Potent effect of KISS1-54 DNA vaccine compared with KISS1-10 DNA vaccine in inhibiting gonadal growth and sexual behavior in male rats

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Abstract: Our purpose was to compare the effect of KISS1-54 and KISS1-10 DNA vaccines in inhibiting gonadal growth and sexual behavior of male rats. Fifteen male rats were randomly divided into KISS1-54 (Group T1), KISS1-10 (Group T2) DNA vaccine groups and control group (Group C), in which the rats were orally given respectively KISS1-54, KISS1-10 DNA vaccines fused tPA signal peptide and the empty vector vaccine at a dose of 5×10^9 CFU/male rat at weeks 0, 3 and 6 of this study. Blood samples were collected before primary immunization and at weeks 3 and 9 after primary immunization. This study showed that both KISS1-54 and KISS1-10 DNA vaccines induced humoral immune response and the antibody titre in T1 group was significantly higher than those in T2 and C groups. Rats in T1 group has significantly lower serum kisspeptin and testosterone levels and slower growth of scrotum width than that in T2 and C groups. The frequency of mounting with females in T1 group was significantly lower than that in control group, and no significant difference was observed between T2 and C groups. The number of vaginal plugs in T1 group was obviously lower than that in T2 and C groups. These results suggest that oral KISS1-54 DNA vaccine was more effective than KISS1-10 DNA vaccine in inhibition of gonadal growth and sexual behavior in rats.

1. Introduction

Kisspeptins, encoded by KISS1 gene, play an important role on the puberty onset of animal and the maintenance of adult reproductive activity [1-2]. Kisspeptins secreted by the hypothalamus can regulate the secretion of hypothalamic gonadotropin-releasing hormone (GnRH) through binding with G-protein coupled receptor 54 (GPR54) [3]. The different length's kisspeptins (54, 14, 13 or 10-amino acids residue peptides) has highly conserved among mammal and share the decapeptide sequence of the common C terminal [4]. In vitro, these kisspeptins has similar affinity for binding GPR54 receptors [5]. However, in vivo, kisspeptin-54 has a higher and prolonged effect than kisspeptin-10 on stimulating LH releasing, which may be due to higher half-life or stronger ability in crossing blood brain barrier [6-7].

Immunization against KISS1 DNA vaccine can effectively inhibit male sexual behavior. Active immunization of KISS1 DNA vaccine encoding 54-amino acids can effectively suppress ram gonadal function and sexual behavior [8-9]. Kisspeptins with different amino acid residue can produce different physiological effect [7,10]. However, It remains unclear whether KISS1-54 DNA vaccine can produce stronger immunocastration in male animals than the KISS1-10 DNA vaccines.

The tissue plasminogen activator (tPA) signal peptide and attenuated *Salmonella* delivery vector can enhance the immune response of DNA vaccines and save costs. tPA signal peptide can transfer more antigens encoded by DNA vaccines to extracellular, which increases the chance that antigen will be captured by antigen-presenting cells (APC) and improve immunogenicity of the DNA vaccines [11]. DNA vaccines delivered by attenuated *Salmonella* do not need the extraction and

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purification of plasmids, thereby simplifying operation and reducing costs [12-13], and the bacterial carrier debris from attenuated *Salmonella* as an adjuvant can up-regulate expression of antigen, thereby improving immune response of DNA vaccines [14]. The attenuated *Salmonella choleraesuis* C500 delivery vector with deletion of *asd* (aspartate-semialdehyde dehydrogenase) and *crp* (cAMP receptor protein) has been widely used into construction of many DNA vaccines [12]. However, tPA signal peptide and attenuated *Salmonella* has not been applied in the construction of KISS1-54 and -10 DNA vaccines.

The study aims to compare the effect of oral KISS1-54 and -10 DNA vaccines fused tPA signal peptide on rats immunocastration. The immunocastration effect of oral DNA vaccines was evaluated in terms of anti-kisspeptin antibody response, serum kisspeptin and testosterone levels, scrotum length and width, frequency of mounting females and number of female vaginal plugs after mating.

