppacciaro, A., Bacci, M.L., Forni, M., Fioretti, D., Pucci, L., Di-Stefano, C., Lazzereschi, D., Rughetti, A., Ceretta, S., Zannoni, A., Rahimi, H., Moioli, B., Rossi, M., Nuti, M., Rossi, G., Seren, E., Alfani, D., Cortesini, R. and Frati, L. (1999). Human decay accelerating factor transgenic pigs obtained by sperm mediated gene transfer. *Transplantation Res.,* **31**: 972 -974.

Mackay, I.M., Arden, E.K. and Nitsche, A. (2002). Real-time PCR in virology. *Nucleic Acids Res.,* **30(6)**: 1292 -1305.

Maione, B., Lavitrano, M., Spadofora, C. and Kiessling, A.A. (1997). Sperm-mediated gene transfer in mice. *Mol. Reprod. Dev.,* **50**: 406 -409.

Mullins, L.J. and Mullins, J. J. (1996): Transgenesis in the rat and larger mammals. *J. Clin. Invest.,* **97 (7)**: 1557 -1568.

Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G. and Erlich, H.A. (1986). Specific enzymatic amplification of DNA *in vitro*: The polymerase chain reaction. *Cold springer Harbor Symposia on Quantitative Biology.,* **51**: 263 -273.

Nakanishi, A. and Iritani, A. (1993). Gene transfer in the chicken by sperm-mediated methods. *Mol. Reprod. Dev.*, **36**: 258 -261.

Perry, A.C.F., Wakayama, T., Kishikawa, H., Kasai, T., Okabe, M., Toyoda, Y. and Yanagimachi, R. (1999). Mammalian transgenesis by intracytoplasmic sperm injection. *Science,* **284**: 1180 -1183.

Robertson, E., Bradley, A., Kuehn, M. and Evans, M. (1986). Germ-line transmission of

genes introduced into cultured pluripotential cells by retroviral vector. *Nature*, **323 (6087**): 445 -448.

Rottmann, O.J., Antes, R., Hofer, P. and Maierhofer, G. (1992). Lipsome mediated gene-transfer via spermatozoa into avian egg cells. *J. Anim. Breed. Genet*., **109**: 64 -70.

Sambrook, J. and Russel, D.W. (2001). Rapid isolation of yeast DNA. In: Molecular cloning, a laboratory manual (Sambrook, J. and Russel, D.W. eds.). Cold Spring Harbor Laboratory, New York, pp. 631 -632.

Sato, M. (2005). Transgenesis via sperm. *J. Mamm. Ova. Res.,* **22**: 92 -100.

Sato, M., Iwase, R., Kasai, K. and Tada, N. (1994). Direct injection of foreign DNA into mouse testis as a possible alternative of sperm-mediated gene transfer. *Anim. Biotechnol*., **5**: 19 -31.

Schaefer-Ridder, M., Wang, Y. and Hofschneider, P. H. (1982). Liposomes as a gene -carriers: efficient transformation of mouse L-cells by thymidine kinase gene. *Science,* **215:** 166.

Sciamanna, I., Barberi, L., Martire, A., Pittoggi, C., Beraldi, R., Giordano, R., Magnano, A.R., Hogdson, C. and Spadafora. C. (2003). Sperm endogenous reverse transcriptase as mediator of new genetic information. *Biochem. Biophys. Res. Commun.,* **312:** 1039 -1046.

Shemesh, M., Gurevich, M., Harel-Markowitz, E. and Benvenisti, L. (2000). Gene integration into bovine sperm genome and its expression in transgenic offspring. *Mol. Reprod. Dev*., **56**: 306 -308.

Sine, F.Y.T., Bartley, A.L., Walker, S.P., Sin, I.L., Symonds, J.E., Hawke, L. and Hopkins, C.L. (1993). Gene transfer in chinook salmon (*Oncorhynchus tsawytscha*) by electroporating spermin presence of pRSV-lacZ DNA. *Aquaculture,* **117**: 57-69.

Solter, D. (2000). Mammalian cloning: advances and limitations. *Nat Rev Genet*., **1**: 199-207.

