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RESEARCH ARTICLE

Effect of Active Immunization Using Gonadotropin-Releasing Hormone 2-Multiple Antigen Peptide with Different Adjuvants on the Reproductive Functions of Male Rats

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A B S T R A C T

This study was aimed to compare the effects of different adjuvants on male reproductive function in rats immunized using GnRH2-MAP. Male Sprague-Dawley rats were assigned to control, ISA 201 adjuvant and Gel 01 adjuvant groups along with GnRH2-MAP. The serum testosterone and antibody titers were measured every 2-week by ELISA, the testes were weighed and histologically analyzed. mRNA expression levels in the hypothalamus, pituitary, and testes were determined by RT-qPCR. Among the three groups, the ISA 201 adjuvant group exhibited the lowest testosterone concentration ($P<0.05$), the highest antibody titer ($P<0.05$), and testis atrophy. *GnRH* mRNA expression in the hypothalamus; *GnRH-R*, *FSH-β*, and *LH-β* expression in the pituitary; and *FSH-R*, *LH-R*, and *3β-HSD* expression in the testes were significantly lower in this group ($P<0.01$). Additionally, several other genes showed significant differential expression in the ISA 201 adjuvant group than in the other two groups. It is concluded that the GnRH2-MAP vaccine emulsified with ISA 201 adjuvant is more effective than that administered with Gel 01 adjuvant.

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INTRODUCTION

It is well known fact that the traditional castration methods many times induce serious wound, stress and infection to livestock. Then, many studies have found that exogenous GnRH can stimulate the production of specific antibodies that neutralize endogenous GnRH (Han *et al*., 2015). GnRH-I, one of the 3 GnRH types (GnRH-I, GnRH-II, GnRH-III), is sythesized and secreted by hypothalamus, leading to regulate the process of reproduction, which were used to inhibit the expression of all downstream hormones (luteinizing hormone and testicular steroids) (Wagner and Claus, 2004). From so many previous studies, it is clear that the decade peptide, GnRH-I, namely pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, it couldn't content our demand in stability and continuity of GnRH castration vaccines. GnRH2, in this study, was synthesized by two GnRH-I monomers and linked to the unique radioactive structure of MAP to synthesize the Gonadotropin-releasing hormone 2-mutiple antigen peptide (GnRH2-MAP) vaccine in the laboratory, recently. In order to enhance the immunogenicity, most vaccines are always supplemented with adjuvants which enhance the immunocastration effect. It is disappointing that the most effective GnRH vaccine has not been studied due to many kinds of antigen and adjuvant.

There is a wide variety of adjuvants, in recent years, complete Freund's adjuvant (CFA) and aluminum hydroxide ($Al(OH)_3$) have been the most commonly used ones. For example, boars injected with the maltose-binding protein GnRH6 (MBP-GnRH6) with $Al(OH)_3$ adjuvant demonstrated reduced scrotal circumference, antibody concentrations, and testosterone levels compared to the MBP controls (Jiang *et al*., 2015). In addition, GnRH lipopeptide vaccine plus CFA induced significantly high serum immunoglobulin G (IgG) titers *in vivo*. Both

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adjuvants are associated with a higher immune response, but CFA demonstrated a better immune response than $Al(OH)_3$ (Goodwin *et al*., 2014). In the case of vaccines supplemented with CFA and Titermax, both adjuvants induced similar T levels but the former produced a relatively strong inflammatory response (Cribbs *et al*., 2003).

In brief, previous reports have demonstrated that different types of adjuvants exert different effects on immunocastration. Although there are many studies about GnRH vaccines supplemented with adjuvants, only three tests just studied the effect of different adjuvants to GnRH immunocastration vaccine until now. One study (Finnerty *et al*., 1994) compared the effects of two adjuvants on a single GnRH vaccine. Another research (Ferro *et al*., 2004) found that 85/15-poly (lactide-co-glycolide acid) and triacetein (PLGA) was the most effective five adjuvants analyzed in their study. Moreover, modified Freund's complete adjuvant was less efficient than CpG ODN 2006 in Freund's incomplete adjuvant, which was administered to female rats with two booster injections and higher antibody concentration (Conforti *et al*., 2007). Moreover, there was a recent study proved that GnRH2-MAP vaccine can affect the reproductive function of Sprague-Dawley (SD) male rats by influencing the endogenous GnRH (Yao *et al*., 2018).

However, GnRH2-MAP lacks research on immunological castration. In the current study, we have chosen two commercially available adjuvants to enhance the immunogenicity of GnRH2-MAP. The water-in-oil adjuvant Montanide ISA™ 201 VG (ISA 201) is a compound of mannitol from plants and the emulsifier refined from pure oleic acid with several advantages, and it can prolong antigen-release time without inflammatory response at the site of injection. Montanide™ gel 01(Gel 01) is a type of water-soluble dispersion synthesized by polyacrylic acid without any animal-origin components. It is easy to inject and disperse and is associated with improved safety and requires a lower dosage.

