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Kisspeptin recombinant oral vaccine: A master gene vaccine inhibiting the reproductive physiology and behavior of ram lambs

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ABSTRACT

The *KISS1* gene product, kisspeptin, stimulates gonadotrophic steroid hormone (GNRH) neuronal signaling through the G-protein coupled receptor, *kiss1r*. Disturbance of this signaling pathway causes hypogonadotropic hypogonadism in mammals. As part of this cutting-edge research project, we analyzed the efficacy of an oral kisspeptin recombinant vaccine on the reproductive physiology and behavior of ram lambs. Ten 56-day old ram lambs were randomly divided into treatment and control groups to receive the experimental recombinant vaccines, C500/pKS-asd or C500/pVAX-asd (aspartate-β semialdehyde dehydrogenase), respectively. The vaccines were orally administered at day 0, 28 and 56 and blood samples were taken and scrotal circumference data recorded at 14-day intervals (days 0, 14, 28, 42, 56, 70, and 84). At the end of the experimental period, day 98, sexual behaviors were assessed, scrotal circumferences were measured, and blood samples were collected. Testicular samples were also collected after the animals were sacrificed. Anti-kisspeptin antibody and testosterone serum levels were measured by indirect ELISA. Results demonstrated that the levels of anti-kisspeptin antibodies were significantly higher in the treatment group compared to controls ($P < 0.05$, $P < 0.01$ and $P < 0.001$). However, serum testosterone levels were lower in the treatment group ($P < 0.01$). Interestingly, vaccine administration contributed to a significant reduction ($P < 0.01$) in sexual behavior propensity. These results suggest that the kisspeptin recombinant oral vaccine regulates and inhibits the reproductive physiology and behavior of ram lambs.

1. Introduction

Plasmid recombinant DNA vaccines were established more than two decades ago [1]. The first of these vaccines was found to induce an active host humoral immune response that neutralized the fecundity of mammals [2,3] and suppressed fertility [4]. Recombinant vaccines consist of a specific DNA sequence encoding an immunogenic protein that can be expressed in bacterial or mammalian cells. This gene-specific sequence is characterized, purified, and cloned into an expression carrier. These recombinant plasmids contain a gene-of-interest and a promoter region which is used to initiate the transcription of the target protein within the animal that has received the oral vaccine [5]. The host cellular machinery allows *in vivo* manufacturing of the target protein, thereby, bypassing the need to manufacture and purify the protein *in vitro*.

Immunocastration is a process whereby the immune system is stimulated to produce specific antibodies against endogenous gonadotropin releasing hormone (GNRH). In previous studies, the development of GNRH DNA-based vaccines has been investigated in various animals. In these studies, engineered GNRH DNA vaccines induce strong resistance responses, decreased serum testosterone levels, and suppress fertility [6–8]. Consequently, anti-fertility vaccines have been thought to break immune tolerance by selectively targeting antigens to decrease the risk of immunological cross-reactions and interference with physiological processes. These vaccines are thought to accomplish this through regulating the hormonal cascade and therefore controlling reproduction.

The KISS-1, which encodes kisspeptin, is critical for the onset of puberty and regulates adult fertility [9]. Kisspeptin signaling is well established in GNRH-producing neurons and plays a pivotal role in the internal control of the hypothalamus-pituitary-gonadal axis (HPG), linking follicular development, ovulation, spermatogenesis, and steroidogenesis [10,11]. In fact, mutations in the

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G-protein coupled receptor, *kiss*1r, the receptor for kisspeptin, are linked to hypogonadotropic hypogonadism [12,13].

As a result, a kisspeptin recombinant vaccine was genetically engineered and subcutaneously administered to lambs. As expected, it led to decreased steroid hormone and testosterone release, which affected reproduction [14]. Use of the kisspeptin recombinant vaccine, as a novel method for immunocastration, also resulted in alterations in gonadal characteristics and sexual behavior in ram lambs [14]. To the best of our knowledge, the use of a kisspeptin recombinant plasmid for an oral vaccine and its efficacy on influencing the reproductive physiology of ram lambs has not been previously assessed. Therefore, the current research project aims to fill this gap and examine the feasibility and efficacy of an orally administered kisspeptin recombinant vaccine on the reproductive physiology and behavior of ram lambs.

2. Materials and methods

2.1. Kisspeptin bacterial plasmid for oral vaccine

The kisspeptin immunocastration vaccine was prepared using an antibiotic-free plasmid. Professor Ai-zhen Guo at Huazhong Agricultural University kindly supplied the *Escherichia coli* C500 strain. The subcloned kisspeptin gene into the pVAX1 vector to create the recombinant pVAX-KISS1 vector and the subcloned HBsAg-S gene PCR into the pVAX-KISS1-S (pKS) plasmid were previously constructed in our laboratory, (Fig. 1).

