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ORIGINAL ARTICLE

Sperm Biology

Active immunization with GnRH-tandem-dimer peptide in young male rats reduces serum reproductive hormone concentrations, testicular development and spermatogenesis

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GnRH sterilization vaccines have been developed for various practical and clinical reasons. However, conjugation of GnRH peptide to carrier protein has many drawbacks, hampering the further commercialization of GnRH vaccines. In this study, a new nonconjugated GnRH vaccine, D-Lys6-GnRH-tandem-dimer peptide (TDK), emulsified in Specol adjuvant was investigated for its immunocastration efficacy in young male rats. Prepubertal male rats were randomly allocated into three groups (n = 12): control (no treatment), surgically castrated or immunized against 100 μg TDK in Specol adjuvant at 6 weeks of age (with a booster 8 weeks later). Blood samples (for antibody titers and hormone concentrations) were collected at 2-week intervals until rats were killed (18 weeks of age). Compared to intact controls, active immunization against TDK reduced ($P < 0.05$) serum concentrations of testosterone, inhibin B, LH and FSH, prevented the onset of spermatogenesis at puberty. Furthermore, mRNA expressions of GnRH receptor, *LH-β* and *FSH-β* in the pituitary, LH receptor, FSH receptor, inhibin α, β_A and β_B subunit in the testes were decreased in immunocastrated rats compared to intact *controls* ($P < 0.05$). These results demonstrate for the first time that GnRH-tandem-dimer peptide emulsified in Specol is a promising veterinary sterilization medicine.

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Keywords: GnRH; nonconjugation; rat; reproductive axis; sterilization

INTRODUCTION

Active immunization against GnRH, disrupts hypothalamic-pituitary-gonadal axis, inhibits secretion of gonadotropins, induces atrophy of gonadal tissues, and terminates gametogenesis, thereby resulting in infertility of both male and female mammals.[1] Accordingly, active immunization against GnRH has been an animal-friendly alternative to surgical castration to improve meat quality, growth performance, animal handling, as a tool to prevent (or terminate) unwanted pregnancy, to prevent undesired sexual behavior, to treat sex hormone dependent disorders, and to control the population of wildlife and wandering species.[2,3]

GnRH is a small peptide, which consists of only 10 amino acids and has no immunogenicity by itself. Thus raising an effective immune response against such a small peptide requires amplifying its immunogenicity. The most often used strategy is to conjugate a single copy of native GnRH molecule or modified polyvalent GnRH peptide to a carrier using chemical conjugation techniques.[2] However, such methods unavoidably create by-products, especially the nonconjugated carrier proteins, which require extensive purification to finally obtain a homogenous protein.[4] This drawback largely hampers the commercialization of GnRH vaccines as well as their further practical applications.

Oonk *et al.* (1998) designed a tandem decapeptide, with D-Lysine replacing L-Glysine at position 6 of the decapeptide, resulting in G6K-GnRH-tandem.[4] Surprisingly, immunization against such a short peptide, G6K-GnRH-tandem nonconjugated to carrier protein in Freund's adjuvant was tested to be effective in inhibiting testicular development of piglets.[4] In order to further improve its immunogenicity, G6K-GnRH-tandem was dimerized, resulting in the G6K-GnRH-tandem-dimer (TDK).[4] Immunization against TDK, nonconjugated to carrier protein, in Freund's adjuvant was tested to be much more effective than G6K-GnRH-tandem in inhibiting testicular development of piglets.[4] Based on these results, it appears that TDK alone possibly could be as a potential sterilization antigen. If so, this nonconjugated antigen, could largely avoid drawbacks resulting from conjugating GnRH peptide to carrier protein as mentioned above. However, in Oonk and his co-workers' study, they only examined the effect of active immunization against TDK on testis development and serum LH, the most important parameters on GnRH-immunocastration, such as antibody titers, testosterone and spermatogenesis were not systematically investigated. Moreover, in that study, the antigen was emulsified in Freund's adjuvant, which usually causes harsh injection-site reactions and thus is undesired or even

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unacceptable in case of companion animals or animals used for meat production.[2] Therefore, whether immunization against TDK emulsified in a mild and acceptable adjuvant is also effective in suppressing reproductive functions of animals, requires further investigation.

The present study, using a rat model, determined the efficacy of TDK emulsified in a practically acceptable adjuvant Specol[4] on antibody response, hormone level, testicular development and spermatogenesis, as well as mRNA expressions of reproduction-related genes in pituitary-testicular axis.