Therefore, in this study, the effects of ISA 201 and Gel 01 adjuvants on the GnRH2- MAP vaccine castration in male rats were compared, which is expected to provide a viable vaccine for castration.

MATERIALS AND METHODS

Experimental animals: Twenty-day-old male SD rats with similar weight (35.0±3.0g) were obtained from the Experimental Animal Center of Anhui Medical University and housed at 22°C in a fixed 12:12 h light: dark cycle with *ad libitum* access to food and water in a comfortable environment. The study was implemented strictly in accordance with the guidelines formulated by the China Control on Animal Care.

Immunization products and procedures: The amino acid sequence of GnRH2 was designed according to Oonk's description (Oonk *et al*., 1998). Then GnRH2-MAP antigen was synthesized using Fmoc chemistry (Pau *et al*., 2007). All experimental rats were randomly divided into ISA 201 adjuvant group (n=36), Gel 01 adjuvant group (n=36) and control group (n=36). The ISA 201 adjuvant group was inoculated with a mixture of ISA 201 adjuvant and GnRH2-MAP; Gel 01 adjuvant group was inoculated with a mixture of Gel 01 auxiliary and GnRH2-MAP; control group was inoculated with a mixture of physiological saline and GnRH2-MAP ,per rat was injected with mixture at a dose of 1 ml of which GnRH-MAP was 100 μg (Han *et al*., 2015; Yao *et al*., 2018). The first immunization was administered via subcutaneous injection in many sites of the back of 20 days rats, followed by two booster doses at 2-week intervals, with a total immunization of 3 times per rat.

Collection and processing of samples: Six SD rats were randomly selected from each group every 2-week until the end of the experiment (lasts 84 days). After anesthetized, the rats were sacrificed and blood, testes, as well as hypothalamus and pituitary, were collected. Blood samples were centrifuged for the separation of sera and stored at -20°C. The pituitary, hypothalamus and left testis of the rat were weighed and measured. All tissues were stored at -80°C. The right testis were made into paraffin sections and subjected to hematoxylin and eosin (HE) staining. All sections were photographed with a camera (Motic, HongKong, China) fitted in the microscope.

Detection of antibody titer and Testosterone (T) concentration: Serum anti-GnRH antibody and T titers were quantified using a sandwich Enzyme linked immunosorbent assay (ELISA) kit for antibodies (sensitivity, 1.0 mIU/ml; Lianshuo, Shanghai, China) and T (sensitivity, 0.1 nmol/l; Yuanye, Shanghai, China). After preparing standard, sample and blank wells at room temperature, the standard substance was added to the standard wells while serum (diluent, 1:50) was added to the sample wells (50 ul/well). Then, horseradish peroxidase-conjugated anti-mouse IgG (IgG-HRP) was added (100 ul/well), incubated for 1 hour at 37°C. Thereafter, it was washed with phosphate-buffered-saline and stand for 1 minute (5 times). Then, 3,3',5,5'-tetramethylbenzidine (TMB A, 50 μl each of B solution) was added to the wells and incubated at 37°C for 15 minutes, then 50 μl of H_2SO_4 was added to terminate the reaction. The T concentration was determined by ELISA according to the same procedure (Yuanye, Shanghai, China).

RNA extraction and Real-Time quantitative PCR: Experimental procedure was performed using the methods described in Fang *et al.* (2010). Total RNA was extracted from the hypothalamus, pituitary, and testis using Trizol reagent (Invitrogen, Carlsbad, USA). Total RNA was quantified by absorbance at 260 nm using an ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies, Willmington, USA). Total RNA quality was assessed by formaldehyde agarose gel electrophoresis. The total RNA reverse transcription into cDNA was performed using a EnergicScript® First-Strand cDNA Synthesis kit (ShineGene, Shanghai, China) according to the manufacturer's instructions and then used for subsequent Real-Time quantitative PCR.

All the primers (Table 1) were designed using the Primer Express 2.0 (ABI, USA). qPCR was performed using a ShineSybr® Real-Time qPCR MasterMix Kit (ShineGene, Shanghai, China) and FTC2000 (Funglyn, Toronto, Canada). The reaction mixture was prepared in the wells of a 96-well plate, and each well contained 30 pmol each of forward and reverse primers, 1× Hotstart Fluo-PCR mix, and 1 μl of cDNA. The final reaction volume was 50

µl.The PCR conditions were as follows: an initial denaturation step of 94°C for 4 min followed by 35 cycles of 94°C for 20 sec, 60°C for 30 sec, and 72°C for 30 sec with signal detection after every cycle. Relative mRNA quantification was performed using the relative standard curve method (see details on ABI User Bulletin #2, https://www.sogou.com/tx?query=ABIUserBulletin%232&hdq=sogou-site-706608cfdbcc1886&ekv=3&ie=utf 8&).