The recombinant plasmids pKS-asd and pVAX-asd were transformed into competent *E. coli* x6097 cells stressed with *Salmonella enterica serovar Choleraesuis* strain (C500), by electroporation, to create the C500/pKS-asd and C500/pVAX-asd lines (Fig. 1). C500/pKS-asd and C500/pVAX-asd are referred to as the experimental and control vaccines, respectively. The C500/pKS-asd and C500/pVAX-asd strains were in Luria-Bertani (LB) broth and grown overnight to an optical density (OD) of 0.5 at 650 nm (OD 650). The cells were then accumulated by centrifugation at 3000 rpm and incubated at 4 °C for 10 min. Prior to their use for vaccination, the number of bacterial cells were verified by dilution of the inoculum onto LB agar. The bacterial cells were then adjusted spectrophotometrically to a dose of 5×10^{10} CFU/ml for both the treatment and control groups, diluted in phosphate-buffered saline (PBS) for the vaccinations.

2.2. Ethical statement and management of experimental animals

A total of ten 56-day old male Hu with body weight 17.62 ± 0.48 kg were used for these experiments. The ram lambs were randomized into the treatment and control groups (n = 5) based on their body weight and scrotal circumference. The sheep were ear tagged, fed three times per day, and given free access to water and salt. All experimental animals were carefully observed for the occurrence of any ill health condition. The granted research animals and all experimental procedures were approved by the Institutional, Animal Care and Use Committee of Huazhong Agricultural University.

2.3. Vaccination of experimental animals

The prepared vaccines were administered orally using a straw syringe (5 ml of 5×10^{10} CFU/ml C500/pKS-asd or 5×10^{10} CFU/ml C500/pVAX-asd). For each ram lamb, the same vaccine treatments were repeated at days 28 and 56, after the initial vaccination. Fourteen days into the experiment, blood samples were collected from all of the animals via jugular venipuncture. Blood samples were centrifuged at 3000 rpm, 4 °C, for 10 min. The plasma samples were collected and then stored at −20 °C for future use.

2.4. Detection of anti-KISS1 antibody concentrations

An indirect ELISA was performed to determine the titers of anti-kisspeptin antibody. The resulting OD values were measured using an ELISA reader (Bio-Rad iMark Microplate Reader) at 450 nm.

2.5. Serum testosterone measurement

Total serum testosterone levels were measured using the Sheep T ELISA kit (Cusa Biotech, Wuhan, China), per the manufacturer's recommended protocol.

2.6. Vaccine impacts on libido and testicle structure

The willingness of ram lambs to reproduce with females was evaluated beginning on day 84 post-immunization through the end of the experimental period (day 98). For seven days, the rams were allowed to run with an estrous female for 30 min. Visual

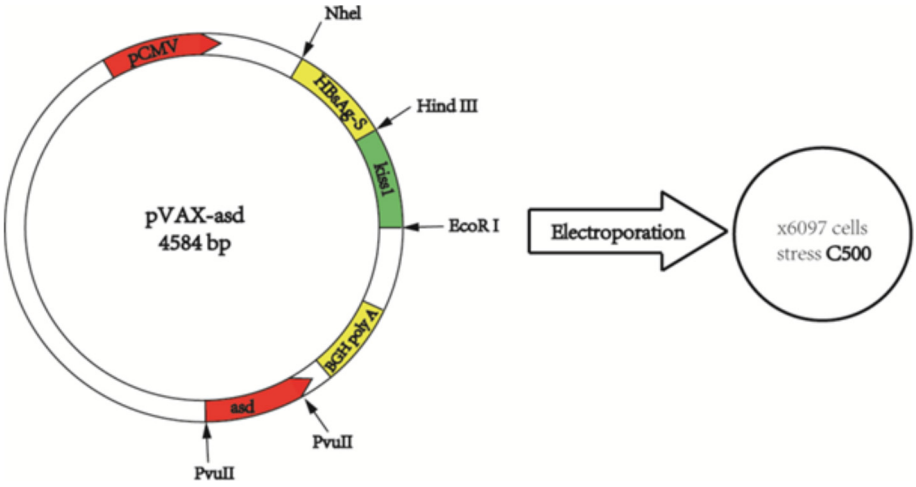

Fig. 1. Schematic diagram of the plasmid pKS-asd. Shows antibiotic-free Kisspeptin DNA vaccine encoded with kisspeptin, HBsAg-S gene and transfection to *Salmonella enterica serovar Choleraesuis* strain (C500).

observation of the sexual behavior of the treatment and control animals was performed daily from 7 AM to 11 AM over the entire experimental period. The mounting frequencies, sniffing, and butting were assessed.

2.7. Histological examination of testes

At the end of the experimental period, all animals were slaughtered by cutting their jugular vein with a knife after low voltage electrical head stunning at 250 V for 3 s. Testes tissue samples were collected from every experimental animal and fixed with 10% (v/v) buffered formalin. Hematoxylin and eosin (H&E) stain was performed for histological and morphological analyses.

