Infertility in mice induced by the rhesus monkey chorionic gonadotropin β-subunit glycoprotein (rmCGβ) using DNA immunization

Yun Chen, Zhe Liu, Ying Yang, You-Zhen Chen and Jing-Pian Peng
State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China

Abstract

The recombinant eukaryotic expression vector pCMV4-rmCGβ, inserted full-length cDNA of the β-subunit of rhesus monkey chorionic gonadotropin (rmCGβ), as DNA immuno-contraceptive against CGβ glycoprotein, has previously demonstrated the biological expression of rmCGβ *in vitro* and *in vivo*. The plasmid DNA of pCMV4-rmCGβ was inoculated into BALB/c mice at different doses and routes as DNA immuno-contraceptive to understand its antifertility effect. The results of immune responses indicated that the intradermal inoculation is the optimal pCMV4-rmCGβ DNA delivery method for BALB/c mice, and the dose of 10 μg should be enough to elicit immune response. With different doses from 10–50 μg, marked reductions in the fertility of the female mice after two intramuscular inoculations of pCMV4-rmCGβ DNA were seen, while the similar level of humoral immune responses were induced. With the dose of 20 μg of pCMV4-rmCGβ DNA, the mice showed reduction in fertility from intraperitoneal, and intradermal to intramuscular inoculating method. The antifertility effect of antiserum from immunized mice confirmed that the antibodies elicited by pCMV4-rmCGβ DNA could prevent pregnancy in female mice. At the same time, the full-length cDNA of β-subunit of mouse chorionic gonadotropin (muCGβ) was cloned from placenta and sequenced for the first time (GenBank Accession No. AF333067). Sequence analysis showed that muCGβ shares 99.6% homology with rmCGβ and 90.6% with hCGβ respectively. The results indicated that the infertility of BALB/c mice induced by pCMV4-rmCGβ contraceptive should be further studied as a CGβ DNA contraceptive. (Mol Cell Biochem **231**: 89–96, 2002)

Key words: rmCG β-subunit, DNA immuno-contraceptive, BALB/c mice, infertility, muCGβ

Introduction

Human chorionic gonadotropin β-subunit (hCGβ) vaccines have demonstrated the feasibility of effectively eliciting antibodies in women and inhibiting fertility in both human and non-human primates [1–3]. Evidence is available to indicate that interference of hCG function by antibodies can disrupt pregnancy prior to implantation, and antibodies specific for hCG would not affect the function of other hormones in non-pregnant women [4]. Because of the physiological and temporal specificity, the hCGβ as antigen to develop immuno-contraceptives have been explored for more than 20 years, and some prototype vaccines have reached the stage

of clinical trials. Three main types of hCGβ vaccines have been subjected to clinical trials [5], including hCG β-subunit conjugated to tetanus toxoid (βhCG-TT) [6], hCGβ subunit-ovine luteinizing hormone (LH) α-subunit heterodimer conjugated to tetanus toxoid (HSD-TT-DT) [7], and hCGβ-subunit C-terminal 37 residue synthetic peptide conjugated to diphtheria toxoid (CTP-DT) [8]. The high effects in preventing pregnancy (one pregnancy in 1224 cycles of exposure to pregnancy) were observed in HSD-TT-DT vaccine phase II trial [7]. The antifertility effect of HSD-hCG vaccine is reversible and low titers of antibodies below the protective threshold have no apparent side effects on the progression of pregnancy and on the early development of

pregnancy [9]. However, none of these vaccines can establish effective immunity with a single inoculation, and none of these vaccines are suitable in their current form for manufacture and distribution for widespread application in family planning. Current vaccines require 2–4 immunizations to establish effective antibody level [5]. Learning from these previous studies, current vaccines might be lacking the antigen components to simulate crucial cellular immunity. To achieve a successful immuno-contraceptive, other methods are needed.

DNA inoculation technology has recently been demonstrated as a potent means to generate both humoral and cellular immune responses in various animals and human [10–11]. To study the feasibility of a human DNA immuno-contraceptive by using the hCGβ-subunit gene, we chose the rhesus monkey chorionic gonadotropin β-subunit (rmCGβ) to understand the immune response, antifertility effect of CGβ DNA immuno-contraceptive, because rmCGβ is biologically close to hCGβ, and the antifertility results may be better implicated for hCGβ vaccine used for human trials in the future [12]. In previous work, we cloned the rmCGβ cDNA sequence and found that rmCGβ (GenBank Accession No. AY011015) and hCGβ (GenBank Accession No. G180436) share 90.6% homology at cDNA level, and 79.5% homology at amino acid level [13]. A powerful bio-functional eukaryotic expression vector PCMV4-rmCGβ was also constructed. In this paper, we report the results of immune responses and antifertility of BALB/c mice after inoculating PCMV4-rmCGβ DNA with varied injection doses and varied methods.

