

—Original—

# Construction and evaluation of the novel DNA vaccine harboring the inhibin $\alpha$ (1–32) and the RF-amide related peptide-3 genes for improving fertility in mice

Xingang DAN<sup>1,2</sup>), Li HAN<sup>1</sup>), Hasan RIAZ<sup>3</sup>), Xuan LUO<sup>1</sup>), Xiaoran LIU<sup>1</sup>), Zhenglu CHONG<sup>1</sup>), and LiguO YANG<sup>1</sup>)

<sup>1</sup>Key Lab of Education Ministry of China in Agricultural Animal Genetics, Breeding and Reproduction, Huazhong Agricultural University, Wuhan 430070, P.R. China

<sup>2</sup>The Agricultural College of NingXia University, Yinchuan 750021, P.R. China

<sup>3</sup>Department of Biosciences, COMSAT Institute of Information Technology, Sahiwal, 57000, Pakistan

**Abstract:** To further improve fertility of animals, a novel gene *RFRP-3* (RF-amide related peptide-3, *RFRP-3*) was used to construct DNA vaccines with *INH*  $\alpha$  (1–32) (inhibin, *INH*) fragment for the first time. The aim of this study was to evaluate the effects of novel DNA vaccines on fertility in mice. Synthesized *SINH* and *SRFRP* (*INH* and *RFRP* genes were separately ligated to the C-terminus of the small envelope protein of the hepatitis B virus (HBV-S) gene) fragments were inserted into multiple cloning site of pIRES vector to develop p-SINH/SRFRP. The synthesized tissue plasminogen activator (TPA) signal sequence was then inserted into the p-SINH/SRFRP to construct p-TPA-SINH/TPA-SRFRP. Meanwhile, p-SINH was prepared and considered as positive control. Forty Kunming mice were equally divided into four groups and respectively immunized by electroporation with p-SINH, p-SINH/SRFRP and p-TPA-SINH/TPA-SRFRP vaccine (three times at 2 weeks interval) and saline as control. Results showed that the average antibodies (P/N value) of anti-*INH* and anti-*RFRP* in mice inoculated with p-TPA-SINH/TPA-SRFRP were significantly higher ( $P < 0.05$ ) than those inoculated with p-SINH/SRFRP and the positive rates were 100% (anti-*INH*) and 90% (anti-*RFRP*) respectively, at 2 weeks after the third immunization. Litter size of mice immunized with the three recombinant plasmids was higher ( $P < 0.05$ ) than that of the control, and litter size of mice immunized with p-TPA-SINH/TPA-SRFRP significantly increased ( $P < 0.05$ ) compared with p-SINH. These results suggested that the p-TPA-SINH/TPA-SRFRP harboring *INH* and *RFRP* genes was successfully constructed and had good immunogenicity, and might effectively increase litter size.

**Key words:** DNA vaccine, fertility, inhibin, mice, *RFRP-3*

---

## Introduction

The release of inhibin inhibits the secretions of gonadotropin, follicular development and spermatogenesis. Immunoneutralization of endogenous inhibin is considered to be an effective method for induction of super-

ovulation [13, 16, 25, 41]. DNA immunization usually leads to *in vivo* expressions of the target antigen by a plasmid DNA [17]. This process is reported to be safe and easier on large-scale production and storage compared with the use of purified proteins [28]. The small envelope protein of the hepatitis B virus (HBV-S) is used

---

(Received 8 May 2015 / Accepted 14 July 2015 / Published online in J-STAGE 2 Octobr 2015)

Address corresponding: Li Guo Yang, College of Animal Science and Technology and College of Animal Science and Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, P.R. China

as a carrier molecule for DNA vaccine, which are regarded as safe and can induce high immune responses [23, 39]. Therefore, in our previous studies, the INH- $\alpha$  (1–32) fragment was inserted into the HBV-S gene to construct two DNA vaccines (pcIS and pcISI), which effectively induced immune responses [10, 42]. However, the effect of these vaccines on improving fertility, particularly in large animals, was inadequate. The limitation could be due to other key factors that inhibit gonadotropin secretion and follicular development, which may result in low fertility.

A novel hypothalamic neuropeptide was found in quail and termed gonadotropin-inhibitory hormone (GnIH) for inhibiting gonadotropin release from cultured quail anterior pituitary gland [33]. The GnIH precursor is composed of 173 amino acid residues and cleaved into three mature peptides, namely, GnIH, GnIH related peptide-1 and GnIH related peptide-2, which possess an LPXRF amide sequence at their C-termini. Subsequently its analog was found in mammals, and named as RF-amide related peptide (RFRP) possessing LPXRF amide sequence at the C-terminus, and most mammalian *RFRP* genes encode two peptides, namely, RFRP-1 and RFRP-3 [7, 37]. GnIH and RFRP-3 are the most effective in acting in the hypothalamus-pituitary-gonad axis. In addition to the mRNA expression of *GnIH* (*RFRP-3*) in the hypothalamus, its expression was also found in other organs such as, ovary, testis, uterus, kidney, eye, and adrenal gland [19, 26, 30, 44]. *GnIH* (*RFRP-3*) plays major physiological roles by binding to its specific receptor, G-protein-coupled receptor147 (GPR147), which is also known as Npffr1 or OT7T022 [4, 12]. GnIH could inhibit the release of LH and FSH from the pituitary glands of chicken and quail [5, 38]. Intravenous administration of RFRP-3 decreased the level of peripheral blood gonadotropin in gonadectomized male rats and inhibited testicular steroidogenesis and spermatogenesis in adult mice [1, 27]. In sheep, intravenous injection of RFRP-3 reduced LH pulse amplitude and suppressed LH and FSH secretion [8]. In addition, it has also been shown that GnIH suppressed follicular development and steroidogenesis in chicken [2, 22]. These findings suggest that GnIH may direct or indirect inhibit follicular development and ovulation. We infer that if innate INH and GnIH (*RFRP-3*) are simultaneously neutralized through specific immunization in animals, then it might further improve litter size of animals.