MATERIALS AND METHODS

Antigen and vaccine formulation

The GnRH antigen used in the present study was prepared as described by Oonk *et al.* (1998).[4] Briefly, the G6k-GnRH-tandem peptide (HWSYkLRPGQHWSYkLRPGC) was synthesized as described[4] and subsequently dimerized by dissolving it in 20% DMSO in water. The dimerized peptide (TDK) was dialyzed (MW cut-off 3000) against water and lyophilized. The obtained TDK was stored at −80°C until immunization. One day prior to immunization, TDK was emulsified in Specol adjuvant (ID-Lelystad formula) as described.[4]

Rats and immunization

Thirty-six adult male Sprague–Dawley (SD) rats (102–183 g, 4 weeks old) were purchased from the HuaXi Laboratory Animal Center of Sichuan University and randomly allocated to one of three groups (n = 12): control (no treatment), surgically castrated or immunized against TDK in Specol adjuvant. The surgical castration procedures were performed 2 weeks before the start of the experiment. These rats were individually housed, given *ad libitum* access to a commercial standard rat chow diet and tap water in a controlled environment with temperature of 21 ± 1°C, a relative humidity of 50%–60% and a 12 h light/12 h dark cycle. All procedures involving rats were approved by the Sichuan Agricultural University Animal Care and Use Committee.

Immunized rats received 100 μg TDK at 6 weeks of age (0 wpv = 0 week postprimary vaccination). The booster administrated 8 weeks later (8 wpv) had the same composition. The vaccine was given intramuscularly in both hind legs (1 ml per leg). All rats were decapitated 12 weeks after primary immunization.

Sample collection and measurements at decapitation

Blood samples (2.0–2.5 ml) were collected from the caudal artery (base of the tail) of each rat, at the start of the study and every 2 weeks thereafter until the end of the study. Blood samples were centrifuged at 2000 g for 15 min at 4°C and sera were stored at −20°C pending analysis of hormone concentrations and antibody titers.

After 12 weeks, all rats were anesthetized with ether and then decapitated. The pituitary gland was removed, and frozen in liquid nitrogen and subsequently stored at −80°C pending analysis of gene expression. Both testes were excised, dissected free of epididymides, weighed as a pair, and then length and width were measured with vernier calipers. Testis volume was calculated using the formula: $v = (4\pi(\text{width}/2)^2(\text{length}/2))/4$ and recorded as an average of both testes. One testis was frozen immediately in liquid nitrogen and stored at −80°C pending analysis of gene expression, whereas the other was fixed in Bouin's solution for histological examination.

Antibody titer and hormone determination

Anti-GnRH titers were determined by a radioimmunoassay (RIA) as our previous descriptions.[5] Antibody titers were expressed as percentage binding of ^{125}I-GnRH at 1:2000 dilution of sera.

Serum concentrations of testosterone, inhibin B, LH and FSH were measured using rat-specific ELISA Kits (Cusabio Biotech Co., Ltd, Wuhan, China). All samples were measured in duplicate. The sensitivity of the assays for testosterone, inhibin B, LH and FSH were 0.06 ng ml^{-1}, 0.5 pg ml^{-1}, 0.15 mIU ml^{-1} and 0.25 mIU ml^{-1}, respectively. The manufacturer reported that both the intra- and inter-assay variation coefficients for all the assays were lower than 15%.

Morphological analyses of testes

One testis from each immunized and each intact rat was prepared for morphological analysis. Testes were postfixed in 1% osmium tetroxide, dehydrated in a graded ethanol series from water through 70% to 100% ethanol in subsequent steps, and embedded in paraffin. Sections (5 μm) were cut and stained with hematoxylin and eosin (H and E) for light microscopy. Five sections of each testis were randomly selected, and the number of germ cells at various developmental stages in seminiferous tubules was counted using morphology analysis software (Images Advanced 3.2, MOTIC, Hong Kong, China). The diameter of five randomly selected round or nearly round profiles of seminiferous tubules were measured on each slide by optically superimposing a stage micrometer calibrated ocular rectile.