Materials and methods

Construction of rmCGβ-subunit eukaryotic expression vector

The mammalian expression vector pCMV4 was chosen to drive the expression of the rmCGβ-subunit cDNA. The full-length rmCGβ-subunit cDNA was cloned from rhesus monkey placenta and inserted into PCR®2.1 clone vector (Introgen), and then was cut from PCR®2.1-rmCGβ vector by Hind III and Xba I (Promega). The rmCGβ cDNA was subcloned into the same sites of eukaryotic expression vector pCMV4. The recombinant pCMV4-rmCGβ was transformed into *E. coli* DH5a strain. The pCMV4-rmCGβ plasmid DNA was purified with Qiagen tip-10,000 Giga column kit (Qiagen) prior to inoculation. The more detail methodology was described, and the expression of rmCGβ *in vitro* cells and *in vivo* tissues was confirmed at previous work [13].

Clone of the β-subunit of muCG (muCGβ)

Touch-down reverse transcription polymerase chain reaction (TDRT-PCR) was adopted to clone the cDNA of muCGβ. The

method was same as the clone of rmCGβ [13]. In brief, total RNA was isolated from the placenta BALB/c mouse. The RT-PCR reaction was made up of 10 µL of AMV/Tfl 5 × reaction buffer, 2 mM of dNTPs mix, 2 mM of MgSO$_4$, 1 mM each of upstream and downstream primers (the upstream primer was 5′-CACGGGCCCGGGGACGCACCAAGG-ATG-3′; the downstream primer was 5′-GCGGATTCAGAA-GCCTTTATTG-3′), 0.1 U/µL of AMV reverse transcriptase, 2 µg of total RNA and nuclease-free water to a final volume of 50 µL, overlaid with 50 µL of mineral oil (all RT-PCR reagents were from Access RT-PCR kit) (Promega). The RT reaction was allowed to proceed at 48°C for 45 min and followed by incubation at 95°C for 5 min prior to the PCR amplification reaction. At the first denaturation step, 0.1 U/µl of Tfl DNA polymerase was added into the 50 µL reaction volume. The 2-cycle TD-PCR amplified program was initiated with cycle times of 1 min at 94°C, 1 min at 65°C (from 65°C to 55°C decreasing 1°C every 2 cycles), and 40 sec at 68°C, then followed by another 20-cycle amplification for 1 min at 94°C, 1 min at 60°C, and 40 sec at 68°C.

The reaction product was purified by using 1 × TAE low-melt agarose gel (Promega), and then was ligated into PCR®2.1 cloning vector (Invitrogen) with T4 DNA ligase (Promega). Fluorescent DNA sequencing reactions with M13 reverse primer and T7 promoter primer were performed to sequence the muCGβ cDNA, and the results showed that muCGβ cDNA shares 99.6% homology with rmCGβ (the details of data shown in the 'Results' section).

Mice inoculations

All BALB/c mice studies were conducted in accordance with the Chinese Academy of Sciences institutional guidelines for the care of animals. The 6–8 week-old healthy female BALB/c mice were divided into 6 groups at random, and each group was comprised of 8 mice. The BALB/c mice of Groups 1, 2 and 3 were intramuscularly (i.m.) inoculated 10, 20 and 50 µg of pCMV4-rmCGβ plasmid DNA, respectively. The BALB/c mice of Group 4 were intraperitoneally (i.p.) inoculated with 20 µg of pCMV4-rmCGβ plasmid DNA. The BALB/c mice of Group 5 were intradermally (i.d.) inoculated with 20 µg of pCMV4-rmCGβ plasmid DNA. The BALB/c mice of Group 6 were i.m. inoculated with 20 µg of pCMV4 mock plasmid DNA as control. The quadriceps muscles of each mouse were multi-spot injected with 50 µL of 0.5% (w/v) bupivacaine-HCl (Sigma) [10] 24 h before DNA inoculation. The boost immunization with same amount and injection regions for each group was performed at 4 weeks post-immunization (p.i.). The sera of mice were collected from the postorbital vein at 4, 6, 8, 10 and 12 weeks p.i., and stored at –20°C prior to detecting the CGβ-specific antibody titers of mice.

