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Gene Expression of GX-12, a New Naked DNA Vaccine for HIV Infection, in Reproductive Organs

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ABSTRACT. GX-12 is a naked DNA vaccine developed by the DongA Pharmaceutical Co. Ltd. and Genexine for the treatment of HIV infection. This study was performed to evaluate the biodistribution and expression of GX-12 mRNA in gonadal tissues, and to investigate the histopathological changes after the repeated intramuscular injection. GX-12 (400 μg/head) was injected into the left anterior tibialis once a week for four weeks. On day 1, 5, 15, 30 and 45 after the final administration, gonadal tissues (testes, epididymis, seminal vesicles, penis, prostate glands, ovaries, vagina, uterus) and the injection site (muscle) were harvested and examined for the expression of mRNA by RT-PCR. In addition, histopathological examination was performed at each time point. At the injection site, mRNA expression of GX-12 was detected only at early time points (1~15 days after injection) but not thereafter. However, in gonadal tissues, mRNA expression was not identified at all time points both in male and female rats. There were no histopathological changes in all reproductive organs and muscle. Based on these results, it is unlikely that the plasmid DNAs of GX-12 was distributed to- and expressed in gonadal tissues, suggesting that the chance of germline integration and transmission is negligible.

Keywords: GX-12, HIV, DNA vaccine, Expression, Reproductive organ.

INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) induced by human immunodeficiency virus (HIV) is one of the most fatal infectious diseases. HIV can be transmitted by blood and blood products, vertically (from mother to child), or sexual activity. Despite public health efforts to prevent the spread of the AIDS the number of new infections is continuing to rise to alarming proportions. The UNAIDS and WHO estimated the global epidemic at 36.1 million infected individuals at the end of the year 2000, 50% higher than originally predicted on the data available a decade ago (Mooij and Heeney, 2002). Although tremendous progress has been made in treating HIV infection in industrialized countries, no current treatment is wholly satisfactory. Furthermore, HIV continues to spread unabated in regions where access to current drugs is limited by insufficient financial resources. Therefore there is an urgent need for the development of a vaccine for HIV which is safe, effective and economically feasible.

In the past 10 years there has been a diversity of approaches to vaccine design such as recombinant proteins, synthetic peptides, and whole-killed and live-attenuated HIV. A relatively recent approach is DNA vaccination, a method of immunization using the genes for viral antigens rather than the antigens themselves. Immunization with naked or formulated DNA plasmids, by intramuscular injection or intradermal gene-gun delivery, elicits both humoral and cellular responses. Several clinical trials to test the potency of candidate DNA vaccines are now in progress. An initial phase I clinical trial of a therapeutic DNA vaccine encoding for Env and/or Rev proteins in asymptomatic HIV-infected patients, has proven the safety and potential immunogenicity of a HIV-directed DNA-based vaccine. Additional phase I clinical trials of several DNA candidate vaccines encoding for Env and Gag/Pol proteins of HIV are conducted, and more trials are being planned (Bojak *et al.*, 2002).

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GX-12 is a naked DNA vaccine developed by DongA Pharmaceutical Company and Genexine for the treatment of HIV infection. GX-12 consists of four separate plasmids: pGX10-GE HX, pGX10-dpol JR, pGX10-VN/TV JR and pGX10-hIL-12m which encode the HIV gag and env genes, pol, several regulatory genes (Vif-Nef-Tat-Vpu) and a human IL-12 mutant gene. This study was performed to evaluate the distribution of GX-12 in reproductive organs and to investigate the histopathological changes in rats after intramuscular injection to assess the reproductive toxicity.