2. Material and Methods

2.1 Preparation of oral DNA vaccines and immunization of male rats

Oral KISS1-54 and -10 DNA vaccines, C500 (pVAX-tPA-HBsAg-S-2KISS1-54-asd) and C500 (pVAX-tPA-HBsAg-S-2KISS1-10-asd), were successfully constructed, in which the tPA signal peptide, *HBsAg-S-2KISS1* fusion gene (two copies of KISS1-54 or KISS1-10 gene were inserted into the hepatitis B surface antigen S gene) and *FLAG* protein label gene were inserted into the pVAX1-asd vector and then were electroporated into the mutant attenuated *Salmonella choleraesuis* C500 with deletion of *asd* and *crp* gene. The oral KISS1-54 and -10 DNA vaccines were identified by PCR and sequencing before animal immunization. Fifteen specific-pathogen-free (SPF) male SD rats (3-weeks-old) were purchased from Chongqing Academy of Chinese Materia Medica (Chongqing, China) and raised in the Experimental Animals House of Southwest University (Chongqing, China) according to the guidelines of the Committee on the Care and Use of Laboratory Animals of China. One week after caging, these male rats were randomly, equally divided into KISS1-54 (Group T1), KISS1-10 (Group T2) vaccine groups and control group (Group C) and were separately administered orally KISS1-54, -10 DNA vaccines and C500 (pVAX-asd) empty vector vaccine with a dose of 5×10^9 CFU. These rats were boosted twice with an interval of three weeks and all rats were orally administered 1 mL of 7.5% sodium bicarbonate solution to neutralize gastric acid at 30 min before immunization. Before primary immunization and at weeks 3 and 9 after primary immunization, serum samples were obtained by retroorbital bleeding and centrifugation at 1157 × g and 4 °C for 10 min.

2.2 Detection of anti-kisspeptin antibody

Serum specific anti-kisspeptin antibody titres was detected by an indirect enzyme-linked immunosorbent assay [8,12]. Kisspeptin-54 and kisspeptin-10 proteins were artificially synthesized and used as the coating antigen (Apeptide Company, Shanghai, China). In brief, synthetic kisspeptin-54 or kisspeptin-10 proteins were coated the 96-well plate with 100 ng/well at 4 °C overnight. After washing, these antigens were blocked with 1% bovine serum albumin at 37 °C for 1 h. Then, serum samples were serially diluted in PBST (1:25, 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600 and 1:3200) and then 100 μL were added to every well. These pre-immune serum samples were used as a negative control. Then, HRP-conjugated goat anti-rat IgG antibodies (Abbkine, Inc., Redlands, CA, USA) were added to the plate and then were incubated at 37 °C for 1 h followed by Tetramethylbenzidine substrate solution at 37 °C for 25 min. The reaction was terminated by the 2 M H_2SO_4 and the absorbance values were detected at 450 nm by an ELISA reader (Hercules, CA, USA). Endpoint titre was expressed as the reciprocal of highest serum dilution which has greater absorbance than the mean plus two standard deviations of the negative control samples at similar dilution [12].

2.3 Detection of hormone, scrotal length and width

Serum kisspeptin and testosterone concentrations were measured by radioimmunoassay (Beijing Sino-UK Institute of Biological Technology, Beijing, China). The intra- and inter-assay coefficients of variation were both less than 15%. All male rats were fasted 12 h and then scrotal length and width was measured with vernier caliper through pushing the testicles to the bottom of scrotum at weeks 0, 3 and 9 after primary immunization [15].

2.4 Detection of sexual behavior

Estrous female rats induced by administrating of estradiol benzoate were individually placed in the cage of male for observing sexual behavior of all subject male rats for five days, the mounting frequency of male rats and the number of vaginal plugs of mated female rats were recorded [16].

2.5 Statistical analysis

All data was analysed using ANOVA and Duncan's multiple-range test by SAS 8.1 system software (SAS Institute, Inc., Cary, NC, USA). The level of statistically significance was taken as $p < 0.05$ and all results were expressed as mean ± SD.