2.8. Evaluation of reproductive organs

After correct straining, the ram's testicular circumference was measured using a caliper and tape according to [15]. The testicular length was measured by placing the constant arm of the caliper at the proximal groove and the sliding arm on the distal end of the testis. Testicular data for each experimental animal were recorded every 14 days.

2.9. Statistical analyses

Results were expressed as the mean ± standard deviation (Mean ± SD). Unpaired Student's *t*-test analyzed statistical analyses of the anti-kisspeptin antibody, testosterone levels, scrotal circumference, testicular breadth, length and weight, and sexual behavior. All assessments were achieved using a statistical analysis software program, SAS version 9.1. The threshold for statistical significance was set at $P < 0.05$.

3. Results

3.1. General health status of the experimental animals

The feed intakes, behaviors, and activities of all of the experimental ram lambs were within the range of good health condition throughout the experimental duration. None of the animals showed adverse medical signs in their digestive, respiratory organs or signs of oral inflammation. These observations implied that the recombinant plasmid vaccines were non-toxic to the animals.

3.2. Immune response of the rams to the oral vaccine

Immunization with the recombinant vaccine resulted in a robust immune reaction, as measured anti-kisspeptin antibody production by indirect ELISA. The specific serum antibodies started to be detected in the treatment group at day 28 post-immunization, and titers steadily increased to a peak at day 84 post-vaccination (Fig. 2). Unpaired Student's *t*-test results revealed that a higher anti-kisspeptin antibody titer in the treatment rams ($P < 0.05$, $P < 0.01$ and $P < 0.001$), starting from day 28 to the end of the experimental period. The kisspeptin recombinant oral vaccine, therefore, induced an effective anti-kisspeptin IgG response in the treated rams, showing that the vaccine can play a role in immunocastration (Fig. 2).

3.3. Immunization effect on reproductive organ tissue

Vaccination resulted in a lower scrotal circumference in the treatment ram lambs (Supplementary Fig. 3). The scrotal circumference of the control group expanded over the experimental period and increased with the increasing age of the ram lambs.

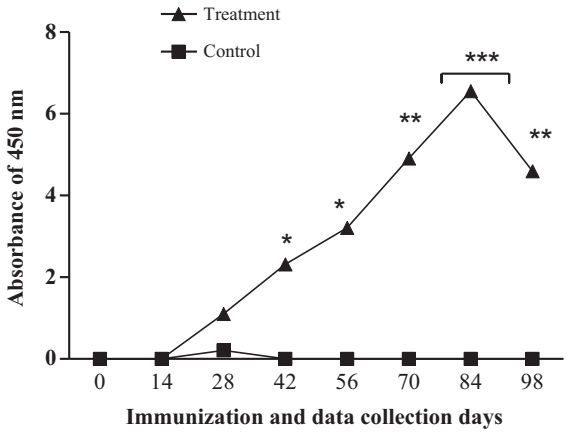

Fig. 2. Anti-kisspeptin antibody titer ranges in ram lambs following vaccination. Anti-kisspeptin antibody titers measured at the absorbance of 450 nm. The results (Mean ± SD) shown are one star, two stars and three stars at $P < 0.05$, $P < 0.01$ and $P < 0.001$. Black square and black triangle indicate control and treatment ram lambs, respectively.

Comparatively, the scrotal circumferences of the treatment group slowly increased 19.82 ± 3.18 cm than control group 22.16 ± 4.79 cm, ($P < 0.05$) through experimental period. These data show that the scrotal circumference of the treatment group was considerably lower than the control group. Moreover, the testes breadth, weight and length of the control group were significantly higher than the treatment group ($P < 0.05$) Table 1 below.

In comparison to the control group, the treatment rams shown decreased testicular breadth and length, as illustrated in Table 1. The analysis of this data demonstrated that immunization had a notable effect on testicular length, weight and breadth ($P < 0.05$). Alterations in testicular weight were measured and the treatment group presented with the smallest size (Supplementary Fig. 4A).

The testicular samples collected from treated animals were completely devoid of spermatids (Fig. 5). The microscopic assessment confirmed a significant failure of spermatogenesis inside the seminiferous tubules, which contained highly dispersed spermatogonium and spermatocytes in the treatment group compared with the control ram lambs (Fig. 5B). The epithelial height of the seminiferous tubules from the treatment ram lambs was also reduced compared with that of the controls (Fig. 5A).

3.4. Serum testosterone concentration

The serum concentrations of testosterone were lower in the treatment rams and higher in the control rams throughout the experimental period. The testosterone levels of the rams in the control group increased starting at day 28 and peaked at 70 days post-immunization. Serum testosterone levels showed slight increases in the treatment group with, increases after vaccination and with increasing age of the animals (Fig. 6).

The unpaired Student's *t*-test results revealed a highly significant difference between the treatment and control groups

Table 1
The effect of oral kisspeptin recombinant vaccine on the testicles parameters of the rams.