MATERIALS AND METHODS

Test Materials

The DNA vaccine, GX-12, was supplied as a sterile solution for intramuscular administration (Lot No. GX01-1101). It consists of four separate plasmids (pGX10-GE HX, pGX10-dpol JR, pGX10-VN/TV JR, pGX10-hIL-12m) which were constructed by inserting the HIV-1 gag-env, pol, regulatory genes (Vif-Nef-Tat-Vpu) and a human IL-12 mutant gene into pGX10 plasmid vectors. Master cell banks (MCB) of recombinant *E. coli* for each plasmid were established by transforming *E. coli* DH5α with the respective plasmids via $CaCl_2$ treatment (Sambrook *et al.*, 1989), and working cell banks (WCB) were subsequently produced from each MCB. For production of each plasmid, one vial was taken from the WCB to create a seed culture which was used as the inoculum for a 15 l fermentation. The cells were harvested from the fermentation broth by centrifugation and subjected to alkaline lysis. The plasmid DNA was purified from the lysate by PEG precipitation, ion exchange chromatography and gel filtration chromatography. The four plasmids produced by this method were mixed to equal ratios and dialyzed against sodium phosphate buffer. The total DNA concentration was adjusted to 4 mg/2 ml to give the final GX-12 formulation. Each 2 ml dose contains the following active ingredients; pGX10-GE HX 1 mg, pGX10-dpol JR 1 mg, pGX10-VN/TV JR 1 mg, pGX10-hIL-12m 1 mg.

Animals and Experimental Groups

Five-week old SPF rats (Sprague-Dawley) were purchased from Charles River Laboratories (USA). The animals were quarantined and acclimatized for 7 days. After acclimation period, the animals were selected and grouped by a random sampling method based on the body weight (Table 1). The ear tagging method was used to distinguish the animals within the cage. The experiment was conducted in the room number MR-VIII in Animal Care Area of DongA Pharmaceuticals Research Center. Throughout the experiment, the environment was maintained under the conditions of a 12-hour light/dark cycle (lighting: 07:00 to 19:00), the temperature range of 23±2°C, the relative humidity of 60±10%, a ventilation frequency of 15~20 times per hour and a light intensity of 150~600 Lux.

Table 1. Experimental groups

Groups	Dose (μg/kg)	No. of animals	Day of necropsy
T1	Sodium phosphate buffer	Male : 45 Female : 45	Day 1~45
T2	GX-12 400 μg/head	Male : 5 Female : 5	Day 1
T3		Male : 5 Female : 5	Day 5
T4		Male : 5 Female : 5	Day 15
T5		Male : 5 Female : 5	Day 30
T6		Male : 5 Female : 5	Day 45

Administration of Test Substance

Doses of 400 μg/head (approximately 2 mg/kg, 30 times of the anticipated clinical dose) of GX-12 were injected into the left anterior tibialis of male and female rats. The administration was performed weekly for four weeks, and the injection volume was adjusted to approximately 0.1 ml/head. In the control group, sodium phosphate buffer (pH 7.0), a vehicle of the test substance, was administered in the same way.

Tissue Sampling

To assess the tissue distribution of the plasmids, rats were sacrificed at various time points after the final administration; on day 1, day 5, day 15, day 30 and day 45. Testes, epididymis, seminal vesicles, penis, prostate glands and muscle (injection site) of the male rats, and ovaries, vagina, uterus and muscle (injection site) of the female rats were harvested. To minimize the influence of plasmid DNA in blood circulating through the tissues at the time of sampling, the samples were thoroughly washed several times with saline and blotted dry. The samples were then placed in cryotubes separately, frozen in liquid nitrogen, and stored at –70°C until usage.

Preparation of mRNA from Tissue Samples

mRNA was isolated from various tissues of the GX-12-treated and control rats using the SV Total RNA Isolation System (Promega, USA). 200~300 mg of each

Table 2. Number of animals expressing GX-12 mRNA in reproductive organs or muscle

		Control group					GX-12 treated group				
		Day 1	Day 5	Day 15	Day 30	Day 45	Day 1	Day 5	Day 15	Day 30	Day 45
Male	Muscle	0/5	0/5	0/5	0/5	0/5	4/5	3/5	3/5	0/5	0/5
	Testes	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	Epididymis	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	Seminal vesicle	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	Prostate glands	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	Penis	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Female	Muscle	0/5	0/5	0/5	0/5	0/5	3/5	3/5	2/5	0/5	0/5
	Ovary	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	Vagina	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	Uterus	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

Each value represents the No. of animals expressing GX-12 mRNA/No. of total animals.

