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Changes in the reproductive system of the male mice immunized with a plasmid DNA vaccine encoding GNRH-1 and T helper epitopes

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ABSTRACT

Neutralization of Gonadotrophin releasing hormone (GnRH-1) is necessary to control mammalian reproduction. If reproductive capacity and sexual behavior need to be prevented, a contraceptive vaccine that neutralizes GnRH-1 would be preferred. Active immunization against GnRH-1using peptide based vaccine has gained wide spread acceptance and reached to field use. The GnRH-1molecule requires conjugating with a carrier protein along with an adjuvant to act as an antigen. As an alternative to peptide conjugates DNA vaccine can be used. The DNA vaccine that was used in this study containing eight repeated units of GnRH-1 peptides interspersed in eight Th-2 epitopes in a fusion protein. The vaccine was administered into male mice in conjuction with Phosphate buffer saline (Group-1), non ionized surfactant vesicle (Group-2) and bilesome (Group-3) solution. The highest suppression of the epididymal sperm count was seen in Group-2 (0.907±0.1554, p=0.0043) followed by Group-3 (1.790±0.3751, p=0.0009) and Group-1 (2.647±1.3972, p=0.0024), respectively. Testicular weight of the vaccinated mice showed highest suppression in Group-2 (0.105±0.0043, p=0.0003) followed by Group-3 (0.106±0.0049, p=0.0123) and Group-1(0.111±0.0089, p=0.0042). Result of the testicular score count revealed a highest suppression of testicular spermatogenesis (score 7-10) in Group-2 mice testes followed by Group-3 and Group-1 mice. An average, testicular score 10 were 4.00 ±1.73, 5.00 ±3.00, 13.67 ±4.16 and 14.00 ±1.73 for Group-2,3,1 and 4 control mice respectively. This was an indication of suppressed spermatogenesis following vaccination. Epididymal weight of the treated mice showed highest suppression in group-2 (0.021 ±0.0011, p=0.0034). Prostate and seminal vesicles of the all vaccinated mice showed reduced diameter of the glandular alveoli and decreased stored secretions and highest suppression were seen in Group-2 followed by Group-3 and Group-1. Evaluation of GnRH-1 mRNA expression in testicular tissues using GnRH-1 specific primers by RT-PCR successfully detected GnRH-1 mRNA expression in vaccinated mice. This is an indication of the reversibility of the sexual function following vaccination.

INTRODUCTION

Reproduction and sexual behaviour in mammals is governed by various hormonal, neurological and social factors. In this case, hypothalamic GnRH-1 is the key peptide in controlling gonadotrophins release from the pituitary. Research on active immunization against GnRH-I has gained widespread acceptance to control reproduction and behavior of farm mammals, including animals reared for food production, along with companion animals are routinely castrated for a number of reasons. Castration of boars permits increased growth and avoids the unpleasant odour of fat ('boar taint') associated with male hormone breakdown products. Surgery to sterilize larger animals such as horses has a risk of infections and morbidity (Rolf et al., 2008). Various alternatives to surgical procedures have been proposed, among them the use of a specific immunosterilization vaccine targeted at one or more factors of the reproductive system. To be effective, such a sterilization vaccine needs to be reliable, convenient (i.e. less laborious than surgical procedures), cost effective and safe.

GnRH peptide expression has been identified in certain tumors including ovarian (Kang et al.,

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2000, and Ohno et al., 1993), endometrial (Irmer et al., 1995), prostatic (Bahk et al., 1998 and Lau et al., 2001, Ferro, 2002, Lu et al., 2009), and breast cancers (Harris et al., 1991 and Kottler et al., 1997). Further, an autocrine growth-regulatory system that attenuates cancer development through direct effect in the ovary and the endometrium both in vivo and in vitro has been hypothesized based on GnRH production in endometrial and ovarian cancers (Emons and Schulz, 2000, Imai et al., 2000).

More complication is that a minimum of 24 structural variants of GnRH have been described in vertebrates and invertebrates (Guilgur et al., 2006) and are distributed in a diverse group of tissues including mesodermally derived gonadal, placental, mammary and immune tissues and neurectodermally derived peripheral and central nervous system (Whitlock et al., 2006). GnRH-1 in its native form, being a short peptide and a self-hormone, is very poorly immunogenic. Conjugation of the peptide to large carrier molecules such as tetanus toxoid (TT), diphtheria toxoid (DT) and branched polylysine overcomes this problem and induces antibodies in immunized animals (Beekman et al. 1999, Ferro et al., 2001, 2002; Ferro and Stimson, 1997, Ferro et al., 2004, Khan et al., 2007 and Ladd et al. 1990, Sad et al. 1991).