Experimental parameters			
Experimental group	Testes breadth (cm)	Testes weight (gm)	Testes length (cm)
Treatment	4.641 ± 0.056	91.83 ± 1.419	6.640 ± 0.109
Control	5.152 ± 0.1411**	118.1 ± 7.288**	7.400 ± 0.295*

The results Mean ± SD shown one star and two stars the significant difference between groups at $P < 0.05$, $P < 0.01$.

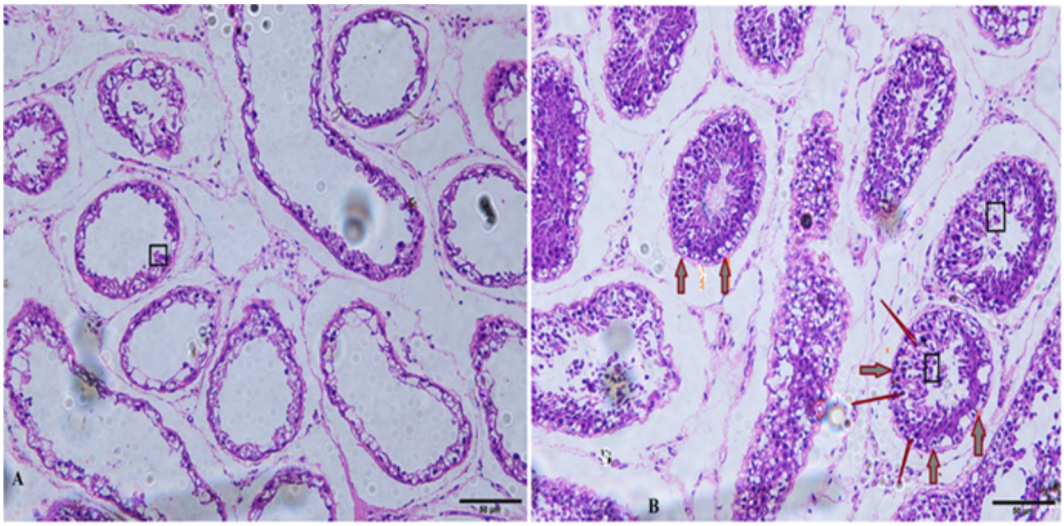

Fig. 5. Light microphotograph examination of the testes. Histological H&E images from treatment (A) and control rams (B), respectively. The spermatogonium (arrowhead), spermatocytes (arrow), and spermatid degree (square). Scale bar represents 50 μm.

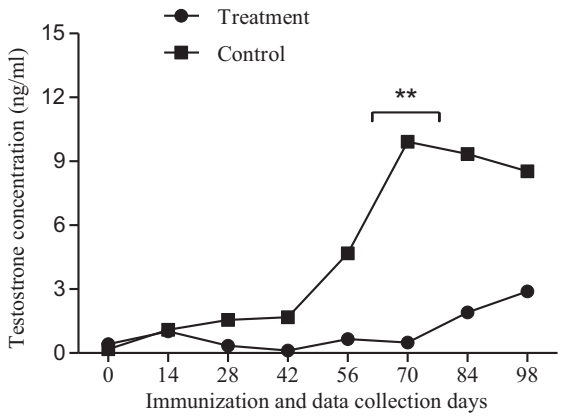

Fig. 6. The serum testosterone concentrations. The results (Mean ± SD) shown are the absorbance at 450 nm. One star and two stars show $P < 0.01$. Black square and black circle indicate control animals and treatment ram lambs, respectively.

(1.18 ± 1.62 ng/ml and 4.698 ± 4.835 ng/ml, respectively, $P < 0.05$). The decreased testosterone concentrations in the treatment group confirmed that the engineered DNA oral vaccine had the capability of interrupting biological hormone expression. Reduced testosterone levels probably exert a major influence on the reproductive organs and aggressive sex behaviors of the treatment group (Table 2).

4. Discussion

In the current study, the kisspeptin vaccine was prepared as an inoculant designed to induce an immune response against the endogenous kisspeptin protein. Vaccines are developed toward self-antigens, including both hormones and proteins to which the recipient is usually immunologically tolerant [16]. GNRH-based immune-castration results in the suppression of gonadotropin hormones, testosterone secretion, retardation of testicular improvement, and sexual conduct in males. It is known that neurons expressing kisspeptin in the hypothalamus are necessary for sexual reproduction, through signaling events that initiate a hormonal cascade in the HPG axis [17–19]. The kisspeptin-based vaccine is a cutting-edge method that is believed to induce immunity against endogenous kisspeptin proteins. Therefore, the vaccination is believed to exert an effect on GNRH as an alternative to surgical castration.