Statistical analyses: Data were analyzed by SAS 9.4 and expressed as mean ± SD. The one-way ANOVA was used for the statistical analysis and the significant differences between the groups was compared used lsd test. Statistical significance was set at $P<0.05$.

RESULTS

Weight of the testes: The weight of the testes were significantly decreased ($P<0.05$, Table 2) in the ISA 201 adjuvant group compared to the control group. It can be seen that the testes in immunized groups had significantly atrophied compared with the control, and the atrophy in the ISA 201 adjuvant-immunized group was sharper than that that in the Gel 01 group.

Serum antibody titers: After the first immunization, the antibody titer of the ISA 201 adjuvant group showed an increasing trend from 20-104 days, and differed significantly ($P<0.05$, Fig. 1) from the control at 34, 48, 62, 90, 104 days of age. Both vaccine groups showed the highest antibody titers at 76 days of age, and at this time point, the antibody titer in the ISA 201 adjuvant group showed a highly significant difference ($P<0.01$, Fig. 1) compared with the control group. The antibody titer decreased from 76–90 days of age, but increased again after 90 days and the difference remained significant ($P<0.05$, Fig. 1). In contrast, the antibody titer in the Gel 01 adjuvant group was only numerically higher than that in the control group, but the difference was not statistically significant ($P>0.05$) between these two groups.

Serum testosterone concentrations: With the reduction in ISA 201 adjuvant group and increase in the control group, based on the level of 20 days, the difference of serum T concentration gradually became significant ($P<0.05$, Fig. 2) between these two groups from 20 to 76 days. The lowest T level occurred at 76 days, and it showed a highly significant difference from the control ($P<0.01$, Fig. 2). The T concentration in the Gel 01 adjuvant group showed a significantly lower compared with the control group at the age of 34 days and 48 days; however, there was only a slight difference in the T concentrations in the Gel 01 adjuvant group and the control group from 62-104 days, but from 76 days of age onwards, the T concentrations in Gel 01 and the control group were similar.

Table 1: Primer sequences used in the present study

Gene	Primer sequences (5'-3')	Gene	Primer sequences (5'-3')
GnRH	Fwd:GCCGCTGTTGTTCTGTTGAC	*LHβ*	Fwd: CATAGTCTCCTTTCCTGTGGC
	Rev: CTGGGGTTCTGCCATTTGA		Rev: CATTGGTTGAGTCCTGGGA
GnIH	Fwd: CCAAAGGTTTGGGAGAACAA	*FSHβ*	Fwd: AGACCAAACACCCAGAAAG
	Rev: GGGTCATGGCATAGAGCAAT		Rev: TCACTATCACACTTGCCACA
Kiss-1	Fwd: TGCTGCTTCTCCTCTGTGTG	*Inhibin B*	Fwd: ATAGCAGACATCGCATCC
	Rev: CCAGGCATTAACGAGTTCCT		Rev: CAGTAGTTCCCGTAGTAGC
MKRN3	Fwd: GAAGAGGAGAAGGAGAAAC	*LH-R*	Fwd: AATTATGCTCGGAGGATGG
	Rev: AACTTGATGCCAGTATGC		Rev: AGATGGATAATATGTAGACTTGG
LEPR	Fwd: AGCAGTCCAGCCTACACTCTTG	*FSH-R*	Fwd: GCCTTGCTCCTGGTCTCC
	Rev:ACCACATACCTCCTCACACTACAC		Rev: CTGTCACCTTGCTGTCTTGG
GPR54	Fwd: GTTATCTGCCGCCACAAG	*3β-HSD*	Fwd: GTGTATGTAGGCAATGTGGC
	Rev: CCTGCTGGATGTAGTTGAC		Rev: ACTGGAATCAAGGTGGAGG
TacR2	Fwd: CCTCTCCTGGTGATGTTC	*17β-HSD*	Fwd: AAGACCGCCGATGAGTTTG
	Rev: TGTAGATGGCGTAAGTTGG		Rev:GGTGGTGCTGCTGTAGAAGAT
Nesfatin-1	Fwd: CCAGACACGGGACTTTATTATG	*StAR*	Fwd: CAACTGGAAGCAACACTCTACA
	Rev: CCGCTCCTTATCTCCTCTATGT		Rev: ACACCTGGCACCACCTTAC
GnRH-R	Fwd: TCTGCAATGCCAAAATCATC	*P450scc*	Fwd:AACGGCACACACAGAATCCAT
	Rev: GTAGGGAGTCCAGCAGATGAC		Rev:AAGAGAGTCGCTGCGTCCTTAG

Note: Fwd, forward primer; Rev, reverse primer.