Therefore, we constructed a novel plasmid DNA vac-

cine p-SINH/SRFRP carrying both *RFRP-3* and *INH* gene by using a commercial mammalian expression vector (pIRES), which contains internal ribosomal entry sites (IRES) and allows the expression of two genes of interest from the same bicistronic mRNA transcript. Because TPA could enhance immunogenicity of antigen and elicit stronger humoral immune responses when it was introduced into DNA vaccine, we constructed another novel DNA vaccine, p-TPA-SINH/TPA-SRFRP fused with TPA, and p-SINH for positive control. All mice were grouped and immunized with p-TPA-SINH/TPA-SRFRP, p-SINH/SRFRP, p-SINH vectors and saline by electroporation. The immunogenicity of p-TPA-SINH/TPA-SRFRP, p-SINH/SRFRP and p-SINH was investigated, and the effects of immunization with these vaccines on the fertility of mice were also evaluated.

---

## Materials and Methods

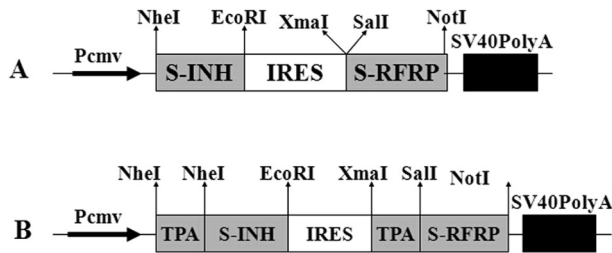
---

### *Construction of recombinant plasmids*

The eukaryotic expression vector pIRES was preserved in our laboratory. The fragment of SINH (swine INH- $\alpha$  (1–32) NM\_214189) was ligated to the C-terminus of *HBV-S* gene) was synthesized by Generay Biotech Co., Ltd., which separately contains *NheI* and *EcoRI* in its ends. Likewise, the fragment of SRFRP (bovine RFRP-3 (BD317666.1) was ligated to the C-terminus of the *HBV-S*) was synthesized, which separately contains *SalI* and *NotI* in its ends. In addition, TPA signal sequences (E02360.1) containing *NheI* and *XmaI/SalI* also were synthesized. The fragments of SINH and SRFRP were separately sub-cloned into pIRES vector using *NheI/EcoRI* and *SalI/NotI* restriction sites and were named p-SINH and p-SINH/SRFRP, respectively (Fig. 1A). Next, two TPA fragments were separately inserted into p-SINH/SRFRP by using *NheI* and *XmaI/SalI* restriction enzymes and the plasmid was named p-TPA-SINH/TPA-SRFRP (Fig. 1B).

### *Enzyme digestion and sequencing of recombinant plasmids*

A single clone of p-TPA-SINH/TPA-SRFRP, p-SINH/SRFRP and p-SINH plasmids was selected and inoculated into the liquid broth medium with ampicillin. After 12 h, the plasmids were extracted through TIANprep Rapid Mini Plasmid Kit, identified by restriction analysis and sequenced by Takara Biotechnology Co., Ltd.



**Fig. 1.** Construction maps of the recombinant plasmid p-SINH/SRFRP and p-TPA-SINH/TPA-SRFRP. (A) p-SINH/SRFRP: the *SINH* gene encoding *HBV-S* and *INH- $\alpha$*  (1–32) fragment, and *SRFRP* gene encoding *HBV-S* and *RFRP-3* gene were separately inserted into the pIRES vector by using different restriction sites. (B) p-TPA-SINH/TPA-SRFRP: TPA signal sequences were inserted into the N-terminal of *SINH* and *SRFRP* genes.