tissue was excised and homogenized in SV RNA Lysis buffer (4 M Guanidine Thiocyanate, 0.01 M Tris, pH 7.5, 0.97% β-mercaptoethanol) using a Dounce homogenizer (Glas-Col., USA). 175 μl of the tissue lysate was added to 350 μl of SV dilution buffer in a 1.5 ml microcentrifuge tube and mixed by inverting 3~4 times. The mixture was incubated in a water bath at 70°C for 3 minutes and then centrifuged for 10 minutes at 12,000~14,000 g. The cleared lysate was transferred to a fresh microcentrifuge tube, and 200 μl of 95% ethanol was added and mixed by pipeting. The mixture was transferred to the spin column assembly and centrifuged for one minute at 12,000~14,000 g. After discarding the filtrate from the collection tube, 50 μl of DNase incubation mix (40 μl Yellow core buffer (0.0225 M Tris, pH 7.5, 1.125 M NaCl, 0.0025% yellow dye), 5 μl of 0.09 M $MnCl_2$, 5 μl of DNase I)) was added to the membrane of the spin basket and allowed to react for 15 minutes at room temperature. The reaction was stopped by the adding of 200 μl of SV DNase stop solution (2 M Guanidine isothiocyanate, 4 mM Tris-HCl, pH 7.5, 57% ethanol) and centrifuging at 12,000~14,000 g for one minute. The membrane was washed by adding 600 μl of SV RNA washing solution and eluted by centrifugation. The membrane was washed again with 250 μl of SV RNA wash solution. The spin basket was transferred from the collection tube to an elution tube. 100 μl of nuclease-free water was added to the membrane, and the RNA bound to the membrane was eluted by centrifugation at 12,000~14,000 g.

Amplification of Plasmid Sequences in the mRNA by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Gag-Env, pol, Vif-Nef-Tat-Vpu and hIL-12m encoding sequences in the purified mRNA were amplified by RT-PCR using the Access RT-PCR system (Promega, USA). The sequences of the primer sets were 5'-AATCCTGGCCTGTTAGAAAC-3' and 5'-TATGTCACTTCCCCTTGGTT-3' for Gag-Env, 5'-TGGCCATTGACAGAAGAAAA-3' and 5'-TCAGGATGGAGTTCATAACC-3' for Pol, 5'-GGGGATGCTAGATTGGTAAT-3' and 5'-TCAG-

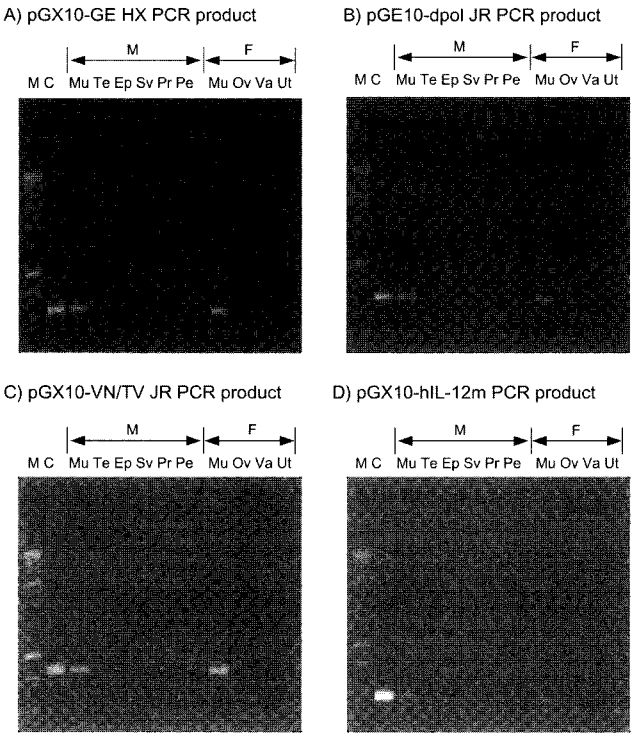

Fig. 1. Results of RT-PCR for the detection of GX-12 mRNA transcription in muscle and reproductive organs on day 1. Lane M, Size marker (1 kb DNA ladder, Promega); Lane C, pGX10-GE HX PCR product (A), pGE10-dpol JR PCR product (B), pGX10-VN/TV JR PCR product (C), pGX10-hIL-12m PCR product (D); Lane Mu, Muscle mRNA; Te, Testes mRNA; Ep, Epididymis mRNA; Sv, Seminal vesicle mRNA; Pr, Prostate glands mRNA; Pe, Penis mRNA; Ov, Ovary mRNA; Va, Vagina mRNA; Ut, Uterus mRNA.