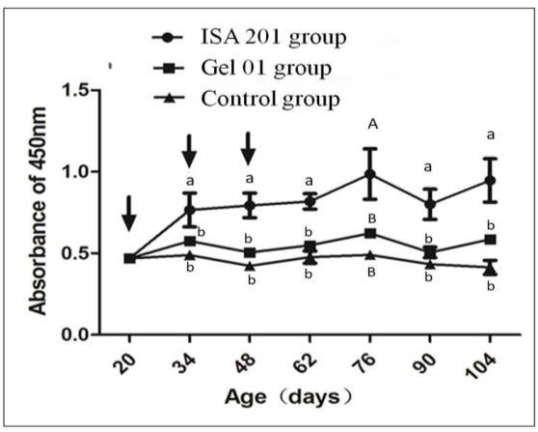

Fig. 1: Serum antibody concentrations in the different groups. Arrows indicated the time of immunization. [a,b] indicate $P<0.05$ and [A,B] indicate $P<0.01$, respectively.

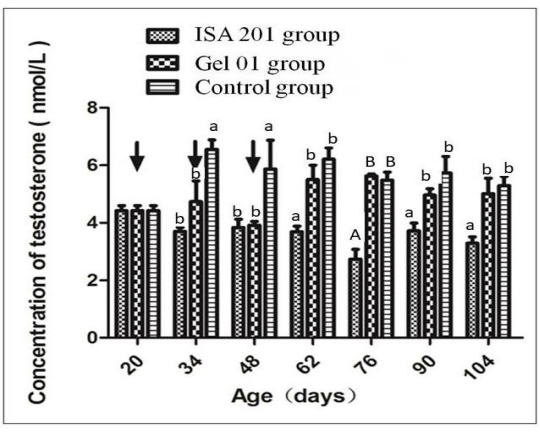

Fig. 2: Changes of the testosterone concentration in the different groups. Arrows indicated the time of immunization. [a,b] indicate $P<0.05$ and [A,B] indicate $P<0.01$, respectively.

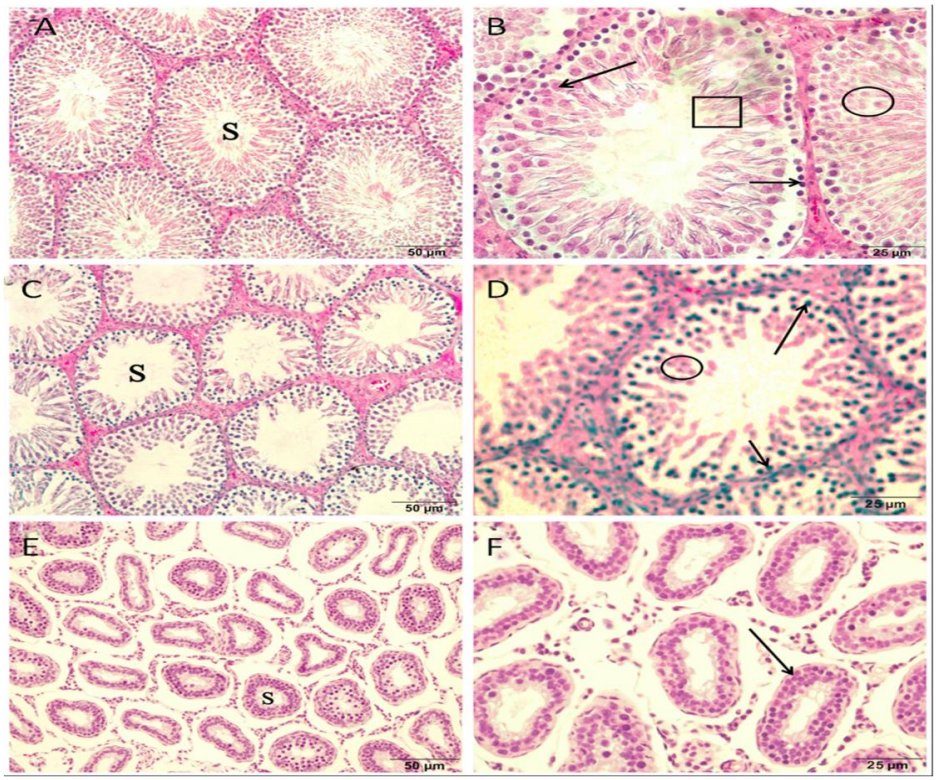

Fig. 3: Histological observation of three groups of rats subjected to different treatments. Testicular tissue of rats in the control group (A, B), GnRH2-MAP with Gel 01 adjuvant-immunized group (C, D), GnRH2-MAP with ISA 201 adjuvant-immunized group (E, F). Seminiferous tubules (S) contain spermatogonia (short arrows), primary spermatocytes (long arrows), spermatids (ellipse area), and spermatozoa (square area). Note: (A, C, E) scale bar = 50 µm; (B, D, F) scale bar = 25µm.