#### Proteins expression of recombinant plasmids in HeLa cells

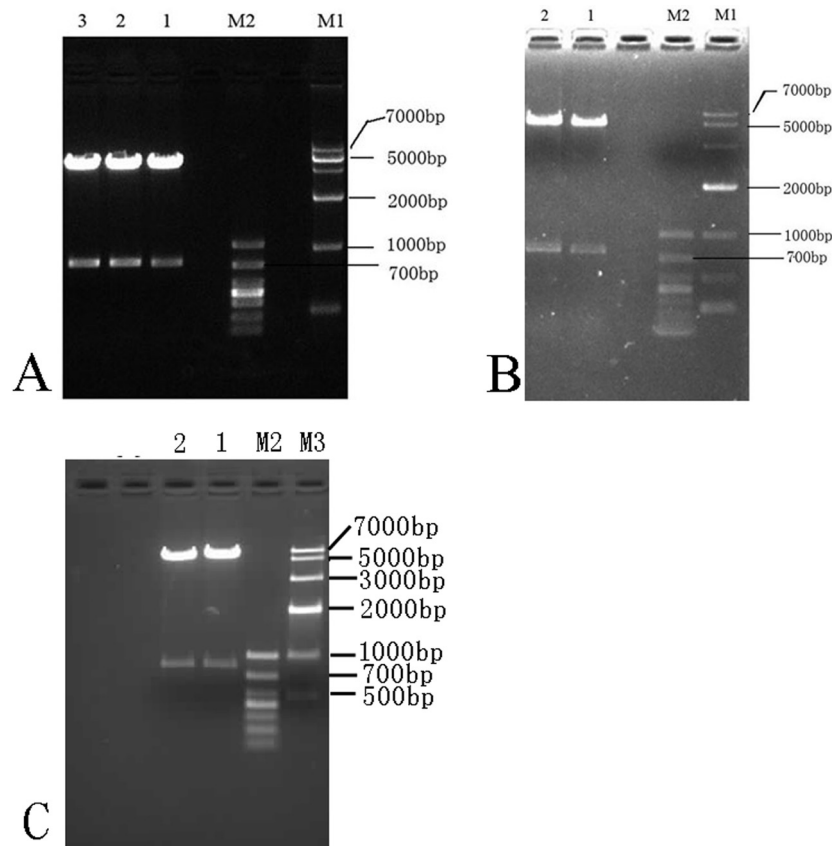
Three recombinant plasmids (p-SINH, p-SINH/SRFRP and p-TPA-SINH/TPA-SRFRP) were transfected into HeLa cells for 48 h. The cells were then washed with cold PBS twice and lysed in RIPA buffer (Beyotime, Nantong, China) containing protease inhibitor cocktail and PMSF (Sigma, ST. Louis, USA). After incubation for 5 min on ice, cell suspension was collected in EP tubes, placed on ice for 20 min, and centrifuged at  $13,000 \times g$  for 15 min. The supernatant was carefully transferred to a new EP tube. Total protein concentration was measured using a BCA kit (Beyotime, Nantong, China). The protein samples were separated on 12% polyacrylamide gel and transferred to PVDF membranes (Millipore, MA, USA). The membranes were blocked in TBST (10 mM Tris (pH7.5), 150 mM NaCl; and 0.05% Tween-20) supplemented with 5% skimmed milk (Sigma-Aldrich, USA) and incubated overnight at 4°C with primary antibodies (anti-inhibin- $\alpha$  1:200, abDSerotec, USA, MCA951S; anti-RFRP 1:500, Santa Cruz, USA, sc-32377, and anti-actin, 1:1,000, Santa Cruz, USA, sc-1616). After incubation, the membranes were washed three times with TBST and then incubated with second antibody (1:3,000, HRP labeled goat anti-mouse, Abbkine, USA, A21010) for 1 h at room temperature. After washing with TBST, the membranes were developed on an ECL Western blot detection system (Beyotime, Nantong, China), and then exposed to X-ray film for visualization of the protein bands.

#### DNA vaccination

Forty Female Kunming mice (6 weeks old) were purchased from the Hubei Center for Disease Control and Prevention (Wuhan, China). All experiments involving mice were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were randomly divided into four groups (10 mice per group). Each group was respectively injected with 20  $\mu\text{g}$  of p-TPA-SINH/TPA-SRFRP (dissolved in 100  $\mu\text{l}$  of 0.9% saline; group P), 20  $\mu\text{g}$  of p-SINH/SRFRP (group S), 20  $\mu\text{g}$  of p-SINH (group T, and positive control), and 100  $\mu\text{l}$  of 0.9% saline (group C, and control) in the quadriceps muscles. After that, two needle electrodes (each spaced by approximately 5 mm) were inserted immediately into the muscle at the site of injection, and electroporation treatment was performed under specific parameters (36 V, with 50 ms duration of electrical pulse duration for six consecutive times at 1 s interval). Mice were immunized thrice (similar to the primary immunization), with an interval of 2 weeks. Blood samples were collected from the tail vein on days 0, 14, 28 and 42. Subsequently, serum was separated after clotting and stored at  $-80^\circ\text{C}$  until further analysis.

#### Detection of antibodies against INH and RFRP

Specific IgG antibodies were detected using indirect ELISA with synthesized INH- $\alpha$  (1–32) and RFRP-3 antigens (Apeptide Co., Shanghai, China) as standard antigen. Briefly, 96-well ELISA plates (Costar-3590, USA) were coated with 100 ng/100  $\mu\text{l}$  antigen and stored at 4°C overnight. The following day, the wells were washed three times with PBS supplemented with 0.1% Tween-20 (PBST) and blocked with 5% (w/v) skimmed milk in PBST for 1 h at 37°C (250  $\mu\text{l}$ /well). Subsequently, 100  $\mu\text{l}$  serum samples (diluted in 1:100 dilutions with PBST) were added into the wells and incubated for 1 h at 37°C. After washing five times with PBST, antibodies were assayed by adding a second antibody (HRP-labeled goat anti-mouse IgG antibody, Abbkine, USA), which was diluted in PBST (1/5,000), and then incubated for 1 h at 37°C. After that, each well was saturated with tetramethylbenzidine [18] and placed in an incubator at 37°C for 20 min. After the reaction was terminated with 2 M  $\text{H}_2\text{SO}_4$  (50  $\mu\text{l}$ /well), optical density values was determined at 450 nm by using a microplate reader (Bio-Rad, Imark680, USA). Each sample was tested in triplicate. ELISA results were analyzed with P/N ratios, where P and N denote the OD values of the tested and



**Fig. 2.** Identification of plasmids by restriction endonuclease digestion. (A) The p-SINH (Lane 1), p-SINH/SRFRP (Lane 2) and p-TPA-SINH/TPA-SRFRP (Lane 3) were separately double digested with *NheI* and *EcoRI*. M1:DL7,000 bp DNA marker. M2:DL1,000 bp DNA marker. The target band was of 789 bp. (B) Lane1& 2: p-SINH/SRFRP was double digested with *SalI* and *NotI*, and the target band was of 778 bp. M1:DL7,000 bp DNA marker. M2:DL1,000 bp DNA marker. (C) Lane1&2: p-TPA-SINH/TPA-SRFRP was double digested with *XmaI* and *NotI*, and the target band was of 847 bp. M3: DNA marker IV. M2: DL 1,000 bp DNA marker.

negative control samples, respectively. P/N ratios higher than 2.0 and OD value over 0.2 were considered positive [10, 43].