Antibody detection by ELISA

The hCGβ-subunit protein co-reaction with antibodies induced by pCMV4-rmCGβ DNA was confirmed at previous work [14], hCGβ-subunit was chosen as coated antigen. Ninety-six wells microtiter plates were coated with 50 μL of 2 μg/mL native hCGβ protein per well in bicarbonate buffer (pH 9.6) (Sigma) and incubated at 4°C overnight. Following blocking each well with 100 μL of 3% BSA-PBST at 37°C for 1 h, 50 μL of sera samples (prepared in blocking buffer as 2-fold dilution) were added. After incubation at 37°C for 2 h, 50 μL of secondary anti-mouse IgG conjugated with horseradish peroxidase (Promega) at dilution of 1:2000 was incubated at 37°C for 2 h. Ten mg of OPD (Sigma) was dissolved in 10 mL of 0.025 M phosphate-citrate buffer (Sigma) and added to each well for the color development. Plate reaction stopped by addition of 2 mol/L of H_2SO_4 was read with a plate reader (Bio-Rad) at 490 nm. Titers were defined as the final dilution giving an optical density of at least 0.1 units above the optical density of the 1:50 dilution of the pre-immune serum. The pre-immune sera normally had an optical density of < 0.1 units.

Fertility assays

Fertility trials were commenced at 12 weeks p.i. by pairing each immunized female mouse with one normal male for 2 weeks. Vaginal plugs were monitored to confirm mating. Number, weight and length of offspring born were recorded.

Ovarian histology

Ovaries were collected from killed mice, fixed in 4% PFA for 2 days. Sections (7–10 μm) were prepared and then stained with hematoxylin and eosin. The development of follicles was observed under a microscope.

Antifertility assay with antisera of immunized mice in vivo

Three microliter of 0.22 μm-filtered antisera at peak antibody titer (1:6,400) of immunized mice were aseptically injected into the left uterine horn of normal pregnant mice on 3.5 days after confirmed mating by vaginal plugs. The same amount of 0.22 μm-filtered mice preimmunized sera was injected into the right uterine horn as control. On the 15th day of pregnancy, the number of implantations in each side of the uterus was recorded.

Statistical analysis

The data of antibody titers were log-transformed using a ten logarithm to alleviate statistical problems and were presented as the means ± S.E.M. The differences between each treatment level and control were evaluated by Student's *t*-test.

Results

Nucleotide and predicted amino acid sequence of the muCGβ

The full-length cDNA of muCGβ including the leader peptide was 499 nucleotide long (GenBank Accession No. AF333067); coding for 165 amino acids (Tables 1A and 1B). The cDNA sequences analysis shows the muCGβ shares the highest identity (99.6%) to rhesus monkey CGβ and the second highest homology (90.6%) to humans. At the amino acid level, 100% sequence homology to the rhesus monkey and 79.5% sequence homology to the human where the most diversified region occurred on the C-terminal portion of the β-subunits (Tables 1B and 1C). The difference between muCGβ and rmCGβ cDNA was at base +489 (muCGβ was T, rmCGβ was C) and base +492 (muCGβ was A, rmCGβ was G), but did not change the amino acids sequence. It indicates that the BALB/c mouse as an animal model to assess the PCMV4-rmCGβ immuno-contraceptive might gain some valuable results.

Humoral immune responses induced by different doses of inoculation of pCMV4-rmCGβ DNA

The humoral immune responses were induced by different doses of inoculation with pCMV4-rmCGβ DNA. Sera antibodies to hCGβ are shown in Fig. 1. Compared to control group, the female mice of each group elicited high titers of humoral immune responses. The antibody titers increased by 6 weeks p.i., and maintained the immune responses at 12 weeks. The titer reached high to 1:6,400, and these intense immune responses lasted at least 12 weeks. Moreover, the humoral immune responses of female mice inoculated with different doses of pCMV4-rmCGβ DNA from 10–50 μg were induced with parallel increase of titer, and there was no great difference in antibody titers. This suggests that 10 μg of pCMV4-rmCGβ DNA should be enough to elicit immune responses in BALB/c mice.