Table 2: Testis weight of ISA 201 group, Gel 01group and Control group male SD rats at different ages after vaccination (mean ± SD)

Age (days)	ISA 201 group (g) n=6	Gel 01group (g) n=6	Control group (g) n=6
34	0.33±0.05[a]	0.32±0.03[a]	0.45±0.05[b]
48	0.71±0.08[a]	0.77±0.11[a]	1.22±0.05[b]
62	1.03±0.11[a]	1.50±0.08[b]	1.67±0.06[b]
76	1.49±0.08[a]	1.77±0.05[a]	1.91±0.03[b]
90	1.23±0.22[a]	1.81±0.03[b]	1.96±0.09[b]
104	1.36±0.28[a]	1.89±0.06[b]	1.94±0.04[b]

[a]means a significant difference between the two groups *(*P<0.05). Different immunized adjuvants groups versus control group, respectively. The rats were injected thrice at 2-week intervals.

Histological observation of the testes: Histological observation of normal testes structure (Fig. 3A, B) revealed a complete layer of spermatogenic cells. Gel 01 adjuvant testicular sample (Fig. 3C, D) lumen enlargement and sperm count decreased. Furthermore, histological changes in the seminiferous tubules were particularly evident in the ISA 201 adjuvant group (Fig. 3E, F), where the seminiferous tubules showed severe atrophy. All levels of spermatogenic cells showed loose regular arrangement, and the spermatogonia and spermatocytes near the wall were the only visible cells. No sperm was present in the lumen, which had almost become vacuoles.

mRNA expression in hypothalamus, pituitary and testis: The *GnRH,* Gonadotropin-inhibitory hormone (GnIH*),* kisspeptins-1(*Kiss-1),* and Makorin3(*MKRN3)* mRNA expression levels in the hypothalamus (P<0.05, Fig. 4A); the Gonadotropin-releasing hormone Receptor *(GnRH-R),* Follicle-stimulating hormone-*β(FSH-β),* and Luteotropic hormone–*β(LH-β)* mRNA expression levels in the pituitary (P<0.05, Fig. 4B); and the *FSH-R, LH-R, inhibin B,* P450 side chain cleavage *(P450scc),* and *3β*-Hydroxysteroid dehydrogenase *(3β-HSD)* levels in the testes (P<0.05, Fig. 4C) were significantly downregulated in the ISA 201 adjuvant group as compared to the Gel 01 adjuvant and control groups. In contrast, G Protein-Coupled Receptors (*GPR54*) and Tachykinin receptor 2(*TacR2*) expression levels (P<0.05, Fig. 4A) in the hypothalamus and the Steroidogenic acute regulatory protein *(StAR)* and *17β-HSD* levels in the testes (P<0.05, Fig. 4C) were significantly upregulated in the ISA 201 adjuvant group compared to the corresponding levels in the other two groups. Changes in the expression levels of *GnRH, TacR2, GnRH-R, FSH-β, LH-β, FSH-R, LH-R, 3β-HSD,* and *17β-HSD* mRNA were extremely significant between the groups (P<0.05, Fig.4). Moreover, Leptin receptor (*LEPR)* mRNA expression in the hypothalamus and *Nesfatin-1* mRNA expression in the hypothalamus-pituitary-testis (*HPT*) axis were all not significant (P>0.05, Fig. 4). Besides, there were no differences in appearance between the Gel 01 adjuvant and control groups (P>0.05, Fig. 4).

DISCUSSION

It is well known that GnRH-I is synthesized locally and plays a vital role in the standard feedback regulation mechanism of the hypothalamic-pituitary-gonadal (HPG) axis (Wang and Swerdloff, 2010; Avendano *et al*., 2017). Active immunization with exogenous GnRH will induce a dramatic immune response to inhibit testicular function either via the HPG axis or directly on the gonads.

In this study, it was found that ISA 201 is better than the Gel 01 adjuvant. In present study, antibody titer had reached a significant castration level at the time when the second sampling was done. The increasing trend maintained 8 weeks until up to the peaking level in 76 days, which may be attributed to an accumulation of the antigen dosage. Corresponding to the high peaks of antibody after the third immunization, T concentration showed the significant decrease, which was regulated through the feedback process of HPG axis.