Quantitative analysis of mRNA expressions

Total RNA was isolated from the pituitary and testis using the TRIzol reagent (Invitrogen Co., Carlsbad, CA, USA), according to the manufacturer's instructions. Quantitative and qualitative analyses of isolated RNA were assessed from the ratio of absorbance at 260 and 280 nm and agarose gel electrophoresis. First-strand cDNA was reverse-transcribed using PrimeScript® RT reagent kit with gDNA Eraser (TaKaRa Bio, Co., Ltd, Dalian, China). Quantitative real-time PCR was analyzed in triplicate on CFX96 Real-Time PCR detection system (Bio Rad, Hercules, CA, USA) with SYBR® green Ⅱ . The PCR contained 40 ng cDNA, 500 nmol l^{-1} each of forward and reverse primers, and 2X SYBR® premix Taq™ (TaKaRa Bio Co., Ltd.). The primer sequences of target and reference genes are listed (**Supplementary Table 1**). The PCR cycling conditions were: initial denaturation at 95°C (1 min), following by 40 cycles of denaturation at 95°C (5 s), annealing at 58°C–61°C (25 s; **Supplementary Table 1**) and a final melting curve analysis to monitor purity of the PCR product. The cycle threshold value was analyzed (CFX96 detection system) and transformed to a relative quantity using a standard curve method. Relative gene expression levels were normalized to those of the eukaryotic house-keeping gene *β-actin*. Outcomes were expressed as fold changes relative to average mRNA levels of genes in intact control groups.

Determination of immunocastrates and nonresponders

Immunized rats with substantial postvaccination serum antibody and physiological responses were defined as "immunocastrates", whereas nonresponders were immunized rats with evidently lower postvaccination serum antibody titers and nearly no physiological responses (i.e., similar serum testosterone concentrations, testes weight and volume compared to intact controls at decapitation). There were three nonresponders.

Statistical analyses

Data were analyzed with the Statistical Analysis System, Version 9.2 (SAS institute, Cary, NC, USA). The experimental treatment was divided into three levels: immunization, surgical castration and control. Since three rats from immunized group did not respond to the treatment, a fourth level (nonresponders) was added to the treatment factor. The effects of treatment group on

testes weight and volume, testicular morphometric data, growth performance and amounts of mRNA were performed by one-way ANOVA using the General Linear Models (GLM) procedure. When applicable, multiple comparisons were performed by Duncan's method. The effects of treatment group on serum anti-GnRH antibodies, testosterone, inhibin B, LH and FSH were evaluated with a Mixed-Models ANOVA. For serum anti-GnRH antibody titers, serum concentrations of testosterone, inhibin B, LH and FSH, the model included the fixed effects of treatment, sampling occasion and their interaction, and the random effects of rat within treatment. Since all serum analyses were performed repeatedly, sampling occasion was analyzed with a repeated statement using various alternatives for the covariance structure (the model with the smallest AKaike's Information Criterion values was chosen). For serum anti-GnRH antibody titers, the covariance structure was Variance Components, whereas for serum concentrations of inhibin B, testosterone, LH and FSH, it was autoregressive of order 1. Serum GnRH antibody titers and concentrations of inhinbin B and testosterone were log-transformed to normalize their distribution. Back-transformed means without further correction were reported. Other outcome variables were normally distributed and did not require further transformation. Results were reported as mean ± s.d. When serum concentrations of testosterone, LH and FSH were below assay limits, the corresponding assay limits were used as their concentrations. For all statistical analyses, differences were considered significant at $P < 0.05$.

RESULTS

Animal health

Two rats from surgical castrates were dead at 2 and 6 wpv, respectively. Data from the two rats were thus excluded from statistical analyses.

Antibody titers

Serum anti-GnRH antibody titers were affected by treatment, sampling occassion, and their interaction ($P < 0.0001$). Anti-GnRH antibody titers in all immunized rats at 0 wpv and intact controls throughout the experiment were nondetectable (**Figure 1**). In immunocastrates, antibody titers increased after vaccination and differed at 4 wpv ($P < 0.001$). Following the booster vaccination, antibody titers in immunocastrates sharply increased ($P < 0.001$) and reached high binding at decapitation (4 weeks after booster vaccination). In nonresponders, anti-GnRH antibody titers remained substantially lower than immunocastrates from 4 to 12 wpv ($P < 0.001$).