Table 1. (A) Sequence alignment of β-subunit cDNA of hCG, rmCG and muCG

```
hCG    ATGGAGATGTTCCAGGGGCTGCTGCTGTTGCTGCTGCTGA          40

rmCG   ---------c-----------------g-----------          40

muCG   ---------c-----------------g-----------          40


hCG    GCATGGGCGGGACATGGGCATCCAAGGAGCCGCTTCGGCC          80

rmCG   -----------g--c---------g---------g-----          80

muCG   -----------g--c---------g---------g-----          80


hCG    ACGGTGCCGCCCCATCAATGCCACCCTGGCTGTGGAGAAG         120

rmCG   --t----------------------------cc------         120

muCG   --t----------------------------cc------         120


hCG    GAGGGCTGCCCCGTGTGCATCACCGTCAACACCACCATCT         160

rmCG   ----c----------------------------------         160

muCG   ----c----------------------------------         160


hCG    GTGCCGGCTACTGCCCCACCATGACCCGCGTGCTGCAGGG         200

rmCG   -------------------------tg--g----------c        200

muCG   -------------------------tg--g----------c        200


hCG    GGTCCTGCCGGCCCTGCCTCAGGTGGTGTGCAACTACCGC         240

rmCG   ----------c-ag----c---------c-----------         240

muCG   ----------c-ag----c---------c-----------         240


hCG    GATGTGCGCTTCGAGTCCATCCGGCTCCCTGGCTGCCCGC         280

rmCG   --g------------------------------------         280

muCG   --g------------------------------------         280


hCG    GCGGCGTGAACCCCGTGGTCTCCTACGCCGTGGCTCTCAG         320

rmCG   ct------g-------------gttc-------------         320

muCG   ct------g-------------gttc-------------         320
```

Table 1. (A) Continued

```
hCG    CTGTCAATGTGCACTCTGCCGCCGCAGCACCACTGACTGC         360

rmCG   -----gt---------------------t-------t         360

muCG   -----gt---------------------t-------t         360


hCG    GGGGGTCCCAAGGACCACCCCTTGACCTGTGATGACCCCC         400

rmCG   --------------------t------------------         400

muCG   --------------------t------------------         400


hCG    GCTTCCAGGACTCCTCTTCCTCAAAGGCCCCTCCCCCCAG         440

rmCG   a-c------c-----------------a------------         440

muCG   a-c------c-----------------a------------         440


hCG    CCTTCCAAGCCCATCCCGACTCCCGGGGCCCTCGGACACC         480

rmCG   --c------t------g------t--a---ag-a----a-         480

muCG   --c------t------g------t--a---ag-a----a-         480


hCG    CCGATCCTCCCACAATAAA                              499

rmCG   ---t-------g-------                              499

muCG   ---t----t----------                             499
```

Table 1. (B) Alignment of β-subunit putative protein of hCG, rmCG and muCG

```
hCG    MEMFQGLLLLLLLSMGGTWASKEPLRPRCRPINATLAVEK          40

rmCG   ---l-----w-------ar--r-----l---------a--          40

muCG   ---l-----w-------ar--r-----l---------a--          40


hCG    EGCPVCITVNTTICAGYCPTMTRVLQGVLPALPQVVCNYR          80

rmCG   -a-------------------m----a---pv----r---          80

muCG   -a-------------------m----a---pv----r---          80


hCG    DVRFESIRLPGCPRGVNPVVSYAVALSCQCALCRRSTTDC         120

rmCG   e------------p--d----vp-----r--------s--         120

muCG   e------------p--d----vp-----r--------s--         120
```

Table 1. (B) Continued

```
hCG     GGPKDHPLTCDDPRFQDSSSSKAPPPSLPSPSRLPGPSDT    160

rmCG    -------------hl-a-----d----p----g-le-a-n    160

muCG    -------------hl-a-----d----p----g-le-a-n    160


hCG     PILPQ                                       165

rmCG    -f---                                       165
```

Table 1. (C) Homology of β-subunit cDNA and putative protein of hCG, rmCG and muCG

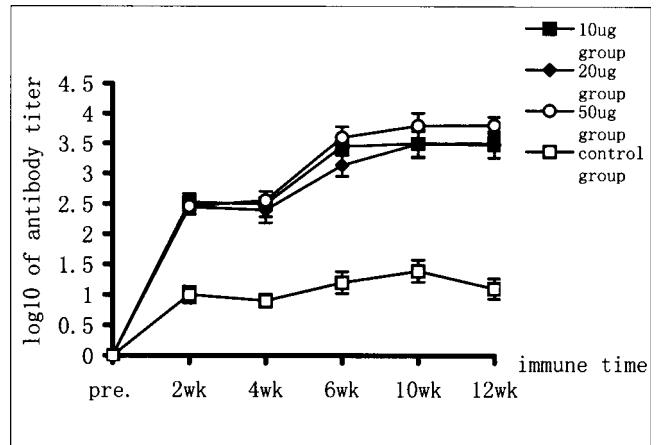| protein cDNA	rmCG	hCG	MuCG
rmCG		79.5%	100%
hCG	90.6%		79.5%
MuCG	99.6%	90.6%	

Fig. 1. Humoral immune responses induced by different doses of inoculation of pCMV4-rmCGβ DNA using ELISA. Sera samples of each group from 2–12 weeks were detected, and the antibody titers were pooled and expressed as log10 titers. The female BALB/c mice of each group were inoculated with 10, 20 or 50 μg of pCMV4-rmCGβ plasmid DNA with intramuscular injection, respectively. Twenty μg of pCMV4 mock plasmid DNA was set as control. At 4 weeks all mice had the boost immunization.