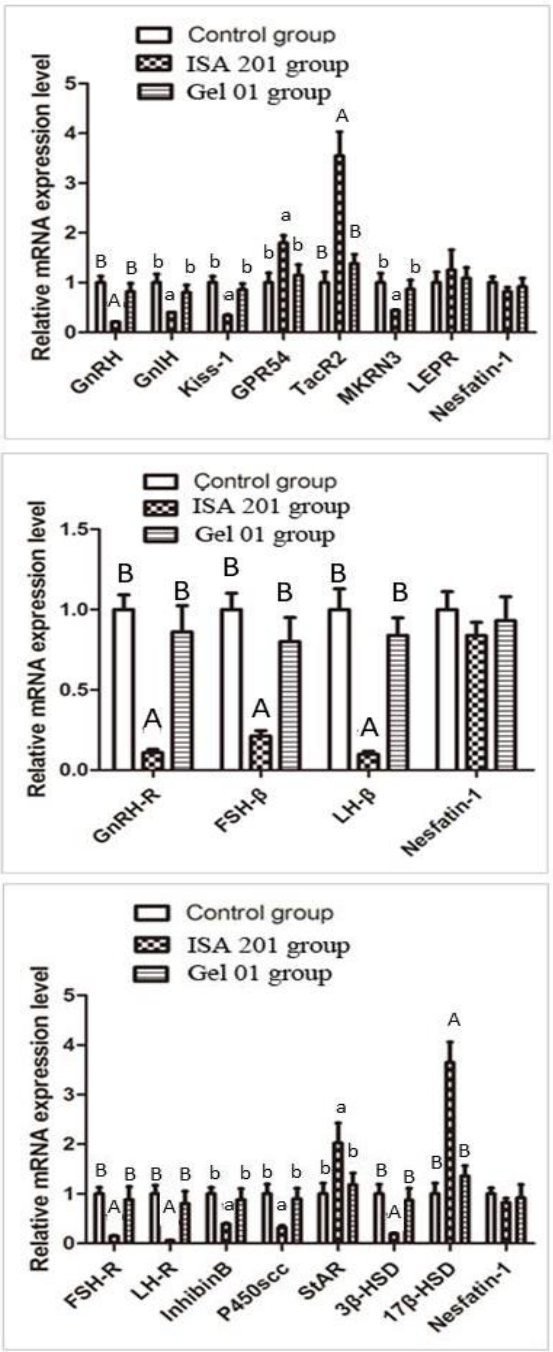

Fig. 4: Comparison of the mRNA expression levels in the hypothalamus, pituitary, and testis in the different groups. [a,b]indicate P<0.05 and [A,B]indicate P<0.01, respectively.

Moreover, microscopic observation of testicular seminiferous tubules revealed that they had significantly atrophied, and there was almost no mature sperm in the lumens of the ISA 201 adjuvant group compared to the Gel 01 adjuvant and control groups. In the present study, no reversibility appeared uptil the late of this experiment, in contrast to many studies detected testosterone concentration with reversibility (Rottner and Claus, 2009). Based on the contradiction between the results of our study and Rottner's report (Rottner and Claus, 2009) it is thought that the reversibility of the T concentration may occur after a more extended term following immune castration. Previous studies reported that the serum LH and FSH levels rapidly increased after GnRH injection, and the LH level quickly reduced after injection of the GnRH antagonist. Therefore, reversibility of the T concentration was attributed to the different sensitivities of LH and FSH to GnRH (Mistry *et al*., 2011).

GnRH in the hypothalamus, LH, and FSH in pituitary and T in testes were synthesized through the control of relevant genes, so it is significant to study the mRNA expression (Avital-Cohen *et al*., 2011).

It is generally accepted that active immunization against GnRH-I can induce a sharp antibody response, neutralizing endogenous GnRH, which disturbs the HPG axis (Bilezikjian *et al*., 2004). It is already known that LH and FSH secretion in the pituitary is regulated by GnIH and leptin (Rohayem *et al*., 2016). The synthesis and release of gonadotropin (LH, FSH) were inhibited by GnIH, which were linked with the GnIH receptors in pituitary; therefore, GnIH gene expression level was in line with that of LH and FSH to maintain a low level. In this study, *LEPR* expression was slightly high but not significant, which may be because the unbroken reproductive axis still maintains its integrity, and the low hormone levels induce an increase in the expression of leptin and leptin receptors (LEPRs) (Rohayem *et al*., 2016). The expression of the *Kiss-1* and *GPR54* gene in the brain is considered to be a catalyst for the initiation of animal adolescence. *Kiss-1* in the hypothalamus can receive the signal from T to stimulate the GnRH neurons by producing kisspeptin, which can activate GPR54 in the hypothalamus to regulate GnRH secretion. Moreover, due to continuous administration, the T concentration was still maintained a low level, continuing to enable the GPR54 so that *Kiss-1* could maintain a significantly low expression and *GPR54* could maintain a significantly high expression level (Navarro *et al*., 2004). *TacR2* is another G protein-coupled receptor with T receptors to act on GnRH neurons such as *GPR54*; thus, it could maintain a significantly high expression level (Millar and Newton, 2013). Moreover, *MKRN3* gene expression can inhibit GnRH secretion. It was significantly downregulated because the GnRH in serum was neutralized by GnRH antibody (Hagen *et al*., 2015).