3. Results

3.1 Vaccine identification and anti-kisspeptin response

Fusion genes of KISS1 DNA vaccines, *tPA-HBsAg-S-2KISS1-54* and *tPA-HBsAg-S-2KISS1-10*, were detected correctly by restriction endonuclease digestion and sequence (Fig. 1). Anti-kisspeptin antibody titres in T1 group were significantly higher than that in T2 and control group at weeks 3 and 9 after primary immunization (Table 1, $p < 0.05$), and antibody titres in T2 group were also significantly higher than that in control group (Table 1, $p < 0.05$).

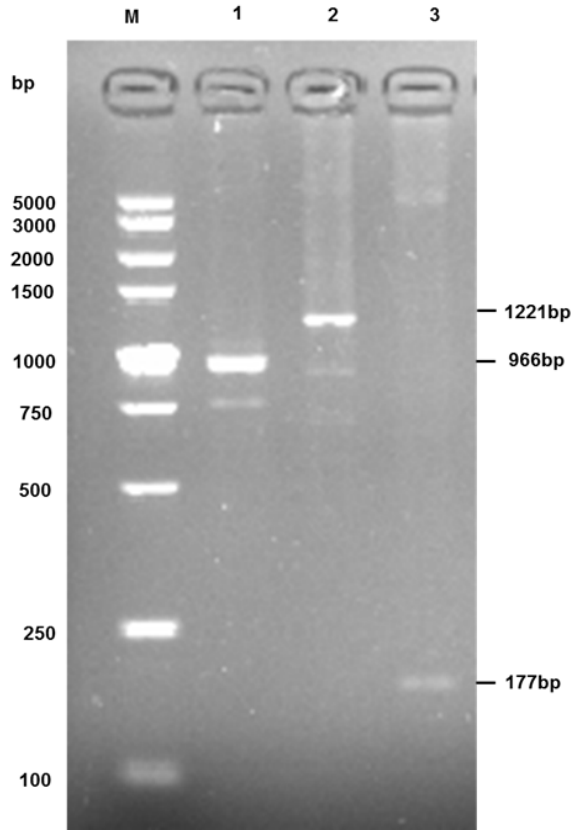

Fig. 1. Identification of recombinant oral KISS1-54, KISS1-10 DNA vaccine and C500 (pVAX-asd) by PCR with *T7* and *BGH* primers. Three bands, *tPA-HBsAg-S-2KISS1-10* (966 bp), *tPA-HBsAg-S-2KISS1-54* (1221 bp) and *multiple cloning sites* (177 bp), are shown in Lanes 1, 2 and 3.

Table 1. Antibody titers in KISS1-54, -10 DNA vaccine groups and control group at week 3 and 9 after primary immunization.

Traits	Group		
	T1	T2	C
3W	100.00±0.00[a]	70.00±27.39[b]	0.00±0.00[c]
9W	250.00±100.00[a]	140.00±54.77[b]	0.00±0.00[c]

Values scripted with different letters denote significant difference between groups ($p < 0.05$).

3.2 Serum kisspeptin and testosterone levels

The kisspeptin concentrations in T1 group were significantly lower than that in T2 group and control group at week 3 after primary immunization (Fig. 2, $p < 0.05$), and the kisspeptin concentrations in T2 group was significantly lower than that in control group at week 3 after primary immunization (Fig. 2, $p < 0.05$). No significant difference was observed among these groups at week 9 after primary immunization (Fig. 2, $p > 0.05$). Testosterone concentrations in T1 group were significantly lower than that in control group at weeks 3 and 9 after primary immunization (Fig. 3, $p < 0.05$), and testosterone concentrations in T2 group was significantly lower than that in control group only at week 3 after primary immunization (Fig. 3, $p < 0.05$).