The best vaccine designs create an immunogen that imitates a pathogenic bacterium. Previous findings have explored the use of the kiss-1 as a target for the development of a DNA immune-sterilization vaccine [14] that efficiently inhibits the release of LH and FSH. The hepatitis B surface antigen S (HBsAg-S) which was amplified from pCMV-S using PCR was used for vaccine synthesis via coupling with the kisspeptin, improving the immunogenicity of the vaccine [14,16,20]. In the current study, we electroporated a recombinant plasmid DNA into a bacterial strain to create C500 (pVAX/pKS-asd).

Antigen delivery systems are considered a promising approach for immune-sterilization, inducing positive antibody responses, reducing serum testosterone levels, preventing gametogenesis, and producing anti-fertility *in vivo*. These antigens have been made "foreign" to the host immune system by coupling them to an endogenous protein-of-interest in the host animal and can block the normal function of the endogenous protein. This interesting method of vaccination triggers targeted effects on the animal reproductive physiology. The antigen dose and assembly can influence the degree of the primary response [21].

The binding of the anti-kisspeptin antibody was assessed and the results demonstrate that ram lambs are capable of generating high anti-kisspeptin antibody titers. These antibodies should result in the depletion of steroid hormones, accompanied by parallel changes in testicular structure and function. Our research results revealed that the serum kisspeptin antibody levels in the experimental group increased from days 56 to 98 post-immunization, and the pick antibody binding was observed at day 84 post-vaccination. The satisfactory performance and quantity of the antigen used in the oral immunization showed its immunogenicity. Our experimental study involved the administration of two doses

Table 2
Effects of oral vaccination on the sexual behavior of ram lambs.

Experimental trait			
Experimental group	Butting	Sniffing	Mounting
Treatment	0	2.00 ± 1.00	1.73 ± 1.43
Control	5.53 ± 3.58**	8.80 ± 2.98**	16.20 ± 9.76**

The results Mean ± SD shown one star and two stars at $P < 0.01$.

at four-week interval and the results demonstrated that these doses were capable of producing a long-lasting immune reaction. Furthermore, the administration of the vaccine at a dose of 3 weeks was likely counterproductive, as the IgA stimulated from the second dose interfered with its uptake [22]. A previous study [23] showed that three immunizations of ram lambs at six-week intervals resulted in a significant reduction in testis size from week 13 onwards, and affected sperm motility in weeks 24 to 26. Similarly, two immunizations of young ram lambs resulted in castrate-like testosterone levels and an 80% reduction in the testis weight at six weeks after the second vaccination [24]. The findings of these studies strongly agree with our study results. The specific antibody was detected at day_42 and reached a peak at day_84, suggesting that the first five immunizations influence the production of antibody.

The repeated administration of plasmid DNA is necessary to increase the lifetime and level of transgene expression [27] and can facilitate plasmid DNA vaccines by efficaciously translating their fusion proteins. In the small intestine, bacteria move across the intestinal epithelial cell and reach the M cells, consequently penetrate in the Peyer's patches [40]. However, some bacteria escape this barrier and reach the Peyer's patches; formed by T lymphocytes and dendritic cells. Dendritic cells present the bacterial antigens to immune cells that provoke activation of T-cell immunity development and B lymphocytes [41,42]. Accordingly, the interaction between B and T-cells is crucial for the development of antibody response to Salmonella proteins and isotype switching of the immune response against antigens.

Oral immunization of antigens induces humoral and cellular responses at both antigen exposure site and other mucosal compartments [43,44], because of the dissemination of antigen-sensitized precursor B and T lymphocytes from intestinal Peyer's patches to the effector sites. After oral immunization, bacteria penetrate the Peyer's patches via M cells and colonize the mesenteric lymph nodes, which contain various antigen-presenting cells. This can generate a range of immune responses, including systemic and mucosal responses at local and distal sites. The orally administered vaccine should have the potential to connect to mucosal surfaces, thereby supplying the vaccine-induced proteins directly into the immune system to synthesize antibodies. Our results indicate that the kisspeptin recombinant oral vaccine carrier was a good vector system for tolerating the oral and gut environment and for attaching to M-cells (gastrointestinal epithelium covering patches of lymphoid tissue), provoking the immune system to release antibodies, and causing immunocastration.