TGGAAATCTGACCCCT-3' for Vif-Nef-Tat-Vpu, and 5'-GGCCATATGGGAACTGAAGA-3' and 5'-GAAGAAGC-TGCTGGTGTAGA-3' for hIL-12m. Each reaction tube contained 50 μl of reaction mixture consisting of 250 μM dNTP, 25 pM each of forward and reverse primer, 5 units of AMV reverse transcriptase, 5 units of T*fl* DNA polymerase, 1 mM $MgSO_4$, AMV/T*fl* reaction buffer and 1 μg of the purified template mRNA. The reverse transcriptase was allowed to react for 1 hour at 48°C, and PCR was subsequently carried out in a RTC-100 thermocycler (MJ Research Co., USA) at the following conditions: denaturation at 94°C for 30 seconds; annealing at 51°C for 1 minute; and extension at 68°C for 2 minutes. After 40 cycles of reaction, the PCR product was analyzed using 1% agarose gel electrophoresis and ethidium bromide staining. The appearance of 570 bp band (Gag-Env), 641 bp band (Pol), 821 bp band (Vif-Nef-Tat-Vpu), and 503 bp band (hIL-12m) was considered as an indication of mRNA expression of the each plasmid sequences.

Histopathology

To investigate the toxic effects of GX-12 on organs, all reproductive organs and muscle of male and female rats were examined histologically. Tissues were fixed in 10% neutral formalin more than a week. The tissues were routinely processed, embedded in paraffin and sectioned at 5 μm. After then, the sections were stained with hematoxylin-eosin for microscopic examination.

RESULTS

Expression of GX-12 mRNA in Reproductive Organs and Injection Site

The mRNA expression of plasmid DNA (GX-12) following intramuscular injection was examined at various time points. As shown in Table 2 and figure 1~5, at early time points (1~15 days) after intramuscular injection mRNA expression was detected at the injection sites (muscle) in some animals (4/5 in male and 3/5 in

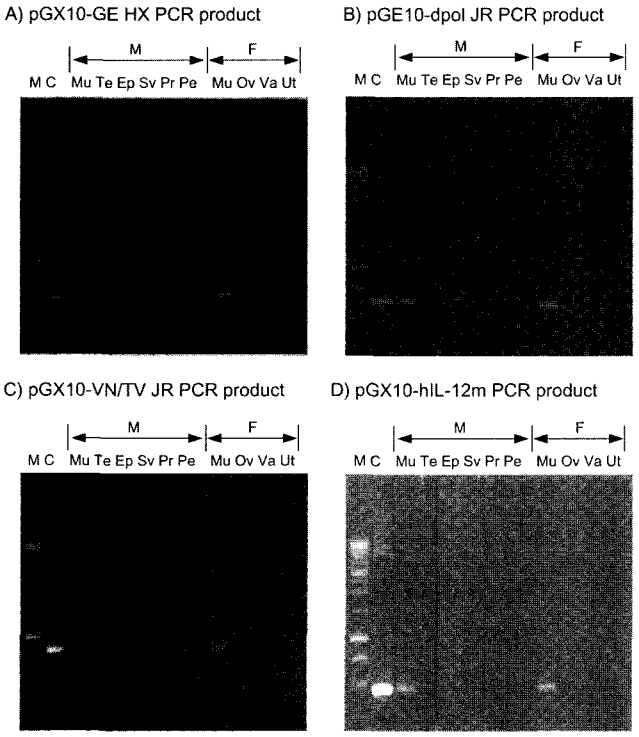

Fig. 2. Results of RT-PCR for the detection of GX-12 mRNA transcription in muscle and reproductive organs on day 5. Lane M, Size marker (1 kb DNA ladder, Promega); Lane C, pGX10-GE HX PCR product (A), pGE10-dpol JR PCR product (B), pGX10-VN/TV JR PCR product (C), pGX10-hIL-12m PCR product (D); Lane Mu, Muscle mRNA; Te, Testes mRNA; Ep, Epididymis mRNA; Sv, Seminal vesicle mRNA; Pr, Prostate glands mRNA; Pe, Penis mRNA; Ov, Ovary mRNA; Va, Vagina mRNA; Ut, Uterus mRNA.