Genetic immunization using plasmid DNA is safe and easier to administer than whole cell vaccination, while large scale production and storage is cheaper, uniform and more rapid than protein based vaccines (Robertson et al., 2001). The advantage that less of the peptide is needed to raise an immune response. Research with animal models has shown that DNA vaccines are safe. Deleterious immune responses, such as autoimmunity and development of tolerance in response to persistent expression of a foreign antigen, have not been observed.

DNA vaccination is a new vaccine approach used to induce an immune response to an antigen protein expressed in vivo. Cell-mediated immunity (Th1 and Th2 responses) and humoral immunity can be obtained following genetic immunization. Production of humoral antibodies is generally weaker than induced by traditional vaccines and most of the DNA vaccine induces cell mediated immunity (Ulmer et al., 2006). One limiting factor in the use of GnRH vaccines is that there is usually considerable variation in the incidence and level of response to immunization. This new approach of DNA vaccination offers new hope because of their low cost and manufacturing stability at ambient temperature.

Clinical trials with DNA vaccines have been conducted for HIV, HBV, HVC, HSV, tuberculosis, influenza and malaria also in hand for the treatment of allergies (Kutzler et al., 2005; Charo et al., 2004). Success with this research will enable us to design DNA vaccine against GnRH-1 to control reproduction and as well as to cure for the breast and prostate cancer patients (Lu et al., 2009).

The effects of immunization on the reproductive system were evaluated by examining the gross morphology, histology of the reproductive organs, decreased amount of sperm concentration and quantitatively measuring changes in gene expression in testes by standard procedure for the isolation of mRNA specific for GnRH-1. The mRNA will be reversed transcribed and cDNA will be used in PCR protocol to evaluate the level of GnRH-1 activity following vaccination. Level of mRNA specific for GnRH-1 will be an indicator of suppressed hormonal activity following immunization. In view of this development, the final objective of this study was to evaluate GnRH-I mRNA expression in testicular tissues using GnRH-I specific primers (Chen et al., 1999) in RT-PCR.

The DNA vaccine is a further improvement of previous study (Khan et al., 2007, 2008) that was administered to male mice encoated in PBS, NISV and Bilosome to determine the efficacy of a GnRH-1 immunization to disrupt fertility in male animal and evaluate the vaccine.

MATERIALS AND METHODS

Samples

The samples were collected from Department of Pathology, BAU, Mymensingh. Vaccinated and control male mice are sacrifized for sperm

concentration count, histopathological examination of male gonadal tissue (testes, epididymis, seminal vesicle and prostate gland). Evaluation of mRNA expression by RT-PCR technique in gonadal tissue (testes). For histopathologic examination samples were collected in 10% neutral buffered formalin. For RT-PCR samples were preserved in -20^{0}C. Before collecting the samples all instruments were sterilized properly.

Sperm analysis

The left cauda-epididymis was excised, weighed and 10 mg epididymis was minced in 10 ml DMEM with fine scissors. After 1h incubation at 37^{0}C in an environment saturated with 5% CO_2 the solution was transferred to a 15 ml centrifuge tube and stood for 5 min in order for tissue fragments to settle down. Supernatant containing sperm was collected in a fresh centrifuge tube and left over tissue fragment were vortexes gently with 4 ml PBS. Pooled supernatants were spun down at 100g for 5 min. The supernatants were removed and pelleted sperm was diluted with 100 µl PBS, pH 7.4. A 90 µl sperm cell suspension was stained with 10µl 1% (w/v) eosin-Y for 30 min. Uniform stained with 10 µl stained samples onto clean, grease-free silanised slides. The slides were air-dried, dehydrated in 90% (v/v) and absolute ethanol, cleared in xylene before being mounted under a cover slip. Sperm on the slides were examined under 20 × and 40 × magnification. A total of 300 spermatozoa/ mouse were examined to explore morphological differences in the head (normal, amporphous, pin-head and banana shaped), hook (knobbed, bent, short on hook) and tail pieces (tail folded overhead, two tails and coiled tails).

Total sperm counts were carried out with a 10 µl sperm suspension diluted in 90 µl PBS, pH 7.4. The sperm suspension was mixed with 0.04% (v/v) Trypan blue solution (1:1) and charged into a Neubauer counting chamber. The total sperm number in each square of a 1 mm^2 area was multiplied by 2 × 105 to obtain the number of sperm/20 mg cauda epididymis.

Tissue processing

The tissues were collected from testes, prostate, seminal vesicle, epididymis of the mice for histopathological examination. Samples were collected at the junction of altered and unaltered tissue immediately upon removal from the body. The collected tissues were fixed in 10% neutral buffered formalin for 7 days. Tissue samples were immersed in at least 10 times their volume of fixative. The histological section on slide was stained with Goldner's trichome stain.