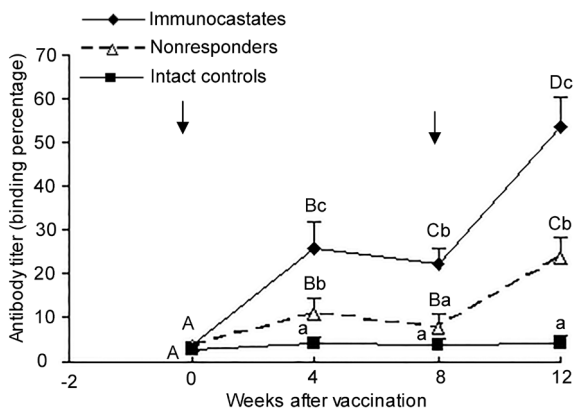

Figure 1: Antibody titers (% binding of ^{125}I-GnRH at a 1:2000 dilution) immunocastrates ($n = 9$), nonresponders ($n = 3$) and intact controls ($n = 12$). Data are mean ± s.d. Arrows indicate primary vaccination and subsequent booster. a–c Within a day, means without a common letter differed ($P < 0.05$). A–D Within a treatment group, means without a common letter differed ($P < 0.05$).

Testes weight and volume

Compared to intact rats, immunization against TDK reduced testis development (**Table 1**). At decapitation, testes in immunocastrates were reduced ($P < 0.05$) to 64% and 62% of the weight and volume of intact controls, respectively. While testes, weight and volume in nonresponders were not significantly affected by immunization (values similar to intact controls).

Growth performance

For the period between the first (day 0) and the second (8 wpv) immunization, average daily weight gain (ADG) of immunocastrated rats was similar to that of intact males ($P > 0.05$), which was higher than that of surgical castrates ($P < 0.05$, **Table 1**). For the second period (8 wpv - decapitation), ADG of immunocastrated rats was higher than that of intact males ($P > 0.05$), but lower than that of surgical castrates ($P < 0.05$). In relation to nonresponders, their ADG was similar to that of intact males from 8 wpv to decapitation ($P > 0.05$), but lower than that of intact males from 0 to 8 wpv ($P < 0.05$) for unknown reasons. However, for the whole experimental period (0 wpv - decapitation), ADG was similar for all the treatment groups ($P > 0.05$).

Serum hormone concentrations

At 0 wpv, serum concentrations of testosterone, inhibin B, LH and FSH were similar in intact male rats and immunized rats ($P > 0.05$; **Figures 2** and **3**). For serum concentrations of testosterone, inhibin B, LH and FSH, there were effects of treatment, sampling occasion, and their interaction ($P < 0.0001$). In intact males, mean testosterone, inhibin B, LH and FSH concentrations increased with age during 0 to 4 wpv ($P < 0.05$) and then varied with sampling occasion ($P < 0.05$). Compared to intact controls, serum concentrations of testosterone, inhibin B, LH and FSH in immunocastrates were only slightly decreased after the primary immunization ($P > 0.05$), whereas following the booster immunization these tested serum hormone concentrations were all substantially decreased and remained lower than those of intact controls from 10 to 12 wpv ($P < 0.05$). In nonresponders, these tested serum hormone concentrations were slightly decreased after immunization, but remained similar to those of intact males throughout the experimental period ($P > 0.05$). Serum concentrations of LH and FSH in surgical castrates increased markedly after surgical castration and then remained consistently higher ($P < 0.05$) than those of intact controls throughout the remainder of the experimental period.

Morphological observation of testes

Testes from intact rats (**Figure 4a** and **4b**) and nonresponders (**Figure 4e** and **4f**) had normal seminiferous tubules (S) containing spermatogenic cells at various stages of development, including spermatogonia, primary spermatocyte, spermatid and spermatozoa, as well as complete interstitial tissues with Leydig cells. In contrast, testes from immunocastrated rats had 33% reduction in diameter of seminiferous tubules compared to intact controls ($P < 0.01$). Furthermore, the number of germ cells at every developmental stage in seminiferous tubules of immunocastrated rats were less than that of intact controls ($P < 0.01$; **Table 1**). In addition, numbers of Leydig and Sertoli cells were also obviously diminished (**Figure 4c** and **4d**).

mRNA expressions

The mRNA expressions for reproduction-related genes in pituitary-testicular axis are shown in **Figure 5**. Compared to intact controls, mRNA expressions of all detected genes, were decreased

Table 1: Effects of treatment on testes characteristics and growth performance of rats[a]