#### *Evaluation of effects on fertility of mice after immunization*

After final blood collection, all mice were housed in different cages (two mice per cage). Male mice were introduced in each cage until female mice became pregnant. Litter size of three successive generations were respectively recorded.

#### *Statistical analysis*

Statistical analysis was performed using SPSS (Version 19.0) software. All data were expressed as mean  $\pm$  SD and differences among groups were determined with

ANOVA and least significant difference test. *P* values <0.05 were considered significant.

---

## Results

---

#### *Construction and identification of recombinant plasmids*

All recombinant plasmids including p-SINH, p-SINH/SRFRP and p-TPA-SINH/TPA-SRFRP were separately identified by double digestion with *NheI* and *EcoRI* endonucleases. The target fragment SINH (789 bp) was confirmed on agarose gel (Fig. 2A). The p-SINH/SRFRP and p-TPA-SINH/TPA-SRFRP plasmids were identified separately by double digestion with *SalI/NotI* and *XmaI/NotI* endonucleases. The target fragments (778 bp for SRFRP and 847 bp for TPA-SRFRP) were sepa-



**Fig. 3.** The fusion proteins of SINH and SRFRP were expressed in HeLa cells. Lanes 1: pIRES; Lanes 2: p-SINH; Lanes 3: p-TPA-SINH/TPA-SRFRP; and Lanes 4: p-SINH/SRFRP.

rately confirmed on agarose gel (Figs. 2B and C). Sequence analysis further showed the successful construction of the three recombinant plasmids.

#### Assay of fusion protein

The protein expression of SINH and SRFRP was analyzed through western blot. The bands of SINH (about 29 kDa) and SRFRP (about 29 kDa) proteins were separately observed using their specific antibodies (Fig.3). The results showed that these proteins were expressed in HeLa cells.

#### Effect of INH immunization on anti-INH level in mouse sera

After immunization with respective vaccines, the level of anti-INH antibodies increased with each immunization in all groups except in the control. Anti-INH antibody levels peaked at 2 weeks after the third immunization. After the primary and second immunizations, mice immunized with the three vaccines (p-SINH, p-SINH/SRFRP and p-TPA-SINH/TPA-SRFRP) developed high levels of antibody. No significant difference was found among groups T, S, and P, but group P developed significantly higher antibody level than the two other groups 2 weeks after of the third immunization ( $P < 0.05$ ) (Table 1). In addition, the percentage of positive-immunized mice for INH was higher in group P (60%) than that in groups T and S (both 40%), 2 weeks after immunization and then peaked after the third immunization (Table 1).

#### Effect of RFRP immunization on the anti-RFRP level in mouse sera

The average level of anti-RFRP antibodies in group P and S reached at peak (Table 2), and the average antibodies level of mice in Group P was significantly higher than that in group S ( $P < 0.05$ ) at 6 weeks after the primary immunization (or 2 weeks after the third immunization). The percentages of positive immunized mice in groups P and S were 40% and 30%, respectively, 2 weeks after

the primary immunization. The percentage of positive anti-RFRP mice gradually increased with each immunization in groups P and S (Table 2).

#### Effect of INH and RFRP immunization on litter size of mice

The mean litter size of three successive generations in all immunized groups was significantly higher ( $P < 0.05$ ) than that of control group (Table 3). Among the vaccinated groups, the p-SINH/SRFRP vaccine caused a slight increase in the mean litter size of mice compared with that of the p-SINH in three successive generations. In addition, the mean litter size in group P evidently increased ( $P < 0.05$ ) compared with that in group T during the entire breeding period. No significant difference was found in litter size of mice between groups P and S (Table 3).

---

## Discussion

---

Previous studies showed that INH could inhibit follicular development and immunization by DNA vaccine encoding inhibin  $\alpha$  (1–32) can increase the quantity of large follicles and enhance litter size of animal slightly [10, 42]. In recent years, numerous studies have investigated GnIH (RFRP-3), which exhibits physiological function similar to that of INH and inhibit gonadotropin secretion, thereby affect the fertility of animals [8, 27]. But so far it has not been reported that the GnIH (RFRP) gene was used to construct a vaccine. In this study, we first constructed double expression DNA vaccines, namely, p-SINH/SRFRP, and p-TPA-SINH/TPA-SRFRP, which could produce better immunogenicity after immunization. The levels of INH and RFRP antibodies in sera were gradually raised with the increase in the number of immunization. The highest positive response rates of immunized mice were obtained with the p-TPA-SINH/TPA-SRFRP vaccine (100% as anti-INH and 90% as anti-RFRP, respectively) 2 weeks after the third immunization. These results have shown that fused protein (SINH, SRFRP) can elicit antibodies more efficiently, which could neutralize endogenous INH and GnIH (RFRP-3), respectively, and may promote follicular development. Interestingly, the positive response rate of mice (whether against INH or RFRP) was relatively higher compared with that reported in a study, in which pcISI vaccine harboring two copies of INH  $\alpha$  (1–32) induced positive immunization in 22.2% of rat popula-

**Table 1.** The P/N value of antibody against INH and ratio of mice with positive antibody after immunization of p-SINH (T), p-SINH/SRFRP (S), p-TPA-SINH/TPA-SRFRP (P), Saline (C)