Sperandio, S., Lulli, V., Bacci, M.L., Fomi, M., Maione, B., Spadafora, C. and Lavitrano, M. (1996). Sperm-mediated DNA transfer in bovine and swine species. *Animal Biotechnol.*, **7**: 59 -77.

Sukardi, S., Curry, M.R. and Watson, P.F. (1997). Simultaneous detection of the acrosomal status and viability of incubated ram spermatozoa using fluorescent markers. *Animal Reproduction Science,* **46:** 89-96.

Symonds, J.E., Walker, S.P. and Sin, F.Y.T. (1994). Electroporation of salmon sperm with plasmid DNA: evidence of enhanced sperm/DNA association. *Aquaculture*, **119**: 313-327.

Thiel, C. and Nix, M. (2006). Efficient transfection of primary cells relevant for cardiovascular research by nucleofection. *Methods Mol. Med.,* **129**: 255 -266.

Torres, M. (1997). The use of embryonic stem cells for the genetic manipulation of the mouse. *Curr. Top. Dev. Biol*., **36:** 99 -114.

Varga, C.M., Wickham, T.J. and Lauffenburger, D.A. (2000). Receptor-mediated targeting of gene delivery vectors: Insights from molecular mechanisms for improved vehicle design. *Biotechnol. Bioeng*., **70**: 593 -605.

Watson, P.F., (**1975**). Use of a Giemsa stain to detect changes in the acrosome of frozen ram spermatozoa. *Vet. Rec*., **97**: 12-15.

Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J. and Campbell, K.H.S. (1997). Viable offspring derived from fetal and adult mammalian cells. *Nature*, **385 (6619):** 810 -813.

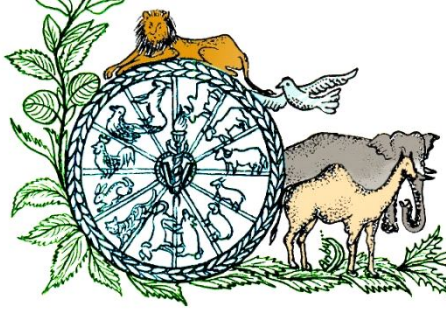
Yap, E.P.H. and Mc-Gee, J.O.D. (1991). Short PCR product yields improved by lower denaturation temperatures. *Nucleic Acids Res*., **19 (7)**: 1713.

Zani, M., Lavitrano, M., French, D., Lulli, V., Maione, B., Sperandio, S. and Spadafora, C. (1995). The mechanisms of binding of exogenous DNA to sperm cells -factors controlling the DNA uptake. *Exp. Cell Res*., **217**: 57 -64.

Zhao, Y.J., Wang, Y., Wang, J., Li, F. B. and Wei, H. (2005). Effect of cryopreservation on the efficiencies of goat sperm in picking up exogenous DNA and *in vitro* producted embryos. *Current Zoology*. **54(6):** 1089 -1097.

Zoraqi, G. and Spadafora, C. (1997). Integration of foreign DNA sequences into mouse sperm genome. *DNA Cell Biol.,* **16**: 291 -300.

Appendix



Appendix-1

CHEMICALS	SOURCES
Agarose (low EEO)	SRL, GIBCO, BRL
Ammonium chloride	SRL
Amyl alcohol	Glaxo laboratories
Bromophenol blue	Hi-media
Bovine serum albumin	Sigma
Chloroform	Qualigens
Chromomycin A_3	Sigma
Cuvette plus	Bio Rad
EDTA disodium salt	SRL
Carboxyfluorescein diacetate	Sigma
Ethanol	Bengal chemicals
Ethidium bromide	SRL
Fluorescein isothiocynate	Sigma
Fructose	Sigma
Glacial acetic acid	Qualigens
Hydrochloric acid	Qualigens
8-Hydroxy-quinoline	Qualigens
Isoamyl alcohol (extra pure AR)	SRL
Isopropanol	SRL
Magnesium chloride	Qualigens
Mannitol	Sigma
Phenol (extra pure AR)	SRL
Propidium Iodide	Sigma
Sodium bicarbonate	SRL
Potassium acetate	Glaxo Laboratories
Sodium acetate	Qualigens
Sodium chloride	SRL
Sodium dodecyl sulphate	SRL
Sodium hydroxide	SRL
Tris base	Qualigens
Tries HCL	SRL
Xylene cyanol	Hi-media
Ham's F-12	Gibco
DMEM	Gibco
Gentamicin	Duchefa
LB Broth	Amresco
Agar – agar	Qualigens