Humoral immune responses induced by different methods of inoculation of pCMV4-rmCGβ DNA

Three different methods, i.m., i.p. and i.d. application were studied to detect the optimal method for rmCGβ DNA-based immuno-contraceptive. Different humoral immune responses were elicited with different inoculating methods in female mice (Fig. 2). The highest titer of immune response and second highest titer of immune response were induced with i.m. method and i.d. method, respectively. While the antibody titers of immune response with i.p. method were less than 1:500. The results indicated that the i.m. inoculation is the optimal pCMV4-rmCGβ DNA delivery method for BALB/c mice.

Antifertility induced by different doses and different methods of inoculation of pCMV4-rmCGβ DNA

The immunized mice in each group were individually paired at 12 weeks. p.i. to determine the fertility compared to controls (Table 2). The results of fertility trail demonstrated that 50 μg of pCMV4-rmCGβ DNA using i.m. inoculation method could reduce significantly the fertility in female mice compare to controls (p < 0.01), and a marked reduction of fertility from low dose to high dose (from 10–50 μg) was found. The number of litters born also showed a slight reduction from the doses from 10–50 μg. At the dose of 20 μg, the mice showed reduction in fertility from i.m., i.d. to i.p. inoculation, but there was no significant difference (p > 0.05).
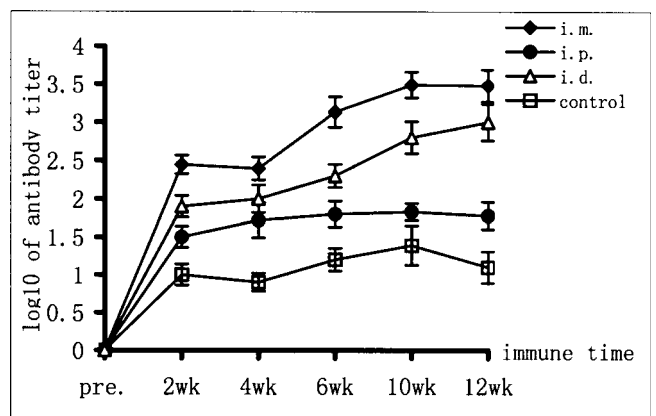

Fig. 2. Humoral immune responses of female mice induced by different methods of inoculation of pCMV4-rmCGβ DNA using ELISA. Sera samples of each group from 2–12 weeks were detected, and the data of the antibody titers were pooled and expressed as log10 titers. The female BALB/c mice of each group were inoculated 20 μg of pCMV4-rmCGβ plasmid DNA with intramuscular (i.m.), intraperitoneal (i.p.) and intradermal (i.d.) injection, respectively. Twenty μg of pCMV4 mock plasmid DNA with i.m. inoculation was set as control. At 4 weeks all mice had the boost immunization.

Table 2. Antifertility of female mice induced by different doses and different methods of inoculation of pCMV4-rmCGb DNA shown.

Treatment groups	Fertility			
	Birthrate N = 8	No. of litters	Litter weight (g)	Litter length (cm)
10 µg PCMV4-rmCGb (intramuscular)	75%	8.75 ± 0.96 n = 5	1.33 ± 0.15 n = 42	2.81 ± 0.20 n = 42
20 µg PCMV4-rmCGb (intramuscular)	62.5%	7.6 ± 1.8 n = 5	1.35 ± 0.18 n = 38	2.90 ± 0.21 n = 38
50 µg PCMV4-rmCGb (intramuscular)	37.5%**	5.67 ± 1.52* n = 3	1.50 ± 0.16 n = 21	2.99 ± 0.26 n = 21
20 µg PCMV4-rmCGb (intraperitoneal)	87.5%	7.0 ± 2.24* n = 7	1.40 ± 0.16 n = 49	2.98 ± 0.2 n = 49
20 µg PCMV4-rmCGb (intradermic)	62.5%	8.0 ± 3.02 n = 5	1.37 ± 0.14 n = 40	2.92 ± 0.19 n = 40
20 µg PCMV4 as control (intramuscular)	100%	9.0 ± 1.73 n = 8	1.24 ± 0.12 n = 61	2.85 ± 0.19 n = 61

Fertility trials were commenced at 12 weeks p.i. by pairing each female with one normal male for 2 weeks. Number, weight and length of kittens born were recorded *p < 0.05; **p < 0.01 compare to control).