Immunocastration was induced and fertility was reduced via antibodies to natural hormones, resulting in an increase in the antibodies and a decrease in serum testosterone. Antigens should remain in the host for a sufficiently long period to allow the vaccine to stimulate specific antibodies and achieve immunocastration. In addition, active immunization against GNRH induces high antibody responses in laboratory animals [28], resulting in a reduction of serum testosterone and the induction of effective castration. Antibodies at sufficiently high levels help to eliminate the reproductive function of ram lambs. We examined whether the kisspeptin recombinant oral vaccine could successfully stimulate immunocastration via effects on serum testosterone secretory cells. Our findings revealed that ram lambs that were orally immunized with C500/pKS-asd presented reduced serum testosterone levels compared with the control rams. A previous study [29] demonstrated that the levels of serum testosterone are substantially decreased in infertile compared with fertile animals. Similarly, another previous study [14] found that a kisspeptin DNA vaccine injected intramuscularly was lower testosterone concentrations and insure immunocastration. The above mentioned results and relevant literature provide many records that the kisspeptin recombinant oral vaccine is a key participant in the manipulation of GNRH communications within the pituitary gland by disturbing the normal level of steroid hormones and regulating fertility. In addition, the total serum testosterone concentrations were decreased, consistent with the treatment group results obtained in a previous study [30,4]. Similarly, active immunization of male mammals species with the prepared proteins can efficiently cause testicular regression, a decline of testosterone discharge, cessation of spermatogenesis, and regulation of fertility [25,26]. In the current research, testosterone levels substantially increased in the control group and reached a peak level at day 70 post-vaccination. Additionally, the serum concentration in the treated rams increased with the increasing days of vaccination and age of the animals. The reduction of testosterone in the blood stream was achieved due to abnormal function of the HPG axis, implying that the kisspeptin recombinant oral vaccines participate in the disruption of testosterone secretion and the overcoming of ram sexual behaviors.

The scrotal circumference is the best and most sophisticated tool to estimate the mass of the reproductive organs [31,32] and the level of testicular development [33] of the animal. The testicle circumference and histological sections were assessed to determine the efficacy of the vaccine on gonadal features. Analysis of the sub-physiological testicular testosterone level revealed a direct relationship between the steroid and spermatogenic output. Our research findings show that the vaccine has a strong effect on the scrotal circumference and densities of spermatogonium, spermatocytes, and spermatids of immunized ram lambs compared with control ram lambs. This research finding agrees with the results obtained in a previous study [34,35], which analyzed GNRH immunization and revealed a marked reduction in spermatogenesis in the experimental group. The withdrawal of gonadotropin secretion by hypophysectomy leads to an arrest of spermatogenesis [36], which results in disorganization of the germinal epithelium and marked degenerative changes. By depriving gonadotropins of their main stimulator, GNRH prevents gonadotropin release and in turn inhibits both spermatogenesis and steroidogenesis. Our findings show that increased antibody levels weaken reproductive features and prevent improvement and function of the testicles in males [37]. The architecture of the testis was maintained, but the germinal epithelium showed disorganization and marked degeneration. Disruption of the HPG axis by neutralization of GNRH leads to a reduction in LH and FSH production and secretion, thereby inhibiting the maturation of the testes in growing animals and the synthesis of testicular steroids [38]. These consequences show that immunization with the kisspeptin recombinant oral vaccine prevents an increase in the testes sizes of ram lambs by inducing antibodies toward the GNRH receptor.

The size of the testes determines the rate of testes development in ram lambs and their sexual performance [39,40]. The active tissue in the testes needs time to respond to the released steroid hormone and become substantially decreased in size after successful immunocastration. Our study shows that the testicular breadth, length, and weight of the treatment group was lower than those of the control group. These findings suggest that oral administration of the kisspeptin recombinant vaccine could interfere with the biological reproductive physiology and resolve testicular degeneration via a GNRH-mediated mechanism, presenting proof of the immediate effects of kisspeptin on GNRH-expressing cells. In short, immunocastration includes a significant increase in antibodies against endogenous sexual hormones and gamete proteins in the bloodstream, thereby blocking their natural purposes. Therefore, these results suggest that the kisspeptin recombinant oral vaccine is a master gene vaccine that regulates the reproductive physiology and behavioral activities of ram lambs.

5. Conclusion

Oral administration of the kisspeptin recombinant vaccine resulted in entry into the host testicular secretory tissues and production of the anti-kisspeptin antibody in the treatment group throughout the experimental trial. The reduction in the size of reproductive organs and damage of testicular histology were observed in the immunized ram lambs. A decrease in sexual behaviors in the treatment group was also a result of the vaccine, demonstrating the effect of decreased testosterone levels in the vaccinated rams. Ultimately, we conclude that the kisspeptin recombinant oral vaccine is a safe vaccine that regulates the reproductive physiology and behavioral performance of ram lambs.

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Conflict of interest

The authors declared no existence of a conflict of interest.

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Authors' contribution

The following authors contributed equally to the manuscript:

Birhanu T. study plan and design, statistical analysis, and manuscript writing; **Jia-yu Z.H.**: animal management, data collection and manuscript preparation; **Xun-ping J.,** and **Gui-qiong L.**: research concept and design, and monitoring of the results and discussion presented in the manuscript; **Yan-guo H.:** research design and contribution of laboratory technic work and **Teketay W.**: manuscript editing.

Data availability

All relevant data are within the paper.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2017.09.001.