EQUIPMENTS	SOURCES
Autoclave	Scientronic instruments
Centrifuge	Hermle Labortechnik
Deep Freezer (-20°C)	Leonard
Distillation plant	Scientronic Instruments
Electro Square Porator	ECM830, BTX
Gel documentation system	Syngene
Horizontal gel electrophoresis	Bangalore Genie Apparatus
Hot air oven	S. P. Scientronic Instruments
Ice box	Torson
Ice flaking machine	Scotsman
Incubator	S. P. Scientronic instruments
Laminar air flow	Kleinzaids Contamination Control Ltd.
Magnetic stirrer	Scientronic instruments
Micropipette (all ranges)	Qualipette
PH meter	Tunco
Power pack	Bangalore Genie
Refrigerator	Godrej
Microwave	Electrolux
Master cycler ep gradient	Eppendorf.
Electroporator	BTX Harvard Apparatus
Vortexer	Scientronic Instruments
Water bath	S.P. Scientific Instruments
Weighing balance (Digital)	Shimadzu
Shaker Incubator	Labcon
Homogenizer	Star Micronic Devices
Nanodrop 1000	Thermo scientific
CO_2 Incubator	Eppendorf
BOD Incubator	Basil
Fluorescent Microscope	Leica
Real Time PCR Mode 7500	Applied Biosystems

LAB WARES/ MISCELLANEOUS ITEMS

LAB WARES

Glass wares

Beakers, conical flasks	Borosil, Schott Duran
Measuring cylinders, Pasteur pipettes	
10 ml pipettes, reagent bottles	

Plastic wares

Polypropylene centrifuge tube (15 ml and 50 ml)	Tarson
Eppendorf tube (1.5 ml and 0.5 ml)	Axygen, USA
PCR tube (0.2 ml)	Axygen, USA
Micro tips (All ranges)	Axygen, USA

MISCELLANEOUS ITEMS

Adhesive tapes	Needles
Aluminium foils	Para film
Autoclave label	Porcelain basin
Black and white films	pH paper
Blotting paper	Racks
Burette	Scissors
Cello-tape	Thermometers
Cotton	Threads
Disposable gloves	Tissue paper filters
Papers	Forceps, Marker pens

Appendix-2

BUFFERS AND SOLUTIONS

1. **0.5 M EDTA solution (pH 8.0)**

EDTA Disodium salt	186.1gm
Double distilled water (up to)	100 ml

Adjust pH 8.0 using Na0H pellets. Autoclaved and stored at room temperature

2. **10% SDS**

SDS (Sodium Dodecyl sulphate)	100 gm
Autoclaved double distilled water (up to)	100 ml

Adjust pH 7.2 using (conc. HCl). Heat in water bath at 60°C to dissolve and then store at room temperature. SDS solution should not be autoclaved.

3. **3 M sodium acetate**

Sodium acetate (anhydrous)	24.6 gm
Double distilled water (up to)	100 ml

Adjust pH 5.5 using glacial acetic acid. Autoclaved in batches of 20 ml

4. **70% ethanol**

Ethanol	70 ml
Autoclaved double distilled water	30 ml

Mix thoroughly and store in amber colored bottle at 4°C.

5. **1 M Tris HCI (pH 8.0)**

Tris HCI	157.6gm
Doubled distilled water (up to)	1000 ml

Adjust pH 8 using NaOH pellets. Autoclave and store at 4°

6. **50 X TAE**

Tris base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

Distilled water was added to make up to a final volume of 100 ml. A working solution of 1x was used.

7. **6 X Gel loading Dye (Sam brook *et al*., 1989)**

a) Type I

Bromophenol blue	0.25%
Xylene cyanol	0.25%
Sucrose in water	40 % (w/v)

Mix and store at 4°C.

b) Type IV

Bromophenol blue	0.25%
Sucrose in water	40% (w/v)

Mix and store at 4°C

8. **Ethidium bromide (10mg/ml)**

Ethidium bromide	10 mg
Autoclaved distilled water (up to)	1 ml

Wrap in aluminium foil and store in dark place at room temperature.