Group	2 weeks		4 weeks		6 weeks	
	P/N value	(%) Ratio of positive antibody	P/N value	(%) Ratio of positive antibody	P/N value	(%) Ratio of positive antibody
T	2.114 ± 0.598 <sup>a</sup>	40 (4/10)	2.301 ± 0.591 <sup>a</sup>	50 (5/10)	2.764 ± 0.869 <sup>b</sup>	70 (7/10)
S	1.943 ± 0.487 <sup>a</sup>	40 (4/10)	2.333 ± 0.667 <sup>a</sup>	50 (5/10)	2.679 ± 0.949 <sup>b</sup>	80 (8/10)
P	2.294 ± 0.431 <sup>a</sup>	60 (6/10)	2.91 ± 1.132 <sup>a</sup>	70 (7/10)	3.578 ± 0.798 <sup>a</sup>	100 (10/10)
C	0.987 ± 0.169 <sup>c</sup>	0 (0/10)	1.1 ± 0.301 <sup>c</sup>	0 (0/10)	1.174 ± 0.379 <sup>c</sup>	0 (0/10)

The same vertical column with different letters indicate significant difference ( $P < 0.05$ ) between groups. 2 weeks=2 weeks after primary immunization ; 4 weeks=4 weeks after primary immunization or 2 weeks of the second immunization; 6 weeks=6 weeks after the primary immunization or 2 weeks after the third immunization. P/N: mean ± standard deviation.

**Table 2.** The P/N value of antibody against RFRP and ratio of mice with positive antibody after immunization of p-SINH/SRFRP (S), p-TPA-SINH/TPA-SRFRP (P), Saline (C)

Group	2 weeks		4 weeks		6 weeks	
	P/N value	(%) Ratio of positive antibody	P/N value	(%) Ratio of positive antibody	P/N value	(%) Ratio of positive antibody
S	1.914 ± 0.345 <sup>a</sup>	30 (3/10)	1.908 ± 0.423 <sup>a</sup>	40 (4/10)	2.16 ± 0.372 <sup>b</sup>	60 (6/10)
P	2.12 ± 0.676 <sup>a</sup>	40 (4/10)	2.167 ± 0.447 <sup>a</sup>	60 (6/10)	2.628 ± 0.68 <sup>a</sup>	90 (9/10)
C	0.987 ± 0.169 <sup>c</sup>	0 (0/10)	1.1 ± 0.301 <sup>c</sup>	0 (0/10)	1.17 ± 0.379 <sup>c</sup>	0 (0/10)

The same vertical column with different letters indicate significant difference ( $P < 0.05$ ) between groups. 2 weeks=2 weeks after primary immunization ; 4 weeks=4 weeks after primary immunization or 2 weeks of the second immunization; 6 weeks=6 weeks after the primary immunization or 2 weeks after the third immunization. P/N: mean ± standard deviation.

**Table 3.** Comparison of litter size of three successive generations after mice immunized with p-SINH (Group T), p-SINH/SRFRP (Group S), p-TPA-SINH/TPA-SRFRP (Group P) and saline (Group C)

Groups	The LS of the first generation	The LS of the second generation	The LS of the third generation
T	12.86 ± 1.86 <sup>b</sup>	13.14 ± 1.67 <sup>b</sup>	13.71 ± 1.31 <sup>b</sup>
S	14.00 ± 2.00 <sup>ab</sup>	14.29 ± 1.70 <sup>ab</sup>	15.14 ± 1.57 <sup>ab</sup>
P	14.71 ± 1.58 <sup>a</sup>	15.57 ± 1.99 <sup>a</sup>	16.00 ± 1.83 <sup>a</sup>
C	10.86 ± 1.68 <sup>c</sup>	11.00 ± 2.00 <sup>c</sup>	11.80 ± 1.57 <sup>c</sup>

The same vertical column with completely different letters indicate significant difference ( $P < 0.05$ ) between groups, and  $P > 0.05$  with same little letters. Data are presented as mean ± standard deviation.

tion, 2 weeks after the primary immunization [42]. This discrepancy may be related to the different inoculation methods employed; the previous study used intramuscular injection, whereas the present study utilized electroporation. Electroporation opens transient “pores” in the cell membrane, which allow macromolecules, such as DNA, to enter into the cytoplasm; the process is followed by the closure of the pores and retention of DNA inside the nucleus [40]. Electroporation can effectively increase the efficiency of gene expression and immunogenicity of DNA vaccines, resulting in strong antibody responses in rhesus macaques and human [3, 45], as confirmed by the current results. Furthermore, in this study, the antibodies level of anti-INH and anti-RFRP in mice immunized with p-TPA-SINH/TPA-SRFRP were

significantly higher than mice immunized with p-SINH/SRFRP at 2 weeks after the third immunization. The phenomenon is probably due to TPA addition into the recombinant plasmid. Previous studies found that DNA vaccine fused TPA can induce significant high levels of IgG antibodies [9, 14, 20, 21].

INH immunization can improve follicular development and litter size of animals [11, 24, 29]. Consistent with these results, in current study, litter size of mice immunized with p-SINH was significantly higher than that of control group. In addition, some studies showed that intravenous injection of GnIH (RFRP-3) reduced the release of FSH and LH [15]. In another experiment, mice injected subcutaneously with GnIH (2 µg/day) daily for 8 days exhibited a decrease in number of fol-

licles and corpora lutea [31]. In the present study, the mean litter size of mice inoculated with p-TPA-SINH/TPA-SRFRP expressing *INH* and *RFRP-3* was significantly higher than that of mice with inoculated p-SINH expressing solely *INH* in the whole breeding. It may be due to the addition of the *RFRP-3*, then resulted in increase of litter size after immunization with the p-TPA-SINH/TPA-SRFRP. Concurrently, it may be also due to the result of the addition of TPA, because the addition of TPA increased antibodies against both *INH* and *RFRP-3* (as mentioned above it). In addition, the DNA vaccine encoding *INH* and *RFRP-3* genes also exhibited certain potency in increasing litter size of mice, because the p-SINH/SRFRP induced slight increase in the litter size of mice compared with that of p-SINH during entire breeding period. Therefore, we infer that the neutralization of endogenous RFRP may promote follicular development and ovulation by either increasing the release of gonadotropins through direct action on the pituitary gland or on the gonads in a paracrine manner [6, 32, 34–36].