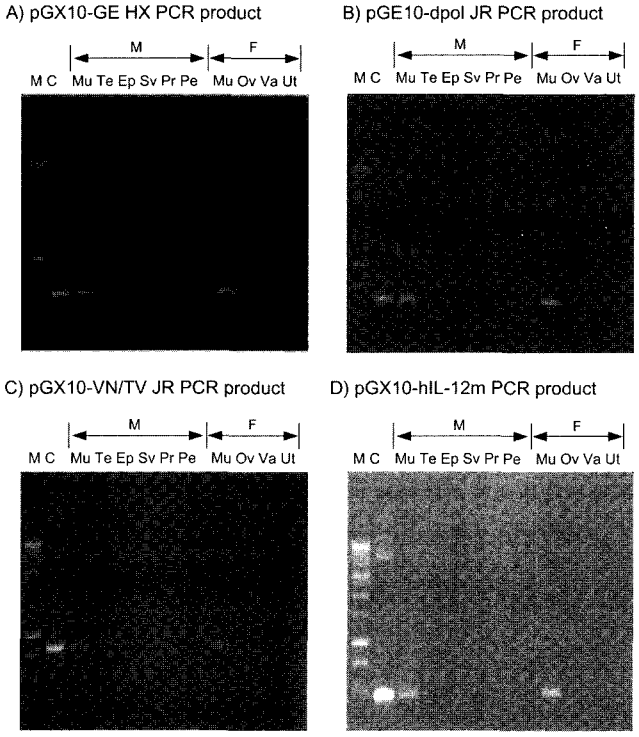

Fig. 3. Results of RT-PCR for the detection of GX-12 mRNA transcription in muscle and reproductive organs on day 15. Lane M, Size marker (1 kb DNA ladder, Promega); Lane C, pGX10-GE HX PCR product (A), pGE10-dpol JR PCR product (B), pGX10-VN/TV JR PCR product (C), pGX10-hIL-12m PCR product (D); Lane Mu, Muscle mRNA; Te, Testes mRNA; Ep, Epididymis mRNA; Sv, Seminal vesicle mRNA; Pr, Prostate glands mRNA; Pe, Penis mRNA; Ov, Ovary mRNA; Va, Vagina mRNA; Ut, Uterus mRNA.