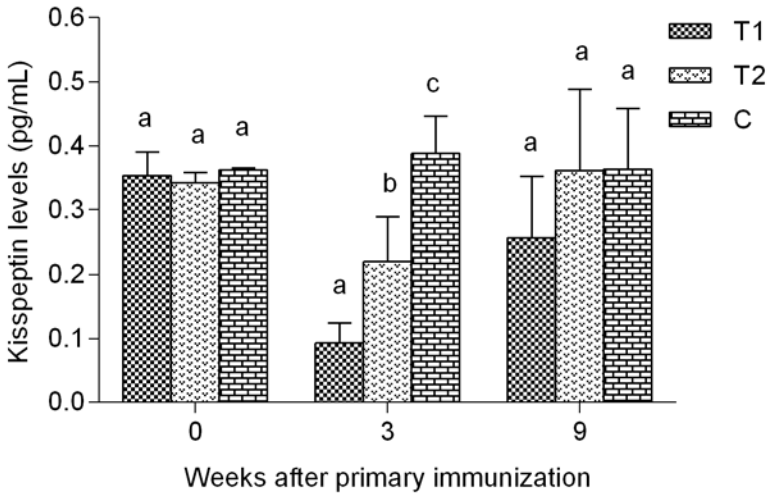

Fig. 2. Serum kisspeptin (pg/mL) concentration in the KISS1-54, -10 DNA vaccine groups and control group at weeks 0, 3 and 9 after primary immunization. Values scripted with different letters denote significant difference between groups ($p < 0.05$).

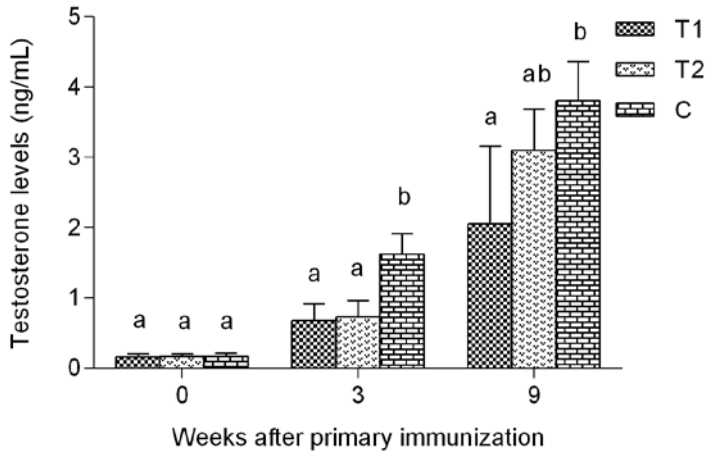

Fig. 3. Serum testosterone (ng/mL) concentration in KISS1-54, -10 DNA vaccine groups and control group at weeks 0, 3 and 9 after primary immunization. Values scripted with different letters denote significant difference between groups ($p < 0.05$).

3.3 Physiological effect of immunization

The scrotum width in T1 group was significantly slower than that in T2 group and control group at weeks 3 after primary immunization (Table 2, $p < 0.05$), and was still significantly slower than that in control group at weeks 9 after primary immunization (Table 2, $p < 0.05$). However, no significant difference was observed between T2 group and control group at weeks 3 and 9 after primary immunization (Table 2, $p > 0.05$). In addition, no significant difference in scrotum length was observed among these groups at weeks 3 and 9 after primary immunization (Table 2, $p > 0.05$).

Table 2. Scrotal length and width in KISS1-54, KISS1-10 DNA vaccine groups and control group at week 3 and 9 after primary immunization.

Traits	Group		
	T1	T2	C
Scrotal width on different immunization weeks			
3W	0.15 ± 0.07^{a}	0.35 ± 0.11^{b}	0.33 ± 0.16^{b}
9W	0.19 ± 0.09^{a}	0.33 ± 0.17^{ab}	0.48 ± 0.23^{b}
Scrotal length on different immunization weeks			
3W	0.33 ± 0.2^{a}	0.32 ± 0.29^{a}	0.50 ± 0.39^{a}
9W	0.28 ± 0.25^{a}	0.28 ± 0.25^{a}	0.56 ± 0.34^{a}

Values scripted with different letters denote significant difference between groups ($p < 0.05$).