In conclusion, novel DNA vaccine p-TPA-SINH/TPA-SRFRP expressing *INH- $\alpha$*  (1–32) and *RFRP-3* gene were successfully constructed, exhibited better immunogenicity, and increased litter size of mice. Further studies will be required to verify whether addition of *RFRP-3* exert a significant effect in improving fertility of animals immunized with the novel p-TPA-SINH/TPA-SRFRP vaccine.

---

### Conflict of interest

---

The authors have no conflict of interest regarding any matter.

---

### Acknowledgments

---

This work was financially supported by National Natural Science Foundation of China (31272446) and Doctoral Fund of Ministry of Education (20120146130003).

---

### References

---

- Anjum, S., Krishna, A., and Tsutsui, K. 2014. Inhibitory roles of the mammalian GnIH ortholog RFRP3 in testicular activities in adult mice. *J. Endocrinol.* 223: 79–91. [Medline] [CrossRef]
- Bédécarrats, G.Y., McFarlane, H., Maddineni, S.R., and Ramachandran, R. 2009. Gonadotropin-inhibitory hormone receptor signaling and its impact on reproduction in chickens. *Gen. Comp. Endocrinol.* 163: 7–11. [Medline] [CrossRef]
- Bolhassani, A., Safaiyan, S., and Rafati, S. 2011. Improvement of different vaccine delivery systems for cancer therapy. *Mol. Cancer* 10: 3. [Medline] [CrossRef]
- Bonini, J.A., Jones, K.A., Adham, N., Forray, C., Artymyshyn, R., Durkin, M.M., Smith, K.E., Tamm, J.A., Boteju, L.W., Lakhani, P.P., Raddatz, R., Yao, W.J., Ogozalek, K.L., Boyle, N., Kouranova, E.V., Quan, Y., Vaysse, P.J., Wetzel, J.M., Brancheck, T.A., Gerald, C., and Borowsky, B. 2000. Identification and characterization of two G protein-coupled receptors for neuropeptide FF. *J. Biol. Chem.* 275: 39324–39331. [Medline] [CrossRef]
- Cicccone, N.A., Dunn, I.C., Boswell, T., Tsutsui, K., Ubuka, T., Ukena, K., and Sharp, P.J. 2004. Gonadotrophin inhibitory hormone depresses gonadotrophin alpha and follicle-stimulating hormone beta subunit expression in the pituitary of the domestic chicken. *J. Neuroendocrinol.* 16: 999–1006. [Medline] [CrossRef]
- Clarke, I.J. 2011. Control of GnRH secretion: one step back. *Front. Neuroendocrinol.* 32: 367–375. [Medline] [CrossRef]
- Clarke, I.J., Qi, Y., Puspita Sari, I., and Smith, J.T. 2009. Evidence that RF-amide related peptides are inhibitors of reproduction in mammals. *Front. Neuroendocrinol.* 30: 371–378. [Medline] [CrossRef]
- Clarke, I.J., Sari, I.P., Qi, Y., Smith, J.T., Parkington, H.C., Ubuka, T., Iqbal, J., Li, Q., Tilbrook, A., Morgan, K., Pawson, A.J., Tsutsui, K., Millar, R.P., and Bentley, G.E. 2008. Potent action of RFamide-related peptide-3 on pituitary gonadotropes indicative of a hypophysiotropic role in the negative regulation of gonadotropin secretion. *Endocrinology* 149: 5811–5821. [Medline] [CrossRef]
- Delogu, G., Li, A., Repique, C., Collins, F., and Morris, S.L. 2002. DNA vaccine combinations expressing either tissue plasminogen activator signal sequence fusion proteins or ubiquitin-conjugated antigens induce sustained protective immunity in a mouse model of pulmonary tuberculosis. *Infect. Immun.* 70: 292–302. [Medline] [CrossRef]
- Han, L., Mao, D.G., Zhang, D.K., Liang, A.X., Fang, M., Moaen-ud-Din, M., and Yang, L.G. 2008. Development and evaluation of a novel DNA vaccine expressing inhibin expressing inhibin alpha (1–32) fragment for improving the fertility in rats and sheep. *Anim. Reprod. Sci.* 109: 251–265. [Medline] [CrossRef]
- Han, L., Zhen, Y.H., Liang, A.X., Zhang, J., Riaz, H., Xiong, J.J., Guo, A.Z., and Yang, L.G. 2014. Oral vaccination with inhibin DNA delivered using attenuated *Salmonella choleraesuis* for improving reproductive traits in mice. *J. Basic Microbiol.* 54: 962–968. [Medline] [CrossRef]
- Hinuma, S., Shintani, Y., Fukusumi, S., Iijima, N., Matsumoto, Y., Hosoya, M., Fujii, R., Watanabe, T., Kikuchi, K., Terao, Y., Yano, T., Yamamoto, T., Kawamata, Y., Habata, Y., Asada, M., Kitada, C., Kurokawa, T., Onda, H., Nishimura, O., Tanaka, M., Ibata, Y., and Fujino, M. 2000. New neu-