9. **Phosphate buffered saline**

NaCl	8gm
KCl	0.2gm
Na_2HPO_4	1.44 gm
KH_2PO_4	0.24gm
Distilled water (up to)	800 ml

Adjust pH to 7.4 with HCI. Add water to 1 liter. Autoclaved and stored at room temperature.

10. **L.B (Luria- Bertani) broth**:

Yeast (0.5%)	0.5 gm
Peptone (1%)	1 gm
NaCl (1%)	1 gm
DDW	up to 100 ml

Autoclave and stored at 4^0 C

11. **L.B (Luria- Bertani) Agar**

Agar – agar	1.1 g
L.B Broth	2.9 g
Distilled water up to	100 ml

Autoclave and store at 4^0c

12. **DEPC treated water**

DEPC	500 µl
Double distilled water	1000 ml

Shake, let stand at room temperature for 6 hr and autoclave at 15 psi at 121^0 C for 20 min.

13. **Resuspension Buffer (R3)**

Tris-HCl, pH 8.0	50 mM
EDTA	10 mM

14. **RNase A** 20 mg/ml in Resuspension Buffer (R3)

15. **Lysis Buffer (L7)**

Sodium Hydroxide	0.2 M
SDS	1% (w/v)

16.	**Precipitation Buffer (N3)**	
	Potassium acetate, pH 5.5	3.1 M
17.	**Equilibration Buffer (EQ1)**	
	Sodium acetate, pH 5.0	0.1 M
	Sodium chloride	0.6 M
	Triton® X-1000.	15% (v/v)
18.	**Wash Buffer (W8)**	
	Sodium acetate, pH 5.0	0.1 M
	Sodium chloride	825 mM
19.	**Elution Buffer (E4)**	
	Tris-HCl, pH 8.5	100 mM
	Sodium chloride	1.25 M
20.	**TE Buffer (TE)**	
	Tris-HCl, pH 8.0	10 mM
	EDTA	0.1M
21.	**Mcllvains buffer**	
	0.1M Citric acid	0.1M
	0.2M Disodium hydrogen phosphate	0.2M
	Magnesium chloride	10mm/ lt
22.	**CMA_3 Solution**	
	CMA_3	0.25mg/ml
	Add in Mcllvains buffer. Store at 4^0C for long time	
23.	**HOS Solution**	
	Sodium citrate	0.735 gm
	Fructose	1.351 gm
	Distilled water was added to make up to a final volume of 100 ml	
24.	**Egg- Yolk-Glucose-Citrate diluents**	
	Sodium citrate dihydrate	3.634 gm
	Glucose	0.80 gm
	Egg yolk	2.5-5 %
	Gentamycin	500µg/ml
	Distilled water was added to make up to a final volume of 100 ml	
25.	**Sperm lysis buffer**	
	SDS	10%
	Tris base	1 M
	EDTA	0.5 M

	NaCl	6 M

To make up the volume up to 10 ml by MQ water.

Heat the aqueous solution up to 65^{0}C for 10 minute and finally add 0.02 ml mercaptoethanol

26. **Fertilization TALP media**

NaCl	114 mM
$CaCl_2.2H_2O$	2.0 mM
KCl	3.2 mM
Na_2HPO_4	0.34 mM
$MgCl_2$ (anhydrous)	0.50 mM
$NaHCO_3$	25.0 mM
Sodium lactate	1.86 µl/ml
Gentamycin	50 µg/ml
Phenol red	10 µg/ml

27. **Sperm TALP media**

Heparin	10µg/ml
Sodium pyruvate	0.25mM
BSA (Fraction V)	3 mg/ml

To make sperm TALP added with fertilization media solution. Adjust pH 7.6-7.8 and Osmolarity 280-300 mOSM.

28. **Hancock's fixative**

NaCl	10 gm
$NaHCO_3$	0.5 gm
Formalin	40%

Distilled water was added to make up to a final volume of 1000 ml

29. **Giemsa working solution**

Stock giemsa solution	3.0 ml
Sorenson's o.1M phosphate buffer solution	2.0 ml
Double glass D.W	45.0 ml

Vitae

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High School	Bihar School of Examination Board (BSEB)	75.4%

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