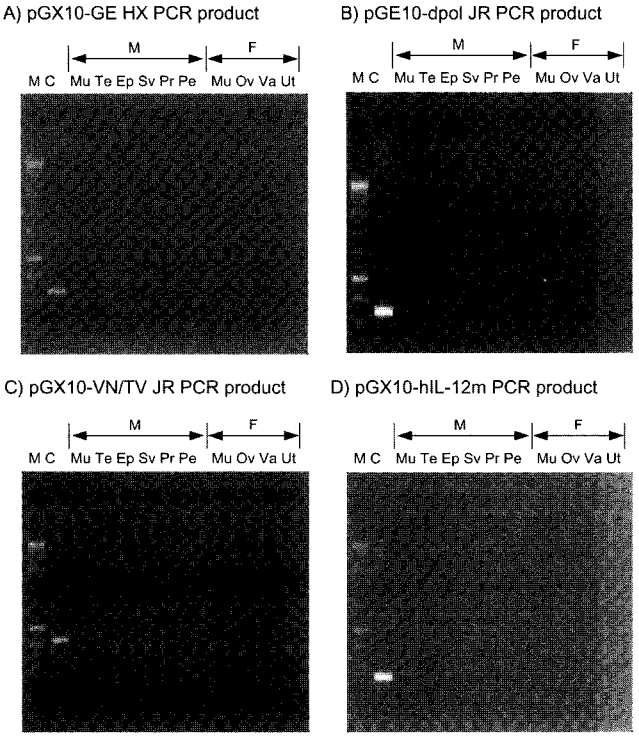

Fig. 4. Results of RT-PCR for the detection of GX-12 mRNA transcription in muscle and reproductive organs on day 30. Lane M, Size marker (1 kb DNA ladder, Promega); Lane C, pGX10-GE HX PCR product (A), pGE10-dpol JR PCR product (B), pGX10-VN/TV JR PCR product (C), pGX10-hIL-12m PCR product (D); Lane Mu, Muscle mRNA; Te, Testes mRNA; Ep, Epididymis mRNA; Sv, Seminal vesicle mRNA; Pr, Prostate glands mRNA; Pe, Penis mRNA; Ov, Ovary mRNA; Va, Vagina mRNA; Ut, Uterus mRNA.

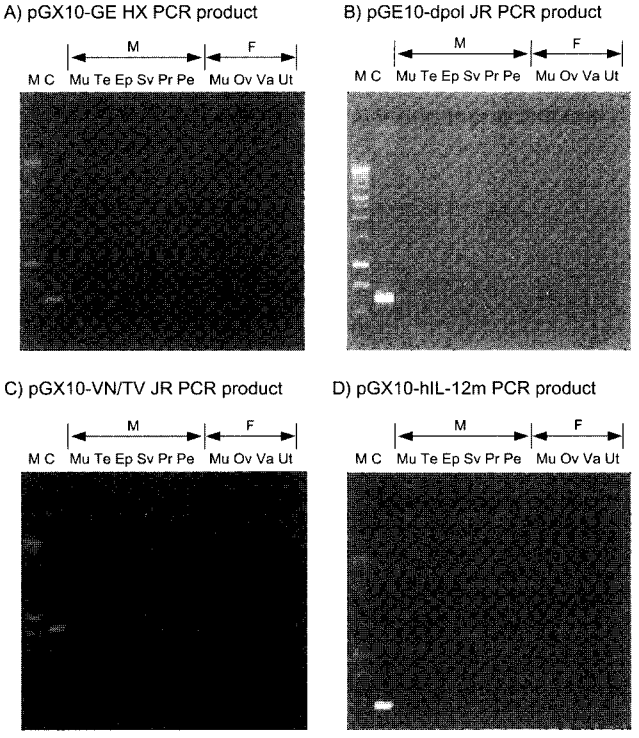

Fig. 5. Results of RT-PCR for the detection of GX-12 mRNA transcription in muscle and reproductive organs on day 5. Lane M, Size marker (1 kb DNA ladder, Promega); Lane C, pGX10-GE HX PCR product (A), pGE10-dpol JR PCR product (B), pGX10-VN/TV JR PCR product (C), pGX10-hIL-12m PCR product (D); Lane Mu, Muscle mRNA; Te, Testes mRNA; Ep, Epididymis mRNA; Sv, Seminal vesicle mRNA; Pr, Prostate glands mRNA; Pe, Penis mRNA; Ov, Ovary mRNA; Va, Vagina mRNA; Ut, Uterus mRNA.

Table 3. Histopathological findings in muscle and reproductive organs in rats

	Male									
	Control group					GX-12 treated group				
	Day 1	Day 5	Day 15	Day 30	Day 45	Day 1	Day 5	Day 15	Day 30	Day 45
Muscle	N	N	N	N	N	N	N	N	N	N
Testes	N	N	N	N	N	N	N	N	N	N
Epididymis	N	N	N	N	N	N	N	N	N	N
Seminal vesicle	N	N	N	N	N	N	N	N	N	N
Prostate glands	N	N	N	N	N	N	N	N	N	N
Penis	N	N	N	N	N	N	N	N	N	N
	Female									
	Control group					GX-12 treated group				
	Day 1	Day 5	Day 15	Day 30	Day 45	Day 1	Day 5	Day 15	Day 30	Day 45
Muscle	N	N	N	N	N	N	N	N	N	N
Ovaries	N	N	N	N	N	N	N	N	N	N
Vagina	N	N	N	N	N	N	N	N	N	N
Uterus	N	N	N	N	N	N	N	N	N	N