Moreover, *InhibinB* was only synthesized by testicular Sertoli cells, which can control FSH secretion in the pituitary. *InhibinB*, which inhibits FSH, was significantly lower in the ISA 201 adjuvant group than in the other two groups. Furthermore, a continuous low level of FSH was maintained by the passive feedback regulation of GnRH (Lomniczi *et al*., 2013). Finally, *Nesfatin-1* is an important and widely distributed molecule, which increases the LH and FSH secretions by affecting their expression in the pituitary, promoting the maturation of the HPG axis (Goebel *et al*., 2009). Similar to the role of *LEPR* in the hypothalamus, all these molecules affect the development of the HPG axis, which retained its integrity; this may explain why *LEPR* and *Nesfatin-1*did not change significantly following treatment with the GnRH vaccine and adjuvant.

Significant downregulation of *P450scc* in the testes in this study may be due to the stimulation of LH and FSH in the pituitary. *Cholesterol expresses P450scc gene in response to irritants from pituitary or hypothalamus.* At the same time, the high expression levels of *StAR* in the mitochondrial membrane facilitate movement of cholesterol from the outer layer to the inner layer. T is

synthesized by androstenedione and androstenediol. The former is catalyzed by *3β-HSD* with a 15.76% conversion rate, while the latter is catalyzed by *17β-HSD* with a conversion rate of less than 5.61%. Thus, the low expression of *3β-HSD* may contribute to the decreased synthesis of T. However, *17β-HSD* expression was highly significant, as this gene can control the synthesis and metabolism of sex hormones (Miller *et al*., 2006). Therefore, the high expression of *3β-HSD* and *17β-HSD* in the ISA 201 adjuvant-immunized group contribute to reduce and maintain the low concentrations of T. All the above findings are presented as a comparison between the ISA 201 adjuvant group and the other two groups. It is clear that the all gene in Gel 01 adjuvant exhibited a similar trend as the ISA 201 adjuvant group, but the results were not as pronounced in the Gel 01 adjuvant group compared with ISA 201 adjuvant group. It should be noted that this study has examined only the choice between ISA 201 and water-solube adjuvants supplemented with GnRH2-MAP antigen. It is unknown whether the adjuvant dosage, immunity times or species variation disturb the effect of GnRH2-MAP vaccine. In addition, little work in this study has been done to testify the duration of immunized effect, reproductive capability and the situation of spermatogenesis after our experiment, which may be considered in the future studies. As we all know that the sexual maturation of SD rats is about 6-8weeks, and that body maturation is about 3 months. Experimental period in the present study, however, is more than 80 days. If mature rats are chosen, the reproductive function of those rats has been in the state of degeneration at the third immunization, which is not helpful to our investigation. It is believed that these existing problems will be solved in the future.

Conclusions: This study shows that GnRH2-MAP is more effective when administered with ISA 201 adjuvant than administered with Gel 01 adjuvant, which was mainly demonstrated in four aspects: Testicular weight was decreased significantly; serum T concentration was maintained at a low level; antibody titers were induced at high levels; and expression levels of related genes were significantly decreased. Thus, GnRH2-MAP administered with ISA 201 adjuvant is expected to be a viable alternative to commercial vaccine for immunocastration.

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Authors contribution: Conceived and designed the experiments: FF and WS; Performed the experiments: RZ; Analyzed the data: WS and RZ; Contributed reagents/ materials/analysis tools: WS, RZ, SJ, TK, ZY, JY, WT, YL, FF. Wrote the manuscript: WS, RZ.

REFERENCES

Avital-Cohen N, Heiblum R, Argov N, *et al*., 2011. The effect of active immunization against vasoactive intestinal peptide and inhibin on reproductive performance of young White Leghorn roosters. Poult Sci 90:2321-31.

Avendano MS, Vazquez MJ and Tena-Sempere M, 2017. Disentangling puberty: novel neuroendocrine pathways and mechanisms for the control of mammalian puberty. Human Rep update 23:737-63.

Bilezikjian LM, Blount AL, Leal AM, *et al*., 2004. Autocrine/paracrine regulation of pituitary function by activin, inhibin and follistatin. Mol Cell Endocrinol 225:29-36.