Testicular score count

Criteria used to quantify testicular spermatogenesis, adapted from the Johnsen method used to quantify human spermatogenesis (Johnsen, 1970) and modified by Peters et al. (2000) for use in dog testes.

Scores	Criteria to quantify the level of spermatogenesis in the tubules
10	Complete spermatogenesis with many spermatozoa. Germinal epithelium organized in a regular thickness with a visible lumen, with 50–80% of the lumen contains spermatozoa
9	Many spermatozoa/spermatids (>10) present but germinal epithelium disorganized, with marked sloughing or obliteration of the lumen
8	Only a few spermatozoa (<5–10) present in the tubules
7	No spermatozoa but many spermatids (10–25) present (lowest standard score) with no visible lumen
6	No spermatozoa and only a few spermatids (<5–10) present
5	No spermatozoa and spermatids in the tubules, but several or many spermatocytes present (>10)
4	Only a few spermatocytes (<5–10) and no spermatids or spermatozoa present
3	Spermatogonia are the only germ cells present
2	No germ cells but only Sertoli cells present
1	No cells in the tubules

RT-PCR

The mRNA of GnRH-1 testes were detected by RT-PCR. In this step first RNA was isolated from collected tissues using Qiagen RNeasy kit (Germany). The RNeasy technology combines the selective binding properties of a silica-gel-based membrane with the speed of microspin technology. High-salt buffer (Guanidine isothiocyanate, GITC) system allows up to 100µg of RNA longer than 200 bases to bind to the RNeasy silica-gel membrane. Contaminants are efficiently washed away and high quality RNA is then eluted in 30µl, or more, of water. Total RNA or mRNA is first reverse transcribed into cDNA that is then amplified by PCR- the RT-PCR technique according to the protocol described by Mario et al., (1995). RT and PCR can be carried out either sequentially in the same tube (1-step RT-PCR) or separately (2-step RT-PCR). In this method for RT-PCR essentially has two parts: (1) Using AMV Reverse transcriptase enzyme one can generate high yields of full-length, first-strand cDNA from mRNA and (2) the cDNA thus generated acts as the template for the subsequent PCR amplification. RT-PCR products were analyzed by electrophoresis on 1% agarose gel, stained with ethidium bromide and examined under UV light using an image documentation system.

Data analysis

The recorded data were compiled and tabulated for statistical analysis. Analysis of variance was done with the help of Computer Package MSTAT. The mean differences among the treatments were determined as per Duncan's Multiple Range Test (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

Sperm concentration

Morphological evaluation of the sperm obtained from the tail of the epididymis did not reveal major defects in their head and tail pieces between the immunized and non-immunized mice.

Sperm obtained from the cauda epididymis of the vaccinated mice showed significant reduction in counts compared to naked pcDNA treatment. The average sperm count was much suppressed in group 1 (0.907±0.1554b×106, p = 0.000) followed by group 3 (1.790±0.3751b×106, p=0.000) and group 2 (2.647±1.3972b × 106, p = 0.000) compared to group 4 control mice (16.333±3.214a×106, p=0.000) (Table 1).

Table 1
Effects of immunization on epididymal sperm counts.

Group of mice	Number of mice	Concentration/20 mg
Control	M1	20
	M2	15
	M3	14
	Mean±SD	16.333±3.2146a
Group 1	M1	0.736
	M2	0.944
	M3	1.04
	Mean±SD	0.907±0.1554b
Group 2	M1	1.046
	M2	3.622
	M3	3.272
	Mean±SD	2.647±1.3972b
Group 3	M1	1.41
	M2	2.16
	M3	1.8
	Mean±SD	1.790±0.3751b
P value		0.000
Level of significance		**

In a column figures with same letter do not differ significantly whereas figures with dissimilar letter Differ significantly (as per DMRT) ** = Significant at 1% level of probability

Epididymal sperm counts revealed the lowest concentration of sperm in group 1 mice, followed by groups 2 and 3 compared to an unaffected sperm count in group 4 control mice. Data represents the mean ± SD for each group NS=p<0.05, ** p<0.01 (Table 1).

Table 2
Effects of immunization on testicular weight of mice.

Group of mice	Number of mice	Testes weight
Control	M1	0.1054
	M2	0.1357
	M3	0.14
	Mean±SD	0.127±0.0189a
Group 1	M1	0.1022
	M2	0.1102
	M3	0.12
	Mean±SD	0.111±0.0089a
Group 2	M1	0.1031
	M2	0.1023
	M3	0.1112
	Mean±SD	0.106±0.0049a
Group 3	M1	0.1023
	M2	0.1102
	M3	0.1033
	Mean±SD	0.105±0.0043a
P value		0.122
Level of significance		NS

In a column figures with same letter do not differ significantly whereas figures with dissimilar letter differ significantly (as per DMRT); NS = Not significant.