Item	*Immunocastrates (n=9)*	*Nonresponders (n=3)*	*Intact controls (n=12)*	*Surgical castrates (n=10)*
Testes at decapitation				
Weight (g)	1.05±0.14[f]	1.65±0.13[e]	1.63±0.25[e]	-
Volume (cm^3)	0.82±0.07[f]	1.43±0.06[e]	1.33±0.23[e]	-
Tubule diameter (μm)	188.6±12.2[f]	276.5±19.4[e]	280.4±18.6[e]	-
Spermatogonium[b]	42.7±5.1[f]	55.6±5.2[e]	57.0±4.6[e]	-
Primary spermatocyte[b]	18.9±2.1[f]	60.0±5.4[e]	62.6±5.0[e]	-
Spermatid[b]	6.8±1.3[f]	431.3±40.6[e]	423.8±38.5[e]	-
Spermatoza[b]	0[f]	106.7±13.0[e]	110.2±11.6[e]	-
ADG[c] (g per day)				
Day 0 - 8 wpv[d]	3.63±0.19[fg]	3.48±0.33[ef]	3.69±0.21[g]	3.36±0.13[e]
8 wpv - Decapitation	1.75±0.13[g]	1.716±0.15[gh]	1.57±0.14[h]	2.38±0.20[e]
Day 0 - Decapitation	3.09±0.13	3.02±0.28	3.07±0.13	3.15±0.12

[a]Values are reported as mean±s.d.; [b]The number of germ cells in seminiferous tubules; [c]ADG: average daily weight gain; [d]wpv: weeks postprimary vaccination; [e–h]Within a row, means without a common superscript letter differed (P<0.05). s.d.: standard deviation

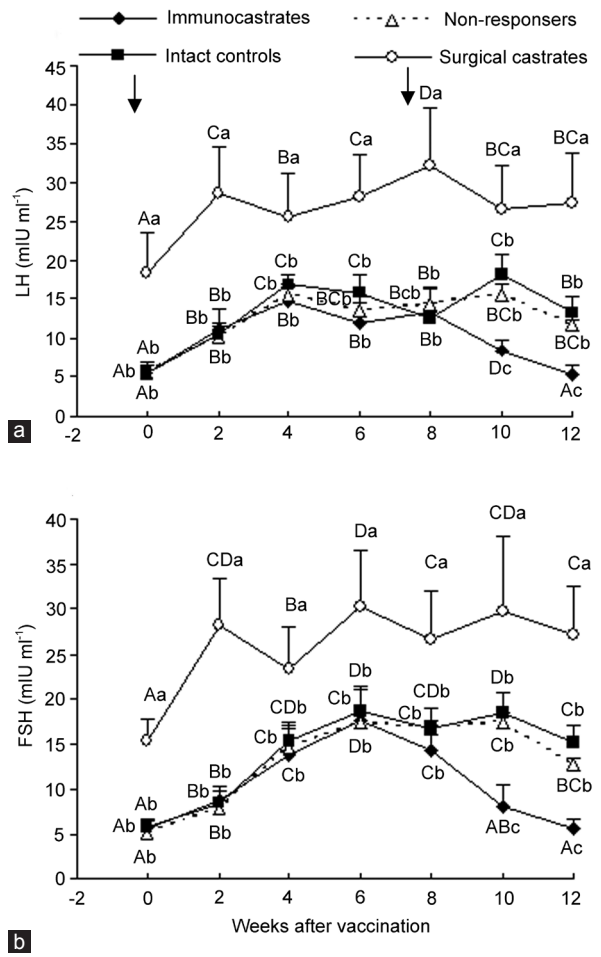

Figure 2: Serum LH (**a**) and FSH (**b**) concentrations in immunocastrates (n = 9), nonresponders (n = 3), intact controls (n = 12) and surgical castrates (n = 10). Data are mean ± s.d. Arrows indicate primary vaccination and subsequent booster. a–c Within a day, means without a common letter differed (P < 0.05). A–D Within a treatment group, means without a common letter differed (P < 0.05).

in immunocastrates (P < 0.05). Conversely, mRNA expressions of *GnRH-R*, *LH-β* and *FSH-β* in the pituitary were increased after surgical castration (P < 0.05). The mRNA expressions of these genes in nonresponders were similar to intact controls (P > 0.05).