Conforti VA, de Avila DM, Cummings NS, *et al*., 2007. The effectiveness of a CpG motif-based adjuvant (CpG ODN 2006) for LHRH immunization. Vaccine 25:6537-43.

Cribbs DH, Ghochikyan A, Vasilevko V, *et al*., 2003. Adjuvant-dependent modulation of Th1 and Th2 responses to immunization with beta-amyloid. Int Immunol 15:505-14.

Fang F, Li H, Liu Y, *et al*., 2010. Active immunization with recombinant GnRH fusion protein in boars reduces both testicular development and mRNA expression levels of GnRH receptor in pituitary. Anim Reprod Sci 119:275-81.

Ferro VA, Costa R, Carter KC, *et al*., 2004. Immune responses to a GnRH-based anti-fertility immunogen, induced by different adjuvants and subsequent effect on vaccine efficacy. Vaccine 22:1024-31.

Finnerty M, Enright WJ, Morrison CA, *et al*., 1994. Immunization of bull calves with a GnRH analogue-human serum albumin conjugate: effect of conjugate dose, type of adjuvant and booster interval on immune, endocrine, testicular and growth responses. J Reprod Fertil 101:333-43.

Goebel M, Stengel A, Wang L, *et al*., 2009. Nesfatin-1 immunoreactivity in rat brain and spinal cord autonomic nuclei. Neurosci Lett 452:241-6.

Goodwin D, Simerska P, Chang CH, *et al*., 2014. Active immunisation of mice with GnRH lipopeptide vaccine candidates: Importance of T helper or multi-dimer GnRH epitope. Bioorg Med Chem Lett 22:4848-54.

Hagen CP, Sorensen K, Mieritz MG, *et al*., 2015. Circulating MKRN3 levels decline prior to pubertal onset and through puberty: a longitudinal study of healthy girls. J Clin Endocrinol Metab 100:1920-6.

Han X, Gu L, Xia C, *et al*., 2015. Effect of immunization against GnRH on hypothalamic and testicular function in rams. Theriogenology 83:642-9.

Jiang S, Hong M, Su S, *et al*., 2015. Effect of active immunization against GnRH-I on the reproductive function in cat. Anim Sci J 86:747-54.

Lomniczi A, Wright H, Castellano JM, *et al*., 2013. A system biology approach to identify regulatory pathways underlying the neuroendocrine control of female puberty in rats and nonhuman primates. Hormones Behavior 64:175-86.

Millar RP and Newton CL, 2013. Current and future applications of GnRH, kisspeptin and neurokinin B analogues. Nat Rev Endocrinol 9: 451-66.

Miller CJ, Geller JI and Rosen L, 2006. Ovarian and adrenal androgen biosynthesis and metabolism. Androgen Excess Disorders Women 14.

Mistry DS, Tsutsumi R, Fernandez M, *et al*., 2011. Gonadotropin-releasing hormone pulse sensitivity of follicle-stimulating hormone-beta gene is mediated by differential expression of positive regulatory activator protein 1 factors and corepressors skil and TGIF1. Mol Endocrinol 25:1387-403.

Navarro VM, Castellano JM, Fernandez-Fernandez R, *et al*., 2004. Developmental and hormonally regulated messenger ribonucleic acid expression of KiSS-1 and its putative receptor, GPR54, in rat hypothalamus and potent luteinizing hormone-releasing activity of KiSS-1 peptide. Endocrinology 145:4565-74.

Oonk HB, Turkstra JA, Schaaper WM, *et al*., 1998. New GnRH-like peptide construct to optimize efficient immunocastration of male pigs by immunoneutralization of GnRH. Vaccine 16:1074-82.

Pau CP, Luo W and McDougal JS, 2007. Chimeric multiple antigenic peptides for simultaneous detection of specific antibodies to HIV-1 groups M, N, O, and HIV-2. J Immunol Methods 318:59-64.

Rohayem J, Sinthofen N, Nieschlag E, *et al*., 2016. Causes of hypogonadotropic hypogonadism predict response to gonadotropin substitution in adults. Andrology 4:87-94.

Rottner S and Claus R, 2009. Return of testicular function after vaccination of boars against GnRH: consequences on testes histology. Animal 3:1279-86.

Wagner A and Claus R, 2004. Involvement of glucocorticoids in testicular involution after active immunization of boars against GnRH. Reproduction 127:275-83.

Wang C and Swerdloff RS, 2010. Hormonal approaches to male contraception. Current Opin Urol 20:520-4.

Yao Z, Si W, Tian W, *et al*., 2018. Effect of active immunization using a novel GnRH vaccine on reproductive function in rats. Theriogenology 111:1-8.