N: Within normal limit.

female on day 1, 3/5 in male and 3/5 in female on day 5, and 3/5 in male and 2/5 in female on day 15). The expression ratios in male and female were not different. On 30 day and 45 days after the administration, mRNA expression was not detected at the injection site. To address the issue of germline transmission, mRNA expression of GX-12 was also examined in male and female reproductive organ samples. Testes, epididymis, penis, seminal vesicles and prostate glands of males, and ovaries, uterus and vagina of females were examined. mRNA expression was undetectable at all time points in both the male and female reproductive organs (Fig. 1~5).

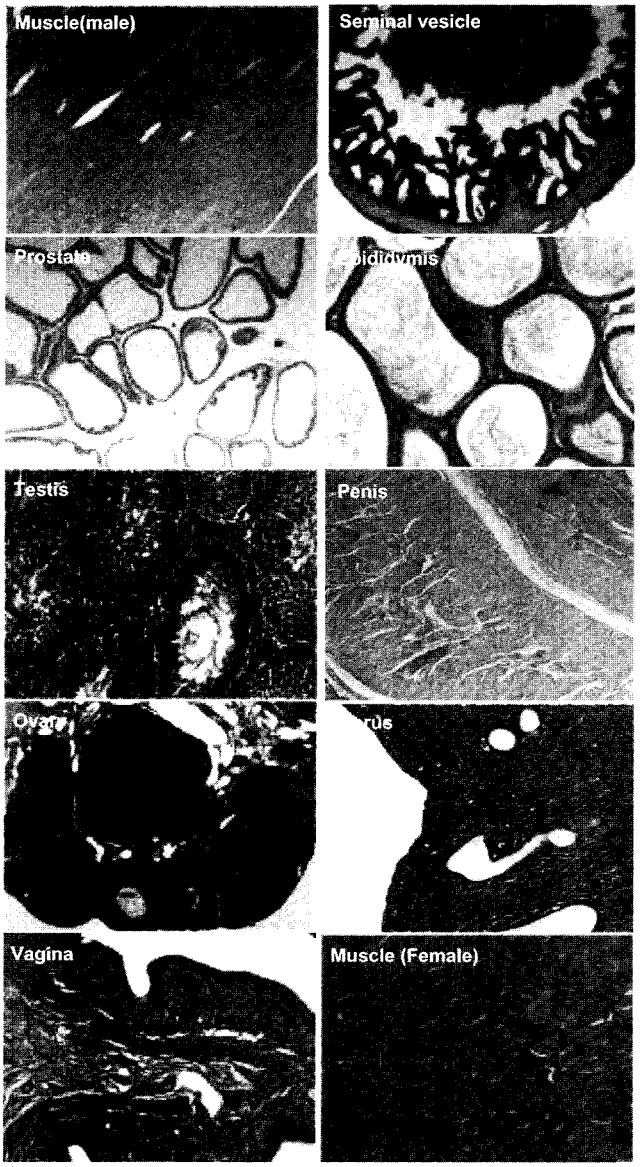

Fig. 6. Photomicrography of reproductive organs in rats after administering the GX-12.

Histopathology

In histopathological examination, there were no treatment related changes in all reproductive organs and the injection site (Table 3). The representative photomicrography is shown in Fig. 6.

DISCUSSION

In this study, we demonstrated that intramusculary injected plasmid DNA, GX-12, was expressed in the site of administration (muscle) until 15 days, and no longer detectable after 30 days post-inoculation. In addition, we demonstrated that GX-12 was not distributed to- and expressed in all gonadal tissues both in male and female rats. There were no histopathological changes related to treatment in all reproductive organs.