Ovaries were collected from fertile and infertile immunized female mice from the treatment group and control group, and were detected. Ovaries from the treatment group and control group all had a normal range of small, medium, and large preovulatory follicles. It indicated that the rmCGβ expressed by pCMV4-rmCGβ did not interfere with the development of follicles and normal ovulatory cycles could be maintained.

To be sure the antifertility induced by pCMV4-rmCGβ in female mouse, 3 µL of antiserum at peak antibody titer (1:6,400) of mice were injected into one side of uterine horns of normal pregnant mice on 3.5 days after confirmed mating by vaginal plugs. The results showed that the number of implantations in the side of uterus received antiserum (1.1 ± 0.4) had significant decrease compared to the control side of the uterus receiving normal sera (4.5 ± 0.8) (n = 16, **p < 0.01 compared to control) (Fig. 3). It confirmed that the antibody elicited by pCMV4-rmCGβ DNA prevented pregnancy in mice.

Discussion

During more than 20 years, several immuno-contraceptives based on hCGβ protein originally isolated from tissues or produced by recombinant DNA techniques or by chemical synthesis have reached the stage of clinical trials. The weak immunogenicity, moderate immune response, and costly procedure make current vaccines do not suit in family planning [5–7]. Talwar *et al.* even constructed live engineered virus recombined hCGβ to assess the antifertility efficacy in female rats [7, 15, 16]. The plasmid DNA vaccine as a kind of safe and highly effective vaccine has been proven in many human pre-clinical and clinical trials [17–19]. The CGβ as contraceptive antigen inserted into eukaryotic expression plasmid vector has not been examined before. In our study,

the recombined expression plasmid DNA inserted interesting reproductive antigen rmCGβ was inoculated into somatic cells to detect the feasibility of pCMV4-rmCGβ DNA as potential contraceptive.

The present study provides evidence for prevention of pregnancy in BALB/c mice by CGβ antibodies. The pCMV4-rmCGβ DNA could generate sufficient immune responses to interfere the function of CGβ to disrupt pregnancy prior to implantation. The highest titer of humoral immune responses elicited by pCMV4-rmCGβ DNA reached 1:6,400, and lasted at least for 12 weeks. The infertility of mouse induced by antibodies elicited by pCMV4-rmCGβ DNA was confirmed by
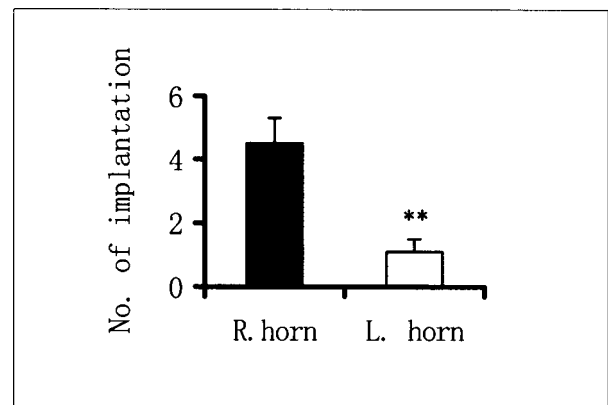
Fig. 3. Antifertility assay with antisera of pCMV4-rmCGβ plasmid DNA immunized mice *in vivo*. Three µL of 0.22 µm-filtered antisera at peak antibody titer (1:6,400) of immunized mice were aseptically injected into the left uterine horn (L. horn) of normal pregnant mice on 3.5 days after confirmed mating by vaginal plugs. The same amount of 0.22 µm-filtered normal mouse sera were injected into the right uterine horn (R. horn) as control. On the 15th day of pregnancy, the number of implantation in each side of the uterus was recorded (n = 16, **p < 0.01 compare to control).