References

[1] Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, et al. Direct gene transfers into mouse muscle in vivo. Science 1990;247:1465–8.
[2] Jinshu X, Jingjing L, Duan P, Zheng Z, Ding M, et al. The immunogenicity of recombinant and dimeric gonadotrophin-releasing hormone vaccines incorporating a T-helper epitope and GnRH or repeated GnRH units. J Immunol Methods 2004;289:111–22.
[3] Gupta JC, Raina K, Talwar GP, Verma R, Khanna N. Engineering, cloning, and expression of genes encoding the multimeric luteinizing-hormone-releasing hormone linked to T cell determinants in *Escherichia coli*. Protein Exp Purif 2004;37:1–7.
[4] Khan MAH, Ogita H, Ferro VA, Kumasawa K, Tsutsui T. Immunization with a plasmid DNA vaccine encoding gonadotrophin releasing hormone (GnRH-I) and T-helper epitopes in saline induces an anti-GnRH-I antibody response and suppresses rodent fertility. Vaccine 2008;26:1365–74.
[5] Rodriguez RE, Wise ME. Ontogeny of secretion of a pulsatile gonadotropin-releasing hormone in the bull calf during infantile and prepubertal development. Endocrinology 1989;124:248–56.
[6] Godfrey SI, Walkden-Brown SW, Martin GB, Speijers EJ. Immunization of goat bucks GnRH to prevent seasonal reproductive and agonistic behaviors. Anim Reprod Sci 1996;44:41–54.
[7] Taylor MF, Akinbami MA, Collins DE. Effect of peripheral and central acting opioid antagonist on the testicular response to stress in rats. Neuroendocrinology 1994;59(4):343–8.
[8] Stadnikov AA, Shelving MM. The morphofunctional character of the hypothalamohypophyseal gonadal system in male rats under emotional pain stress. Morfologiia 1996;110(5):38–42.
[9] Clarkson J, Herbison AE. Postnatal development of kisspeptin neurons in mouse hypothalamus; a sexual dimorphism and projections of gonadotropin-releasing hormone neurons. Endocrinology 2006;147:5817–25. https://doi.org/10.1210/en.2006-0787.
[10] Roseweir AK, Millar RP. The role of kisspeptin in the control of gonadotrophin secretion. Hum Reprod Update 2009;15:203–12. https://doi.org/10.1093/humupd/dmn058.
[11] Tsukamura H, Maeda K. GnRH pulse generation and its adaptation to the manipulation of follicular development and ovulation. Thai J Vet Med Suppl 2011;41:69–72.
[12] de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. PNAS 2003;100:10972–6. https://doi.org/10.1073/ pans.1834399100.
[13] Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno Jr JS, Shagoury JK, Bo-Abbas Y, Kuohung W, Schwinof KM, Hendrick AG. The GPR54 gene as a regulator of puberty. N Engl J Med 2003;349:1614–27. https://doi.org/10.1056/NEJMoa035322.
[14] Han Y, Liu G, Jiang X, Ijaz N, Tesema B, Xie G. KISS1 can be used as a novel target for developing a DNA immune-castration vaccine in ram lambs. Vaccine 2015;33(6):777–82.
[15] Elmore RG, Bierschwal CJ, Youngquist S. Scrotal circumference measurements in 764 beef bulls. Endocrinology 1976;6(5):485–94.
[16] Jones WR. The immunological manipulation of reproduction. In: Immunological fertility regulation. Oxford and London: Blackwell Scientific Publishers; 1983. p. 1–75.
[17] Bliss Stuart P, Navratil Amy M, Xie Jianjun, Roberson Mark S. GnRH signaling, the gonadotrope and endocrine control of fertility. Front Neuroendocrinol 2010;31(3):322–40.
[18] Pinilla Leonor, Aguilar Enrique, Dieguez Carlos, Millar Robert P, Tena-Sempere Manuel. Kisspeptins and reproduction: physiological roles and regulatory mechanisms. Physiol Rev 2012;3:1235–316.
[19] Maruska Karen P, Russell D. Social regulation of gene expression in the hypothalamic-pituitary-gonadal axis. Physiology 2011;26:412–23. https://doi.org/10.1152/physiol.00032.2011.
[20] Liang A, Cao S, Han L, Yao Y, Modern-Ud-Din M, Yang L. Construction and evaluation of the eukaryotic expression plasmid encoding two copies of somatostatin genes fused with hepatitis B surface antigen gene S. Vaccine 2008;26:2935–41. https://doi.org/10.1016/j.vaccine.03.036.
[21] Banatvala J, Van Damme P, Oehen S. Lifelong protection against hepatitis B: the role of vaccine immunogenicity in immune memory. Vaccine 2001;19:877–85.
[22] Ahren C, Wenneras C, Holmgren J, Svennerholm AM. Intestinal antibody response after oral immunization with a prototype cholera B subunit colonization factor antigen enterotoxigenic *E. coli*. Vaccine 1993;11:929–34.
[23] Jeffcoat IA, Lucas JMS, Crighton DB. Effects of active immunization of ram lambs and bull calves against synthetic luteinizing hormone-releasing hormone. Theriogenology 1982;18:65–77.
[24] Schanbacher BD. Responses of ram lambs to active immunization against testosterone and luteinizing hormone-releasing hormone. Am J Physiol 1982;242:201–5.
[25] Khan MAH, Ferro VA, Koyama S, Kinugasa Y, Song M, Ogita K. Immunization of male mice with a plasmid DNA vaccine encoding gonadotrophin releasing hormone (GnRH-I) and T-helper epitopes suppresses fertility in vivo. J Vaccine 2007 [01.089].
[26] Fromme B, Eftekhari P, Van Regenmortel M, Hoebeke J, Katz A, Millar R. A novel retro-inversion gonadotropin - releasing hormone (GnRH) immunogen elicits antibodies that neutralize the activity of native GnRH. Endocrinology 2003;144:3262–9.
[27] Jang JH, Shea LD. Intramuscular delivery of DNA releasing microspheres: microsphere properties and transgene expression. J Control Release 2006;112:120–8.
[28] Turkstra Johan. Active immunization against gonadotropin-releasing hormone: - an effective tool to block the fertility axis in mammals; 2005.
[29] Kotiw M, Johnson M, Pandey M, Fry S, Hazell SL, Netter HJ, Good MF, Olive C. Immunological response to parenteral vaccination with hepatitis B virus surface antigen virus-like particles expressing *Helicobacter pylori* KatA epitopes in a murine *H. pylori* challenge model. Clin Vaccine Immunol 2012;19(2):268–76.
[30] Ramzan F, Qureshi IZ, Ramzan M, Ramzan MH. Kisspeptin-10 induces dose-dependent degeneration in prepubertal rat prostate gland. Prostate 2013;73:690–9.
[31] Notter DR, Lucas JR, McClaugherty FS. The accuracy of estimation of testis weight from "in situ" testis measures in ram lambs. Theriogenology 1981;15:227–34.
[32] Lunstra DD, Ford JJ, Echternkamp SE. Puberty in beef bulls: hormone concentrations, growth, testicular development, sperm production and