Testicular weight of mice

Testicular weights mean ± SD was noted in Group1 (0.111 ±0.0089a) compared to Group 2 (0.106 ± 0.0049a), Group 3 (0.105 ±0.0043a) and Group 4 control (0.127 ±0.0189a) mice respectively and (P =0.122), were not significantly different between the animal groups (Table 2).

Testicular score count

Testicular score count was obtained from the vaccinate and control male mice (Goldner's trichome stain). An average testicular score (number of views±SD) was noted in group 1 mice (5.17±5.28) compared to group 2 (5.00±5.35), group 3 (5.00±5.52) and group 4 control (5.00±5.84) (Table 3).

Table 3
Results of testicular score count.

Scores	Control male mice			Immuno-castrated male mice								
	C-1	C-2	C-3	G-1a	G-1b	G-1c	G-2a	G-2b	G-2c	G-3a	G-3b	G-3c
10	15	15	12	2	5	5	5	0	6	2	6	2
9	6	4	7	2	5	1	3	3	1	0	4	2
8	11	12	11	10	3	11	15	11	15	12	19	10
7	17	13	11	8	15	15	12	10	11	12	6	13
6	0	4	5	28	12	5	13	14	10	15	9	17
5	0	2	4	0	13	4	2	8	5	5	6	4
4	1	0	0	0	2	9	0	4	2	4	0	2
3	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0	0	0	0	0
Mean±SD	5.00 ± 6.05	5.00 ± 6.00	5.00 ± 5.01	5.00 ± 8.86	5.50 ± 5.76	5.00 ± 5.21	5.00 ± 6.02	5.00 ± 5.33	5.00 ± 5.40	5.00 ± 5.85	5.00 ± 5.93	5.00 ± 6.11
Average score	5.00±5.84			5.17±5.28			5.00±5.35			5.00±5.52		
Level of sig	NS			NS			NS			NS		

NS = not significant (P<0.05)
Randomly selected 50 tubular cross sections per animal, stained with Goldner's trichome were studied under 40 x magnifications. All tubular sections in one field of vision were evaluated, each tubule was given a score of 1-10 (Table 2) and results were plotted as a mean of similar score ± SD for each group of animals.

Effect of immunization on epididymis weight of mice

Epididymis weight mean ± SD was noted in Group 1 (0.034 ±0.0062ab) compared to Group 2 (0.027 ± 0.0056b), Group 3 (0.021 ±0.0011c) and Group 4 control (0.035 ±0.0066a) mice respectively. There was significant difference of epididymis weight between untreated control (Group 4) and actively immunized mice during the study period (Table 4).

Table 4
Effects of immunization on epididymis weight of mice.

Group of mice	Number of mice	Epididymis wt
Control	M1	0.0374
	M2	0.0274
	M3	0.0399
	Mean±SD	0.035±0.0066a
Group 1	M1	0.0274
	M2	0.0398
	M3	0.0333
	Mean±SD	0.034±0.0062ab
Group 2	M1	0.0264
	M2	0.0333
	M3	0.0222
	Mean±SD	0.027±0.0056b
Group 3	M1	0.0200
	M2	0.0221
	M3	0.0212
	Mean±SD	0.021±0.0011c
P value		0.047
Level of significance		*

In a column figures with same letter do not differ significantly whereas figures with dissimilar letter differ significantly (as per DMRT); * = Significant at 5% level of probability

Seminal vesicle and prostatic weight of mice

Seminal vesicle and prostatic weight mean ± SD was noted in Group 1 (0.350 ±0.0087b) compared to Group 2 (0.342 ± 0.0015b), Group 3 (0.342 ±0.0015b) and Group 4 control (0.370 ±0.0106a) mice respectively and (P= 0.003), were significantly (at 1% level) different between the animal groups (Table 5).

Table 5
Effects of immunization on seminal vesicle and prostatic weight of mice.

Group of mice	Number of mice	Seminal vesicle + prostate
Control	M1	0.37
	M2	0.36
	M3	0.3811
	Mean±SD	0.370±0.0106a
Group 1	M1	0.3568
	M2	0.3525
	M3	0.34
	Mean±SD	0.350±0.0087b
Group 2	M1	0.3441
	M2	0.3421
	M3	0.3411
	Mean±SD	0.342±0.0015b
Group 3	M1	0.3441
	M2	0.3421
	M3	0.3411
	Mean±SD	0.342±0.0015b
P value		0.003
Level of significance		**

In a column figures with same letter do not differ significantly whereas figures with dissimilar letter differ significantly (as per DMRT); ** = Significant at 1% level of probability. There was significant difference of seminal vesicle and prostatic weight between untreated control and actively immunized mice during the study period (Table 5).