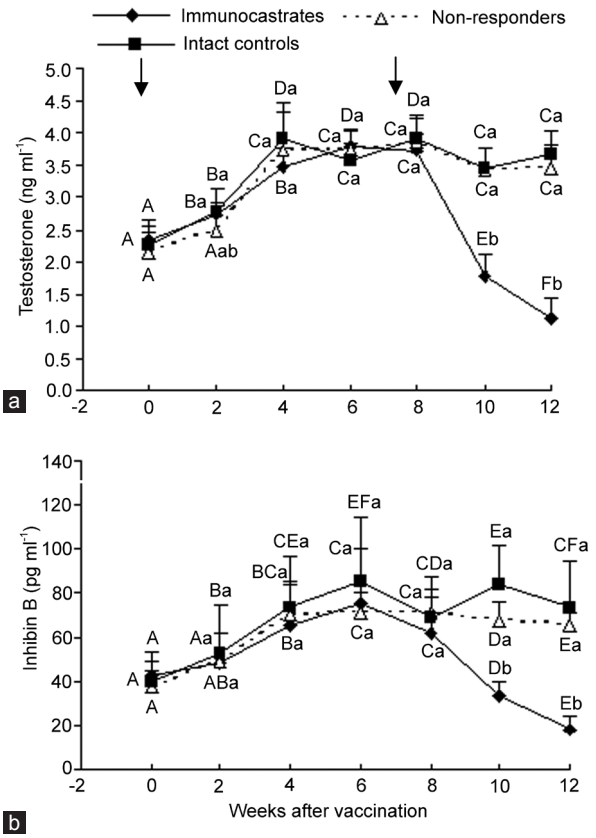

Figure 3: Serum testosterone (**a**) and inhibin B (**b**) concentrations in immunocastrates (n = 9), nonresponders (n = 3) and intact controls (n = 12). Data are mean ± s.d. Arrows indicate primary vaccination and subsequent booster. a and b Within a day, means without a common letter differed (P < 0.05). A–F Within a treatment group, means without a common letter differed (P < 0.05).

DISCUSSION

During the past four decades, various attempts have been made to develop GnRH sterilization vaccines for various practical and clinical reasons. In the present study, detailed information was provided on immune, endocrine and testicular responses to immunization with D-Lys6-GnRH-tandem-dimer peptide (TDK), nonconjugated to carrier in male rats.

Active immunization against TDK induced a good antibody response. Especially, after the booster immunization, there was a rapid