sexual aggressiveness in bulls of different breeds. J Anim Sci 1978;46:1054–62.
[33] Rai J, Pandey SN, Srivastava RK. Testosterone hormone level in albino rats following restraint stress of long duration. J Anat Soc India 2004;53(1):17–9.
[34] Ferro VA, Khan MAH, McAdam D, Colston A, Aughey E, Mullen AB, et al. Efficacy of an anti-fertility vaccine based on mammalian gonadotropin releasing hormone (GnRH-I)-a histological comparison in male animals. Vet Immunol Immunopathol 2004;101:73–86.
[35] Bint Akhtar F, Weinbauer GF, Nieschlag E. Acute and chronic effects of a gonadotrophin-releasing hormone antagonist on pituitary and testicular function in monkeys. J Endocrinol 1985;104(3):345–54. https://doi.org/10.1677/ joe.0.1040345 [PMID: 3882872].
[36] Celis JP, Rodríguez OL, Quintal J. Correlation between scrotal circumference and some zoometric measurements with testis weight in Pelibuey sheep. Tec Peru Mix. 1987;25:85–93.
[37] Hennessy DP, Colantoni C, Dunshea FR, Howard K, Jackson P, Long K, et al. Elimination of boar taint: a commercial boar taint vaccine for male pigs. In: Bonneau M, Lundström K, Malmfors B, editors. Boar taint in entire male pigs, vol. 92. Wageningen Pers, Wageningen: EAAP Publication; 1997. p. 141–5.
[38] Meloen RH, Turkstra JA, Lankhof H, Puijk WC, Schaaper WMM, Dijkstra G, Wensing CJG, Oonk RB. Efficient immune- castration of male piglets by immunoneutralization of GnRH, using a new GnRH-like peptide. Vaccine 1994;12:741–6.
[39] Land RB. The expression of female sex-limited characters in the male. Nature 1973;241:208–9.
[40] Denise MM, Anne M, Stanley F. Persistent bacterial infections: the interface of the pathogen and the host immune system. Nat Rev Microbiol 2004;2:747–65.
[41] Mastroeni P, Simmons C, Fowler R, Hormaeche CE, Dougan G. Infect Immun 2000;68:46–53 [PMC free article] [PubMed].
[42] Yrlid U, Svensson M, Johansson C, Wick MJ. Salmonella infection of bone marrow-derived macrophages and dendritic cells: influence on antigen presentation and initiating an immune response. FEMS Immunol Med Microbiol 2000;27:313–20.
[43] Mestecky J. The common mucosal immune system and current strategies for induction of immune responses in external secretions. J Clin Immunol 1987;7:265–76 [J Clin Immunol 7265276].
[44] Azizi A, Ghunaim H, Diaz-Mitoma F, Mestecky J. Mucosal HIV vaccines: a holy grail or a dud? Vaccine 2010;28:4015–4026.A.