When considering the risk of reproductive toxicity of DNA vaccine, the tissue distribution of the plasmid should be examined, since the exposure of the gonadal tissues to the plasmid, and potential integration into germline DNA could affect the risk of germline transmission. In fact, guideline for reproductive toxicity studies for DNA vaccine was recommended by FDA and KFDA; Gene therapeutic products should be evaluated for migration to gonadal tissues and possible germline alterations in both male and female animals. This may be assessed through the performance of PCR on gonad-derived DNA preparations from male and female animals injected with a plasmid DNA. If the gene is not expressed in gonadal organs, further studies such as fertility and general reproductive study are not necessary. However, if the gene is expressed in gonadal organs, additional studies such as fertility, general reproductive study, maintenance of pregnancy and fetal developmental study were needed (FDA, 1996; KFDA, 2000).

Until now, the levels of systemically administered plasmid in the biological samples have been measured by radiolabelled plasmids. However, thanks to the RT-PCR technique, to determine the mRNA expression of administered plasmid DNA in tissues were performed simply (Ledwith *et al.*, 2000). The application of PCR can therefore be extended to include the detection and quantification of a specific mRNA in cells or tissues. We also used RT-PCR method to investigate the mRNA expression of GX-12 in injection site and gonadal tissues at each experimental time point.

In the injection site, mRNA expression of GX-12 was detected only at early time points (1~15 days after injection) but not thereafter. However, in reproductive organs, mRNA expression was undetectable at all time points in both male and female rats. When comparing

the expression level of HIV DNA vaccine with the previously reported (Ledwith *et al.*, 2000; Manam *et al.*, 2000), we observed a slightly different expression pattern of mRNA in several tissues including gonadal organs. Ledwith et al. reported that HIV DNA vaccine, V1Jns-tPA-gag, a plasmid containing the HIV gag gene persisted in the injection site (quadriceps) at 6 weeks after single injection (Ledwith *et al.*, 2000). At the disital sites including ovaries and testes, the level of plasmid was not detected at 6 weeks postdose (Manam *et al.*, 2000). However, 2 days after intramuscular injection, a low level of plasmid was detected at ovaries. Therefore, they assayed additionally the potential of chromosomal integration against samples that were positive for the presence of plasmid, and reported negative results. In conclusion, they suggested that the plasmid was extrachromosomal occasionally, but it dissipated rapidly, and there is little risk of germline trasmisstion.

In conclusion, mRNA expression of GX-12 plasmids was detected transiently at the injection site, but not detected at gonadal tissues both in male and female rats after repeated administration. Based on these results, it is unlikely that the plasmid DNA of GX-12 was distributed to- and expressed in gonadal tissues, suggesting that the chance of germline integration and transmission is little.

REFERENCES

Bojak, A., Deml, L. and Wagner, R. (2002): The past, present and future of HIV-vaccine development: a critical view. *DDT*, **7**, 36-46.

Food and Drug Administration. (1996): Points to consider on plasmid DNA vaccines for preventive infectious disease indications. Docket No. 96N-0400.

Korea Food and Drug Administration. (2000): Regulatory Guidelines for gene therapy. Notification No. 2000-61.

Ledwith, B.J., Manamm S., Troilo, P.J., Barnum, A.B., Pauley, C.J., Griffiths, II T.G., Harper, L.B., Beare, C.M., Bagdon, W.J. and Nichols, W.W. (2000): Plasmid DNA vaccine: Investigation of integration into host cellular DNA following intramuscular injection in mice. *Intervirol.*, **43**, 258-272.

Manam, S., Ledwith, B.J., Barnum, A.B., Troilo, P.J., Pauley, C.J., Harper, L.B., Griffiths, II T.G., Niu, Z., Denisova, L., Follmer, T.T., Pacchione, S.J., Wang, Z., Beare, C.M., Bagdon, W.J. and Nichols, W.W. (2000): Plasmid DNA vaccines: Tissue distribution and effects of DNA sequence, adjuvants and delivery method on integration into host DNA. *Intervirol.*, **43**, 273-281.

Mooij, P. and Heeney, J.L. (2002): Rational development of ptophylactic HIV vaccines based on structural and regulatory proteins. *Vaccine.*, **20**, 304-321.

Sambrook, J. Frisch, E.F. and Maniatis, T. (1989): Molecular cloning. A laboratory manual 2nd ed. Cold spring harbor laboratories press, New York.