Reproductive organs

Reproductive organs obtained at autopsy were processed for Goldner's trichome staining to evaluate the effect of active immunization against GnRH-1. The testes of the treated animals showed arrested spermatogenesis, depletion of Leydig cells and testicular macrophages. Marked atrophy of the testes leading to testicular failure was characterized by Hypospermia of the seminiferous tubules. The spermatogenic cells of the tubules appeared to become degenerated in compared to nonvaccinated mice testes (Plate 1-a, b).

The head of the treated epididymis was not completely azospermic, there was a reduction in the numbers of spermatozoa occupying the lumen. The concentration of spermatozoa was almost

negligible in thevaccinated tubules of epididymis compared to control mice stained with Goldner's trichome stain (Plate 2 a, b)

The prostate glands were markedly atrophied and to be prepubartal in nature in the immunised animals. The tall columnar folded epithelium of the prostate gland were reduced in height owing to a collapse of the supra nuclear area and loses of folding. The highly alkaline secretory products of untreated prostate, positive to acid eosin; appeared bright red when stained with Goldner's trichome, was diminished or ceased completely in the treated prostate. Epithelial atrophy was always accompanied by the proliferation of the connective tissues around the shrunken tubules, which stained deep green (positive to light green). Marked stromal thickening with collagen fibres without any reactive cells was seen around the shrunken alveoli of both prostate (Plate 3 a, b).

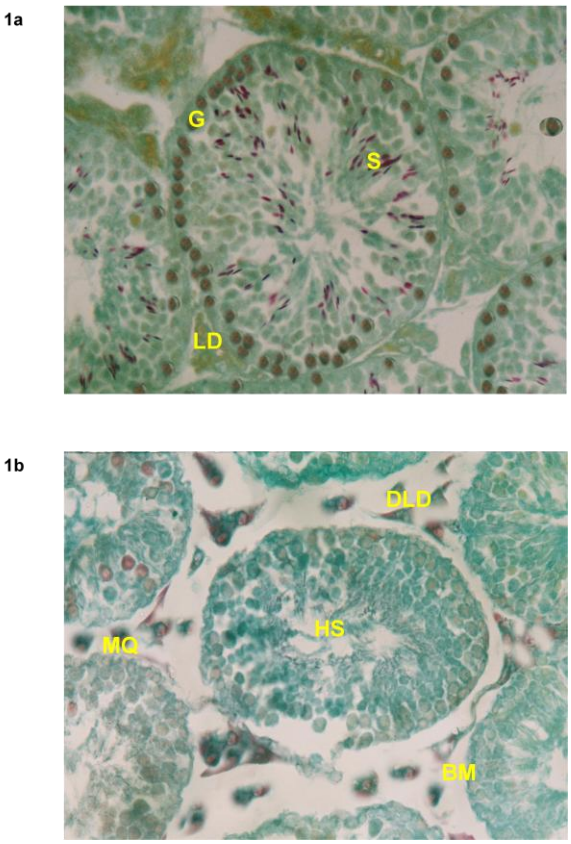


Plate 1 (a, b)
Comparative sections through the mouse testes following Goldner's trichome staining (40x magnification) (a) untreated control, (b) animal immunised with GnRH-1 encoding T-Helper epitopes. Note the presence of spermatazoa (S), germ cells (G) and Leydig cells (LD) in the control section, compared with hypospermia (HS), degenerated Leydig cells (DLD), and tubular infiltration of mononuclear phagocytes (MQ) following treatment. Magnification bar = 10µm.