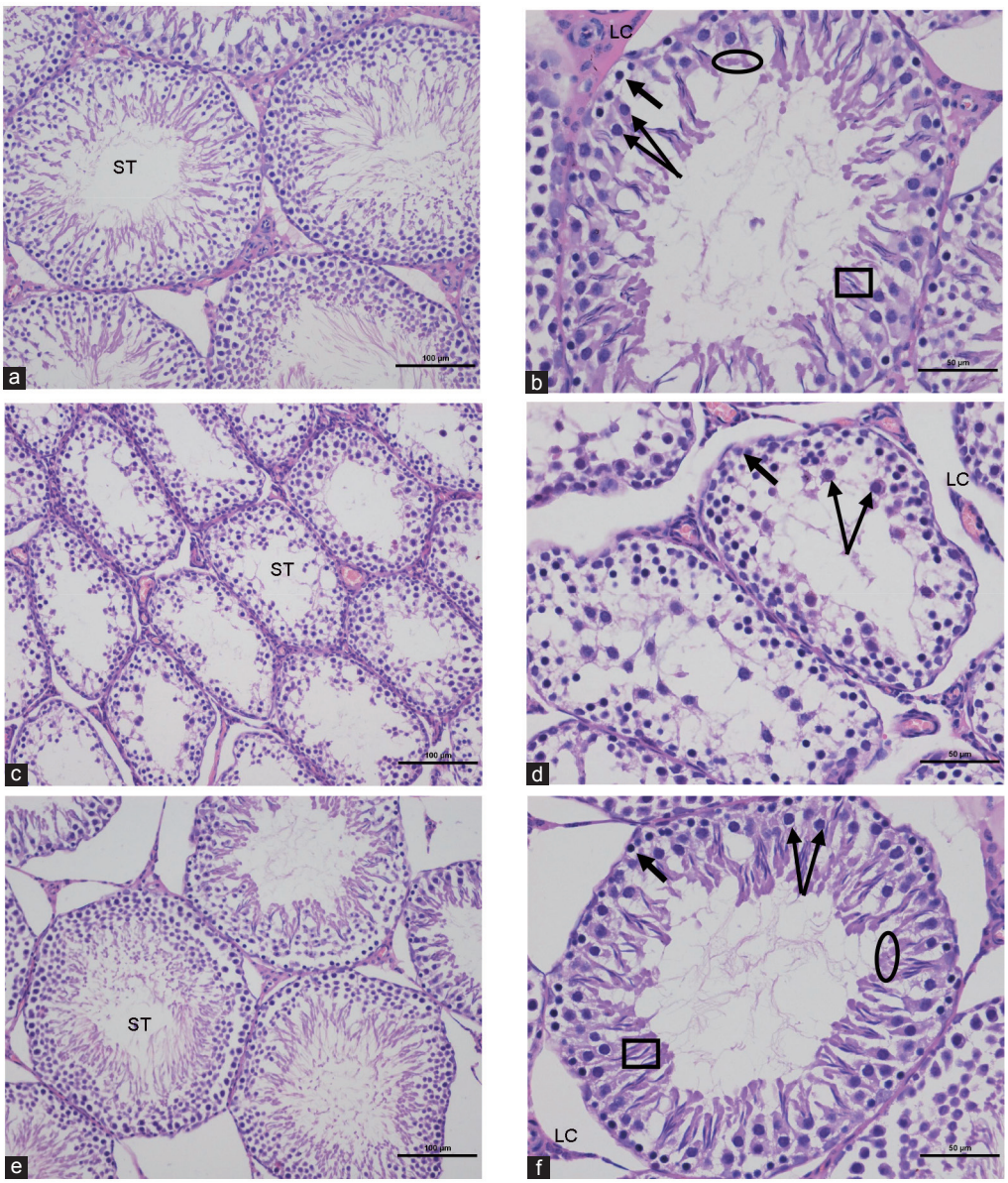

Figure 4: Testicular tissues of a control rat (**a** and **b**), an immunocastrated rat (**b** and **c**), and a nonresponder (**e** and **f**). ST, indicates seminiferous tubules; Cells in spermatogensis are shown as spermatogonia (short arrows), primary spermatocyte (long arrows), spermatid (ellipse), and spermatozoa (square). Note: (**a**, **c** and **e**) – magnification ×200, scale bar = 100 μm; (**b**, **d** and **f**) – magnification ×400, scale bar = 50 μm. Testicular tissues were stained with H and E.

and large increase in serum antibody titers. Although the primary immunization nearly had no obvious effect, with the rapid increase of serum antibody titers after the booster immunization, serum LH, FSH and testosterone concentrations were substantially suppressed, thereby severely restricting testicular development and impairing spermatogenesis. At decapitation, testes were reduced to 64% and 62% of the weight and volume of intact controls, respectively. In agreement with our findings, Oonk *et al.* (1998) documented that active immunization against TDK (or even D-Lys6-GnRH-tandem) was effective in suppressing reproductive functions of piglets.[4] However, in Oonk and his co-workers' study, only its effects on serum LH and testicular development were determined, and the adjuvant used was Freund's adjuvant that is harsh and unacceptable in practical applications.[2] In the present study, we proved that active immunization against TDK emulsified in a mild adjuvant Specol was also effective in inducing antibody production and suppressing reproductive functions in animals. The pattern of biological response following immunization against TDK is similar to that of a commercialized GnRH vaccine, Improvac (Pfizer Ltd.), in which the decreases of serum reproductive hormone concentrations and the suppression of testicular development also occur only after the booster immunization.[6,7] These results apparently demonstrate that G6K-GnRH-tandem-dimer, in nonconjugated form, is an effective antigen against GnRH, which when emulsified in Specol can be applied as a practical veterinary sterilization medicine.

Removal of hypothalamic GnRH input either by passive anti-GnRH immunization[8] or GnRH antagonist administration[9] has demonstrated that continued GnRH stimulation is necessary in maintaining the