- ropeptides containing carboxy-terminal RFamide and their receptor in mammals. *Nat. Cell Biol.* 2: 703–708. [Medline] [CrossRef]
13. Ishigame, H., Medan, M.S., Kawaguchi, M., Fukuda, A., Watanabe, G., Arai, K.Y., and Taya, K. 2005. Induction of superovulation by immunoneutralization of endogenous inhibin in immature rats. *J. Reprod. Dev.* 51: 559–566. [Medline] [CrossRef]
  14. Jiang, C., Magee, D.M., Ivey, F.D., and Cox, R.A. 2002. Role of signal sequence in vaccine-induced protection against experimental coccidioidomycosis. *Infect. Immun.* 70: 3539–3545. [Medline] [CrossRef]
  15. Kadokawa, H., Shibata, M., Tanaka, Y., Kojima, T., Matsumoto, K., Oshima, K., and Yamamoto, N. 2009. Bovine C-terminal octapeptide of RFamide-related peptide-3 suppresses luteinizing hormone (LH) secretion from the pituitary as well as pulsatile LH secretion in bovines. *Domest. Anim. Endocrinol.* 36: 219–224. [Medline] [CrossRef]
  16. Kusina, N.T., Meyer, R.L., Carlson, K.M., and Wheaton, J.E. 1995. Passive immunization of ewes against an inhibin-like peptide increases follicle-stimulating hormone concentrations, ovulation rate, and prolificacy in spring-mated ewes. *J. Anim. Sci.* 73: 1433–1439. [Medline]
  17. Kutzler, M.A. and Weiner, D.B. 2008. DNA vaccines: ready for prime time? *Nat. Rev. Genet.* 9: 776–788. [Medline] [CrossRef]
  18. Liang, A., Cao, S., Han, L., Yao, Y., Moaen-Ud-Din, M., and Yang, L. 2008. Construction and evaluation of the eukaryotic expression plasmid encoding two copies of somatostatin genes fused with hepatitis B surface antigen gene S. *Vaccine* 26: 2935–2941. [Medline] [CrossRef]
  19. Li, X., Su, J., Lei, Z., Zhao, Y., Jin, M., Fang, R., Zheng, L., and Jiao, Y. 2012. Gonadotropin-inhibitory hormone (GnIH) and its receptor in the female pig: cDNA cloning, expression in tissues and expression pattern in the reproductive axis during the estrous cycle. *Peptides* 36: 176–185. [Medline] [CrossRef]
  20. Li, Z., Howard, A., Kelley, C., Delogu, G., Collins, F., and Morris, S. 1999. Immunogenicity of DNA vaccines expressing tuberculosis proteins fused to tissue plasminogen activator signal sequences. *Infect. Immun.* 67: 4780–4786. [Medline]
  21. Luo, M., Tao, P., Li, J., Zhou, S., Guo, D., and Pan, Z. 2008. Immunization with plasmid DNA encoding influenza A virus nucleoprotein fused to a tissue plasminogen activator signal sequence elicits strong immune responses and protection against H5N1 challenge in mice. *J. Virol. Methods* 154: 121–127. [Medline] [CrossRef]
  22. Maddineni, S.R., Ocón-Grove, O.M., Krzysik-Walker, S.M., Hendricks, G.L. 3rd., and Ramachandran, R. 2008. Gonadotropin-inhibitory hormone (GnIH) receptor gene is expressed in the chicken ovary: potential role of GnIH in follicular maturation. *Reproduction* 135: 267–274. [Medline] [CrossRef]
  23. McAleer, W.J., Buynak, E.B., Maigetter, R.Z., Wampler, D.E., Miller, W.J., and Hilleman, M.R. 1984. Human hepatitis B vaccine from recombinant yeast. *Nature* 307: 178–180. [Medline] [CrossRef]
  24. Medan, M.S., Takedom, T., Aoyagi, Y., Konishi, M., Yaza-wa, S., Watanabe, G., and Taya, K. 2006. The effect of active immunization against inhibin on gonadotropin secretions and follicular dynamics during the estrous cycle in cows. *J. Reprod. Dev.* 52: 107–113. [Medline] [CrossRef]
  25. Medan, M.S., Watanabe, G., Sasaki, K., Nagura, Y., Sakaime, H., Fujita, M., Sharawy, S., and Taya, K. 2003. Effects of passive immunization of goats against inhibin on follicular development, hormone profile and ovulation rate. *Reproduction* 125: 751–757. [Medline] [CrossRef]
  26. Oishi, H., Klausen, C., Bentley, G.E., Osugi, T., Tsutsui, K., Gilks, C.B., Yano, T., and Leung, P.C.K. 2012. The human gonadotropin-inhibitory hormone ortholog RFamide-related peptide-3 suppresses gonadotropin-induced progesterone production in human granulosa cells. *Endocrinology* 153: 3435–3445. [Medline] [CrossRef]
  27. Pineda, R., Garcia-Galiano, D., Sanchez-Garrido, M.A., Romero, M., Ruiz-Pino, F., Aguilar, E., Dijcks, F.A., Blumenröhr, M., Pinilla, L., van Noort, P.I., and Tena-Sempere, M. 2010. Characterization of the inhibitory roles of RFRP3, the mammalian ortholog of GnIH, in the control of gonadotropin secretion in the rat: in vivo and in vitro studies. *Am. J. Physiol. Endocrinol. Metab.* 299: E39–E46. [Medline] [CrossRef]
  28. Robertson, J.S. and Griffiths, E. 2001. Assuring the quality, safety, and efficacy of DNA vaccines. *Mol. Biotechnol.* 17: 143–149. [Medline] [CrossRef]
  29. Sasaki, K., Medan, M.S., Watanabe, G., Sharawy, S., and Taya, K. 2006. Immunization of goats against inhibin increased follicular development and ovulation rate. *J. Reprod. Dev.* 52: 543–550. [Medline] [CrossRef]
  30. Singh, P., Krishna, A., Sridaran, R., and Tsutsui, K. 2011. Immunohistochemical localization of GnRH and RFamide-related peptide-3 in the ovaries of mice during the estrous cycle. *J. Mol. Histol.* 42: 371–381. [Medline] [CrossRef]
  31. Singh, P., Krishna, A., and Tsutsui, K. 2011. Effects of gonadotropin-inhibitory hormone on folliculogenesis and steroidogenesis of cyclic mice. *Fertil. Steril.* 95: 1397–1404. [Medline] [CrossRef]
  32. Tsutsui, K., Bentley, G.E., Bedecarrats, G., Osugi, T., Ubuka, T., and Kriegsfeld, L.J. 2010. Gonadotropin-inhibitory hormone (GnIH) and its control of central and peripheral reproductive function. *Front. Neuroendocrinol.* 31: 284–295. [Medline] [CrossRef]
  33. Tsutsui, K., Saigoh, E., Ukena, K., Teranishi, H., Fujisawa, Y., Kikuchi, M., Ishii, S., and Sharp, P.J. 2000. A novel avian hypothalamic peptide inhibiting gonadotropin release. *Biochem. Biophys. Res. Commun.* 275: 661–667. [Medline] [CrossRef]
  34. Tsutsui, K., Ubuka, T., Bentley, G.E., and Kriegsfeld, L.J. 2012. Gonadotropin-inhibitory hormone (GnIH): discovery, progress and prospect. *Gen. Comp. Endocrinol.* 177: 305–314. [Medline] [CrossRef]
  35. Ubuka, T., Son, Y.L., Bentley, G.E., Millar, R.P., and Tsutsui, K. 2013. Gonadotropin-inhibitory hormone (GnIH), GnIH receptor and cell signaling. *Gen. Comp. Endocrinol.* 190: 10–17. [Medline] [CrossRef]
  36. Ubuka, T., Son, Y.L., Tobari, Y., Narihiro, M., Bentley, G.E.,