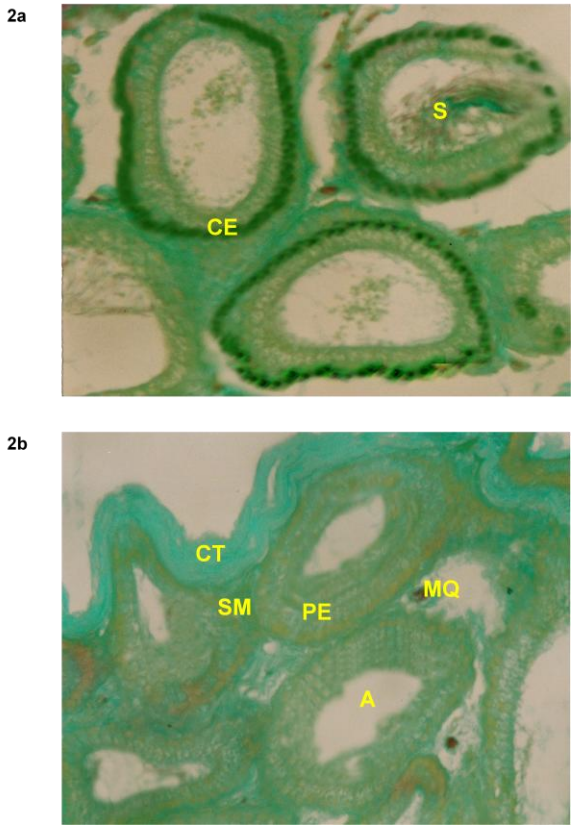
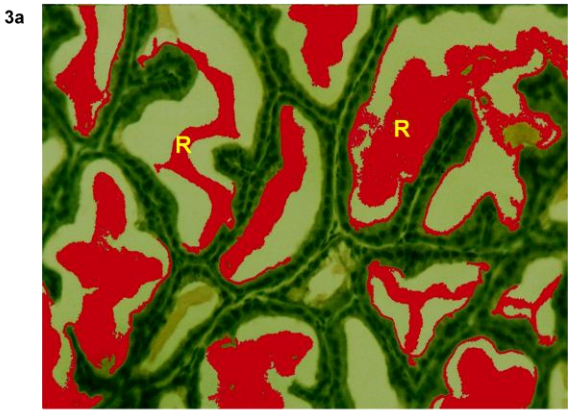


Plate 2 (a, b)
Comparative sections through the mouse epididymis head following Goldner's trichome staining (40x magnification) (a) untreated control, (b) animal immunised with GnRH-1 encoding T-Helper epitopes. Note the presence of spermatazoa (S) and lining columnar epithelial cells (CE) in the control section, compared with azospermia (A), pseudostratificatoin of the epithelial lining (PE), marked thickerning of the smooth muscle lining (SM) and infilitration of loose connective tissue (CT) and mononuclear phagocytes (MQ) following treatment. Magnification bar = 10µm.



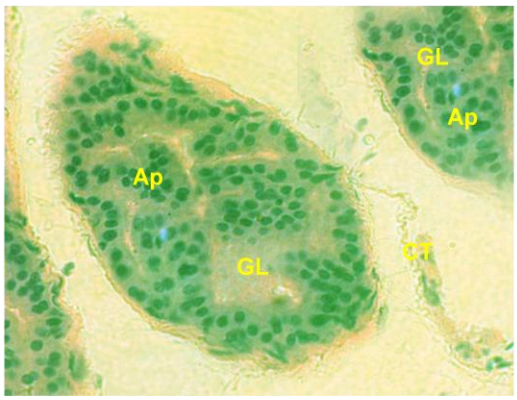
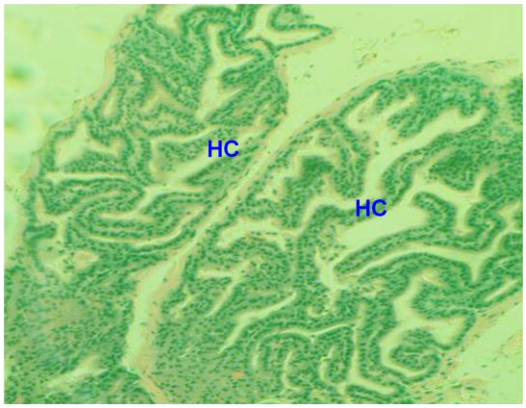


Plate 3 (a, b)
Comparative sections through the mouse prostate gland following Goldner's trichome staining (40x magnification) (a) untreated control, (b) animal immunised with GnRH-1 encoding T-Helper epitopes. Note the presence of brick red colour glandular secretions (R) in the untreated control section and apical cell atrophy (Ap), collapsed glandular lumen (GL), thickened alveolar smooth muscle layer (SM) and varying degrees of loose connective tissue (CT).

Marked stromal thickening with collagen fibres without any reactive cells was seen around the shrunken alveoli of seminal vesicles. Although there was epithelial atrophy in the treated seminal vesicles, the nuclei of the atrophied cells remain high. The lamina propria of the atrophied tubules became thicker with collagenous connective tissues. The honey comb appearance of the tubules as seen in the untreated seminal vesicle disappeared completely with very narrow glandular lumen. The tubular lumen remained empty in most of the tubules found from staining with Goldner's trichome (Plate 4 a, b).