the antifertility assay of antiserum of immunized mice in vivo. Moreover, our study provides evidence that the immune responses were elicited by pCMV4-rmCGβ DNA with varied doses (from 10–50 μg) and varied inoculation methods (i.m., i.p., and i.d. inoculation). The results indicated that the intensive humoral immune response could be induced by pCMV4-rmCGβ in mice with the amount of 10 μg of plasmid DNA. The pCMV4-rmCGβ plasmid DNA with i.d. inoculation, especially with i.m. inoculation does elicit effective immune responses to interfere the pregnancy in mice. The results of immune responses induced by varied doses indicates that the pCMV4-rmCGβ plasmid DNA with i.m. and i.d. inoculation could be easier absorbed by host cells or more efficiently presented by APC cells than with i.p. inoculation, although the transfection efficiency of target tissues is unnecessarily correlate with efficiency of immunization [20]. With i.m. inoculation method, the infertility efficacy of pCMV4-rmCGβ contraceptive in female mice was correlated with the amount of pCMV4-rmCGβ plasmid DNA, while the similar magnitude of immune responses were elicited with different inoculation dose from 10–50 μg. It suggests that the antifertility effects of pCMV4-rmCGβ contraceptive not only depend on the magnitude of the immunity but also some other important factors, such as the route of plasmid delivery, the method of plasmid delivery, as well as the type of immunity [21, 22]. The type of cytokines secreted with different inoculations would appear to preferentially induce antibody responses of the IgG1, IgG2a, IgE, and IgA isotypes, and these factors, especially the capacity to generate mucosal responses near the site of fertility may ultimately be essential for a fully effective fertility control vaccine [23, 24]. In our study, the immunity of mice induced by pCMV4-rmCGβ DNA biases towards IgG1 isotype, and the selective expression of Th2 cytokines (the detail data not shown). It suggests that Th1 immunity would be enhanced in further study with pCMV4-rmCGβ contraceptive.

There are published reports, from Thanavala *et al*., Talwar *et al*. and Stevens *et al*. respectively [5, 7, 25], of both *in vitro* and *in vivo* studies demonstrating the neutralization of the biological action of hCG by β-hCG antibodies. However, the mechanism of action of antibodies raised to the β-CG vaccine in blocking fertility remains unclear [26–28]. The antibodies induced by pCMV4-rmCGβ DNA did prevent implantation in female mice without disrupting folliculogenesis, and this also has been validated by phase I and phase II trials on the hCG vaccine performed by Talwar and associates [7]. Beta subunit of hCG could generate antibodies cross-reactive with the pituitary LH. It has been confirmed that this partial cross-reactivity does not impair ovarian function, and menstrual cycle. Most animals continued to show normal ovulatory cycles indicating that the circulating antibodies did not interfere with the ovulation-inducing action of endogenous LH. In our study, although the low cross-reactive antibodies with

LH were elicited by the mice immunized with pCMV4-rmCGβ DNA (less than 1:600), the anti-LH antibodies were less than 10% compared to the anti-CGβ antibodies at the same time (the detail data not shown), and did not disrupt the folliculogenesis. Compare to 80% of women produced antibodies and high antifertility induced by current protein or recombinant peptide hCGβ vaccine, 62.5% of female mice induced infertility by pCMV4-rmCGβ DNA may be partially caused by the post-translation modification of rmCGβ in mouse. Another possible reason may be correlated with the specific bio-function of muCGβ in mouse. Further optimization of pCMV4-rmCGβ may be required to enhance the efficacy of immune response, such as the use of cytokines as adjuvent [29]. The pCMV4-rmCGβ DNA as female contraceptive should be interesting and deserving further study.

Acknowledgements

References

1. Jones WR, Judd SJ, Ing RMY: Phase I clinic trial of a World Health Organisation birth control vaccine. Lancet 1: 1295–1298, 1988
2. Talwar GP, Singh O, Pal R: A vaccine that prevents pregnancy in women. Proc Natl Acad Sci USA 91: 8532–8536, 1994
3. Stevens VC: Preparation and formulation of a hCG antifertility vaccine, selection of peptide immonogen. Am J Reprod Immunol 6: 307–311, 1991
4. Jones WR: Contraceptive vaccines. Baillieres Clin Obstet Gynaecol 10: 69–86, 1996
5. Stevens VC: Progress in the development of human chorionic gonadotropin antifertility vaccines. Am J Reprod Immunol 35: 148–155, 1996
6. Singh O, Rao LV, Gaur A, Sharma NC, Alam A, Talwar GP: Antibody response and characteristics of antibodies in women immunized with three contraceptive vaccines inducing antibodies against human chorionic gonadotropin. Fertil Steril 52: 739–744, 1989
7. Talwar GP, Singh OM, Gupta SK: The HSD-hCG vaccine prevents pregnancy in women: Feasibility study of a reversible safe contraceptive vaccine. Am J Reprod Immunol 37: 153–160, 1997
8. Dirnhofer S, Klieber R, De Leeuw R: Functional and immunological relevance of the COOH-terminal extension of human chorionic gonadotropin beta: Implications for the WHO birth control vaccine. FASEB J 7: 1381–1385, 1993
9. Singh M, Das SK, Suri S, Singh O, Talwar GP: Regain of fertility and normality of progeny born during below protective threshold antibody titers in women immunized with the HSD-hCG vaccine. Am J Reprod Immunol 39: 395–398, 1998
10. Wang B, Ugen K, Srikantan V: DNA inoculation generates immune responses against human immunodeficiency virus type 1. Proc Natl Acad Sci 90: 4156–4160, 1993