3.4 Sexual behavior changes

Frequency of mounting with female rats in T1 group was significantly lower than that in control group, and no significant difference was observed between T2 group and control group (Table 3, $p > 0.05$). The number of vaginal plugs in T1 group was obviously lower than that in T2 group and control group. However, no significant difference was observed between these groups (Table 3, $p > 0.05$).

Table 3. Effect of immunization with KISS1-54 and KISS1-10 DNA vaccine on rats sex behaviour.

Traits	Mouting	Vaginal plugs
T1	0.25 ± 0.10^{a}	0.25 ± 0.50^{a}
T2	0.28 ± 0.11^{ab}	0.40 ± 0.55^{a}
C	0.40 ± 0.00^{b}	0.60 ± 0.55^{a}

Values scripted with different letters denote significant difference between groups ($p < 0.05$).

4. Discussion

Immunization of KISS1 DNA vaccines can effectively inhibit aggressive and sexual behavior of male animals, which represents a novel and low-cost method on animal castration [8-9]. The kisspeptin 54 has higher and prolonged stimulation of hormone secretion of HPG axis than that kisspeptin 10 [6-7,10]. However, it is still unclear for the immunocastration effect of KISS1-10 DNA vaccine. Therefore, we developed the oral KISS1-10 DNA vaccine and compare its immunocastration effect with oral KISS1-54 DNA vaccine on male rats.

The oral KISS1-54 DNA vaccine induced stronger humoral immune response and more effective suppression on the secretion of kisspeptin and testosterone compared with that oral KISS1-10 DNA vaccine. Antibody titers in KISS1-54 DNA vaccine group were significantly higher than that in KISS1-10 DNA vaccine and control groups. Serum kisspeptin levels in KISS1-54 DNA vaccine group were significantly lower than that in KISS1-10 DNA vaccine and control groups, and the serum testosterone levels in KISS1-54 DNA vaccine group were significantly lower than that in

control group at weeks 3 and 9 after primary immunization. However, the serum testosterone levels in KISS1-10 DNA vaccine group were only significantly lower than that in control group at week 3 after primary immunization. Other research also shows that immunization against KISS1 or GnRH DNA vaccines can induce humoral immune response and then suppress the testosterone secretion [9,17-18]. Our results indicate that KISS1-54 DNA vaccine can inhibit of testosterone secretion more effectively than that oral KISS1-10 DNA vaccine by the neutralisation effect of anti-kisspeptin antibodies on endogenous kisspeptins.

Scrotum length and width were detected to compare the effect of oral KISS1-54 and KISS1-10 DNA vaccines on suppressing gonadal growth. Scrotum width in KISS1-54 DNA vaccine group was significantly lower than that in KISS1-10 DNA vaccine group and control group. However, no significant difference was observed between KISS1-10 DNA vaccine group and control group. Other studies also showed immunization of hormone vaccines can reduce growth rate of the scrotum [9,18]. Our results imply that immunization against KISS1-54 DNA vaccine can more effectively inhibit growth and development of testes than that KISS1-10 DNA vaccine.

Immunization of oral KISS1-54 DNA vaccine can more effectively inhibit sexual behavior of male rats than that KISS1-10 DNA vaccine. The frequency of mounting female rats in KISS1-54 DNA vaccine group was significantly lower than that in control group. However, no significant difference was observed between KISS1-10 DNA vaccine and control groups. The number of vaginal plugs in KISS1-54 DNA vaccine group was obviously lower than that in KISS1-10 DNA vaccine and control groups. However, no significant difference was observed between these groups, which may be due to weak immune response induced by individual animals.

5. Summary

We developed the novel oral KISS1-54 and KISS1-10 DNA vaccines fused tPA signal peptide, and the immunization against KISS1-54 DNA vaccine can more effectively suppress the gonadal growth and sexual behavior of male animals than that oral KISS1-10 DNA vaccine. Further studies should focus on the molecular mechanism of immunocastration of KISS1-54 DNA vaccine.

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