- Kriegsfeld, L.J., and Tsutsui, K. 2014. Central and direct regulation of testicular activity by gonadotropin-inhibitory hormone and its receptor. *Front. Endocrinol. (Lausanne)* 5: 8. [[Medline](#)]
37. Ubuka, T., Son, Y.L., Tobar, Y., and Tsutsui, K. 2012. Gonadotropin-inhibitory hormone action in the brain and pituitary. *Front. Endocrinol. (Lausanne)* 3: 148. [[Medline](#)]
38. Ubuka, T., Ukena, K., Sharp, P.J., Bentley, G.E., and Tsutsui, K. 2006. Gonadotropin-inhibitory hormone inhibits gonadal development and maintenance by decreasing gonadotropin synthesis and release in male quail. *Endocrinology* 147: 1187–1194. [[Medline](#)] [[CrossRef](#)]
39. Valenzuela, P., Coit, D., Medina-Selby, M.A., Kuo, C.H., Van Nest, G., Lyn Burke, R., Bull, P., Urdea, M.S., and Graves, P.V. 1985. Antigen engineering in yeast: synthesis and assembly of hybrid hepatitis B surface antigen-herpes simplex 1 gD particles. *Nat. Biotechnol.* 3: 323–326 [[CrossRef](#)].
40. Wallace, M., Evans, B., Woods, S., Mogg, R., Zhang, L., Finnefrock, A.C., Rabussay, D., Fons, M., Mallee, J., Mehrotra, D., Schödel, F., and Musey, L. 2009. Tolerability of two sequential electroporation treatments using MedPulser DNA delivery system (DDS) in healthy adults. *Mol. Ther.* 17: 922–928. [[Medline](#)] [[CrossRef](#)]
41. Wang, H., Herath, C.B., Xia, G., Watanabe, G., and Taya, K. 2001. Superovulation, fertilization and in vitro embryo development in mice after administration of an inhibin-neutralizing antiserum. *Reproduction* 122: 809–816. [[Medline](#)] [[CrossRef](#)]
42. Wang, S.L., Han, L., Ahmad, S., Cao, S.X., Xue, L.Q., Xing, Z.F., Feng, J.Z., Liang, A.X., and Yang, L.G. 2012. Effect of a DNA vaccine harboring two copies of inhibin  $\alpha$  (1-32) fragments on immune response, hormone concentrations and reproductive performance in rats. *Theriogenology* 78: 393–401. [[Medline](#)] [[CrossRef](#)]
43. Xue, C.L., Liang, A.X., Mao, D.G., Han, L., Liu, X.B., Zhang, J., and Yang, L.G. 2010. Effect of genetic adjuvants on immune response, growth and hormone levels in somatostatin DNA vaccination-induced Hu lambs. *Vaccine* 28: 1541–1546. [[Medline](#)] [[CrossRef](#)]
44. Zhao, S., Zhu, E., Yang, C., Bentley, G.E., Tsutsui, K., and Kriegsfeld, L.J. 2010. RFamide-related peptide and messenger ribonucleic acid expression in mammalian testis: association with the spermatogenic cycle. *Endocrinology* 151: 617–627. [[Medline](#)] [[CrossRef](#)]
45. Zhao, Y.G., Peng, B., Deng, H., Chen, G., Yang, F., Shao, M., Lu, H., Li, Y., Peng, J., Xu, L., and Xu, Y. 2006. Anti-HBV immune responses in rhesus macaques elicited by electroporation mediated DNA vaccination. *Vaccine* 24: 897–903. [[Medline](#)] [[CrossRef](#)]