11. Boyer JD, Cohen AD, Vogt S: Vaccination of seronegative volunteers with a human immunodeficiency virus type 1 env/rev DNA vaccine induces antigen-specific proliferation and lymphocyte production of beta-chemokines. J Infect Dis 181: 476–483, 2000

12. Totowa NJ: In: Methods in Medicine, vol. 29. DNA Vaccines: Methods and Protocols. Humana Press Inc., 1999, pp 439–449

13. Chen Y, Liu Z, Wang B, Peng JP: Identification of beta subunit of the rhesus monkey chorionic gonadotropin (rmCG). Mol Cell Biochem 218: 157–163, 2001

14. Chen Y, Liu Z, Peng JP, Zhang FC, Chen YZ, Wang B: The expression of beta subunit of rhesus monkey chorionic gonadotropin (rmCGβ) DNA in HeLa cells and the immune responses in BALB/c mice inoculated by rmCGβ DNA vaccine. Acta Zoologica Sinica 47: 419–424, 2001

15. Srinvasan J, Om Singh, Chakrabarti S, Talaw GP: Targeting vaccine virus-expressed secretary β-subunit of human chorionic gonadotropin to the cell surface induce antibodies. Infect Immun 63: 4907–4911, 1995

16. Naz RK, Sacco A, Singh O, Pal R, Talwar GP: Development of contraceptive vaccines for humans using antigens derived from gametes (spermatozoa and zona pellucida) and hormones (human chorionic gonadotropin): current status. Hum Reprod Update 1: 1–18, 1995

17. MacGregor RR, Boyer JD, Ugen KE: First human trial of a DNA-based vaccine for treatment of human immunodeficiency virus type 1 infection: Safety and host response. J Infect Dis 178: 92–100, 1998

18. Le TP, Coonan KM, Hedstrom RC: Safety, tolerability and humoral immune responses after intramuscular administration of a malaria DNA vaccine to healthy adult volunteers. Vaccine 18: 1893–1901, 2000

19. Mincheff M, Tchakarov S, Zoubak S: Naked DNA and adenoviral immunizations for immunotherapy of prostate cancer: A phase I/II clinical trial. Eur Urol 38: 208–217, 2000

20. Raz E, Carson DA, Parker SE: Intradermal gene immunization: The possible role of DNA uptake in the induction of cellular immunity to viruses. Proc Natl Acad Sci USA 91: 9519–9523, 1994

21. Böhm W, Mertens T, Schirmbeck R, Reimann J: Routes of plasmid DNA vaccination that prime murine humoral and cellular immune responses. Vaccine 16: 949–954, 1998

22. Sylvia VDLDH, Volker G, Bianca IL: Recent advances in the use of DNA vaccines for the treatment of diseases of farmed animals. Adv Drug Deliv Rev 43: 13–28, 2000

23. Alistair JR, Ian AR: Cytokine enhancement of immune responses important for immunocontraception. Reprod Fertil Dev 9: 91–97, 1997

24. Talwar GP, Raghupathy R: In: Medical Intelligence Unit. (eds). Birth Control Vaccines. R.G. Lands, Austin, TX, pp 149–165

25. Thanavala Y, Hay F, Stevens VC: Affinity, cross reactivity, and biological effectiveness of rabbit antibodies against a synthetic 37 amino acid C-terminal peptide of human chorionic gonadotropin. J Clin Exp Immunol 39: 112–118, 1980

26. Ski QJ, Lei ZM, Rao CV, Lin J: Novel role of human chorionic gonadotropin in differentiation of human cytotrophoblasts. Endocrinology 132: 1387–1395, 1993

27. Miwidsky A, Finci-Yeheskel Z, Yagel S, Mayer M: Gonadotropin-mediated inhibition of proteolytic enzymes produced by human trophoblast in culture. J Clin Endocrinol Metabol 76: 1101–1105, 1993

28. Bagel S, Geva TE, Solomon H: High levels of human chorionic gonadotropin retard first trimester trophoblast invasion *in vitro* by decreasing urokinase plasminogen activator and collagenase activities. J Clin Endocrinol Metabol 77: 1506–1511, 1993

29. Jong JK, Valpani A, Mark LB: *In vivo* engineering of a cellular immune response by co-administration of IL-12 expression vector with a DNA immunogen. J Immunol 158: 816–826, 1997