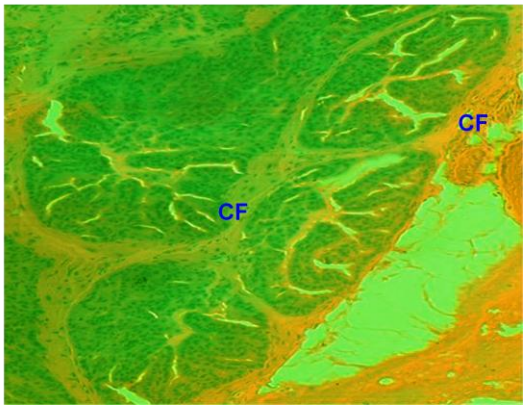


Plate 4 (a, b)
Comparative sections through the rat seminal vesicle following Goldner's trichome staining (x40 magnification) (a) untreated control (b) Immunised with GnRH-1 encoding T-Helper epitopes. Note the honeycomb pattern of the glandular alveoli (HC) in the control section, which however disappeared following treatment. Marked stromal thickening with collagen fibres (CF) without any reactive cell was seen around the shrunken alveoli. Magnification bar = 10µm.

Detection of GnRH transcripts by RT-PCR

To detect the GnRH transcripts in the testis of male mice, RNA were extracted from the testis of mice and a PCR was conducted using forward and reverse primer. Marker lane contains a 100-bp DNA ladder. A product of expected 198bp was successfully amplified. Total RNA was amplified by 60 cycles of PCR using specific GnRH-1 primers. Transcripts of 198bp, which was the size predicted by the GnRH-1 primers, was found in the testis of vaccinated and control male mice. In all a single 198 bp band, corresponding to the size

predicted by the GnRH-1 primers was visualized by ethidium bromide staining. The GnRH-1 mRNA transcript was detected only in the testis all vaccinated and non vaccinated control mice.

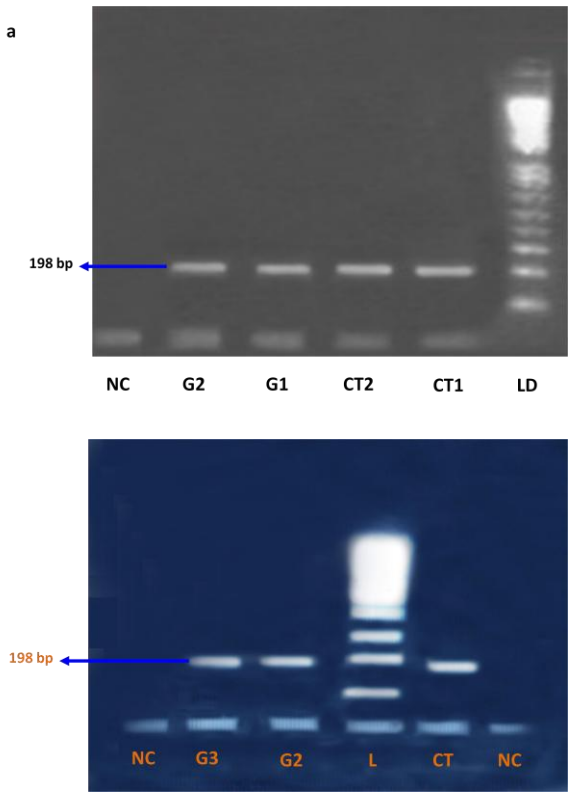


Plate 5 (a, b)
Agarose gel electrophoresis of PCR products using a forward and reverse primer with the RNA obtained from testes of mice. Lane L=Ladder: 100 bp RT, PCR product size marker. 198 bp, NC=Negative control, CT=Control testes, G1=Group 1 testes, G2=Group 2 testes and G3=Group 3 testes.

A positive correlation between suppressed *in vivo* fertility and reduced sperm concentrations in uteri has been reported in mice and pig (Ruiz, et al. 2006; Sharma and Jacob, 2002). Reduced concentration of epididymal sperm at the time point of mating could be an inexpensive indicator of suppressing IVF in mice. This finding indicated that an azospermic state in the vaccinated mice testes/epididymis is not necessarily essential to render them infertile. Practical techniques for assessing semen quality and quantity in order to predict male fertility following GnRH-I immunoneutralisation are still needed.

The findings of the present study testicular weights mean ± SD was noted in Group-1 (0.111 ±0.0089a) compared to Group-2 (0.106 ± 0.0049a), Group-3 (0.105 ±0.0043a) and Group-4 control (0.127 ±0.0189a) mice respectively and (P =0.122), were not significantly different between the animal groups. Epididymis weight mean ± SD was noted in Group 1 (0.034 ±0.0062ab) compared to Group 2 (0.027 ± 0.0056b), Group 3 (0.021 ±0.0011c) and Group 4 control (0.035 ±0.0066a) mice respectively and (P=0.047), were significantly (at 5% level) different between the animal groups. Seminal vesicle and prostatic weight mean ± SD was noted in Group 1 (0.350 ±0.0087b) compared to Group 2 (0.342 ± 0.0015b), Group 3 (0.342 ±0.0015b) and Group 4 control (0.370 ±0.0106a) mice respectively and (P= 0.003), were significantly (at 1% level) different between the animal groups.

Similar finding average mean testicular weights (0.109 ± 0.01, 103 ± 0.01, 0.127 ± 0.01 and 0.119 ± 0.2) of the untreated control, GnRH-I, GnRH-II and GnRH-III immunised mice respectively were not significantly different between the animal groups and accessory sex gland (p= 0.02), average mean, combined prostate and seminal vesicle) weight, compared to untreated control animals (Khan et al., 2008, Khan et al., 2007; Ferro et al., 2004).

Randomly selected 50 tubular cross sections per animal, stained with Goldner's trichome were studied under 40 x magnifications. All tubular sections in one field of vision were evaluated, each tubule was given a score of 1-10 and results were plotted as a mean of similar score ± SD for each group of animals. Average testicular score was noted in group 1 mice (5.17±5.28) compared to group 2 (5.00±5.35), group 3 (5.00±5.52) and group 4 control (5.00±5.84) significantly ($p<0.05$) not differ among each groups.

Since the Johnsens score (Johnsen, 1971) was developed to quantify human spermatogenesis, a modification was introduced to account for the difference in seminiferous tubular architecture

between humans and dogs. In other animals, this feature is not consistent and normally only contain one epithelial stage and the presence of only a few spermatozoa (score-8) or spermatids (score-7) in the tubular cross sections would only be the criteria to score normal tubules (Peters et al., 2000) in dogs. According to the criteria used for assessment of dog testes by Peters et al. (2000), a cross section of testes was given a score of 10 if enough normal, round spermatids and many spermatozoa are present. All other stages were classified according to the regular Johnsens score, as modified by Peters et al., 2000. Subsequently, the average mean score per animal was calculated.
Morphological evaluation of epididymal sperm was also studied to determine suppressed fertility. This study failed to show significant defects in sperm morphology in the vaccinated mice. Two males (one each in groups 1 and 2) appeared infertile in study weeks 12 and 15. These mice showed the highest level of anti-GnRH-I antibody, the lowest epididymal sperm counts. Histological examination of the testes in these mice revealed extremely disorganized spermatogenesis, as indicated by lower sperm cell densities in the tubules and a shift of the testicular score count from 10 to 9.

Similar finding by the Johnsens and Peters method of quantifying the level of spermatogenesis, which gives a score 1-10 according to the spermatogenic cells type in the tubules, was used to assess the quality of treated seminiferous tubules. An average testicular score 8.25 ± 1.35, 7.11 ± 1.54 and 6.56 ± 1.31 were recorded for control and immunised mice sacrificed 2 and 4 weeks after the final boosting respectively. The head and tailpieces of the treated epididymis was not completely azospermic, but there was a reduction in the numbers of spermatozoa occupying the lumen (Khan et al., 2002; 2004; 2007).

In this study, staining with Goldner's trichome, especially designed for staining testes and the excretory ducts revealed that the testes and its entire excretory ducts were azospermic, although scanty lightly stained degenerated tissue debris were seen in the excretory ducts.

Both the prostate and the seminal vesicles were markedly atrophied and to be prepubartal in nature in the immunized animals. The tall columnar folded epithelium of the prostate gland were reduced in height owing to a collapse of the supra nuclear area and loses of folding. The highly alkaline secretory products of untreated prostate, positive to acid eosin; appeared bright red when stained with Goldner's trichome, was diminished or ceased completely in the treated prostate. Epithelial atrophy was always accompanied by the proliferation of the connective tissues around the shrunken tubules, which stained deep green (positive to light green, when stained with Goldner's trichome). Marked stromal thickening with collagen fibres without any reactive cells was seen around the shrunken alveoli of both prostate and seminal vesicles. Although there was epithelial atrophy in the treated seminal vesicles, the nuclei of the atrophied cells remain high. The lamina propria of the atrophied tubules became thicker with collagenous connective tissues. The honey comb appearance of the tubules as seen in the untreated seminal vesicle disappeared completely with very narrow glandular lumen. The tubular lumen remained empty in most of the tubules. Almost similer finding have been found from staining with Goldner's trichome (Ferro et al., 2004; Khan et al., 2002).

CONCLUSION

This is the first DNA vaccine research in Bangladesh. Success with the research would enable us to design future vaccine against HIV, PPR in Goat, Anthrax, and TB in man and animal etc. This data assuming to generate important one that can be used by other molecular researcher in this country and abroad. This study provides fairly good immunization results in flabby testes, which is an indication of GnRH-1 immunoneutralization and arrest of spermatogenesis.

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