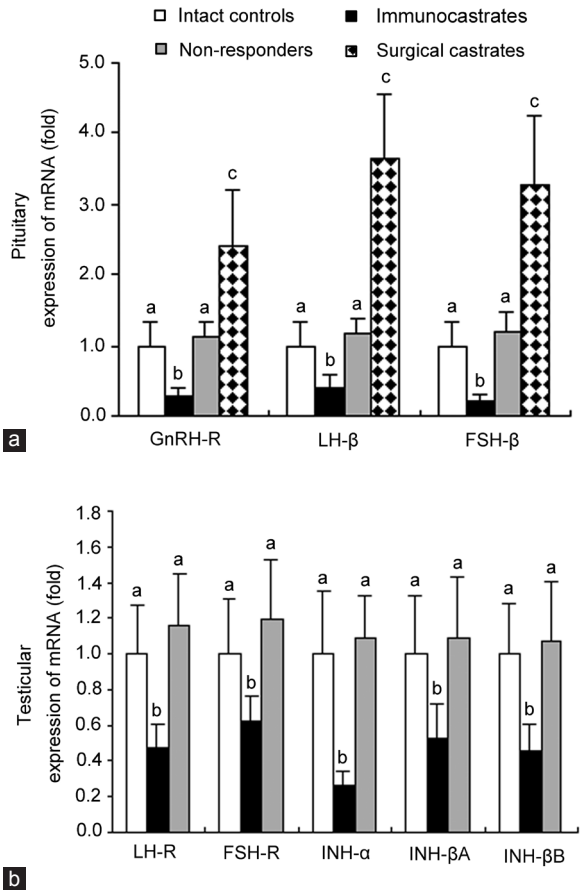

Figure 5: Effects of active immunization against D-Lys6-GnRH-tandem-dimer peptide (TDK) on mRNA expression levels of reproductive genes in the pituitary (**a**) and testis (**b**). Data are mean ± s.d. *GnRH-R*, gonadotropin-releasing hormone receptor; *LH-β*, luteinizing hormone β subunit; *FSH-β*, follicle stimulating hormone β subunit; *LH-R*, luteinizing hormone receptor; *FSH-R*, follicle stimulating releasing hormone receptor; *INH-β*, inhibin alpha subunit; *INH-β_A*, inhinhin beta A subunit; *INH-β_B*, inhinbin beta B subunit. a–c Within an end point, means without a common letter differed ($P < 0.05$).

synthesis of the pituitary GnRH receptor. In the present study, the mRNA expressions of GnRH receptor in the pituitary were decreased in TDK immunocastrated rats, in agreement with results of previous studies using GnRH-carrier conjugates in rats,[5] sheep[10] and rabbits[11] or using recombinant GnRH fusion protein in boars.[12] In parallel with the low concentrations of LH and FSH in serum, the mRNA expressions of LH-β and FSH-β in the pituitary were significantly decreased in TDK immunocastrated rats, as was reported previously in GnRH-immunocastrated rats,[5] sheep[10] and rabbits.[13] These results reflected a substantially diminished pituitary LH and FSH synthesis and secretion by lack of both GnRH and GnRH receptor in GnRH-immunocastrated animals. Therefore, the downregulation of pituitary GnRH receptor was also an important factor responsible for immunocastration.

Due to the reduced input from the pituitary after the booster immunization, testicular spermatogenesis in immunocastrated rats was significantly suppressed compared to intact controls. Histological observations showed smaller seminiferous tubules, and severely arrested spermatogenesis (diminished numbers of spermatogonia and primary spermatocytes, and absence of mature spermatozoa; **Table 1**). Similar histological changes of testes tissues after anti-GnRH immunization were also observed previously in rats,[5] rams,[10,14] pigs[12,15] and bulls.[16] Furthermore, the mRNA expressions of LH receptor and FSH receptor in testes were both significantly decreased, as in agreement with our previous studies in rats[5] and sheep.[10] LH is responsible for initiating testosterone production by acting on Leydig cells, whereas FSH stimulation of the testes starts the process of spermatogenesis by acting on Sertoli cells.[17] Therefore, Leydig cells and Sertoli cells play essential roles in maintenance of steroidogenesis and spermatogenesis within testes. The significantly lower expressions of LH receptor and FSH receptor in the present study suggested that the function of Leydig cells and Sertoli cells within testes was severely disrupted, as substantiated previously in boars.[18] Actually, gonadal functions of GnRH-immunized animals could not be restored by single or multiple administrations of gonadotropin analogs.[19] Therefore, except for the reduced LH and FSH, the compromised ability of testes to produce testosterone and sperm after anti-GnRH immunization was also largely attributed to the dysfunction of Leydig and Sertoli cells within testes.

Serum inhibin B concentrations were decreased in TDK immunocastrated rats after the booster immunization. In agreement with our finding, it was reported that serum inhibin B concentrations were also significantly decreased in rats after GnRH antagonist administration.[20] To the best of our knowledge, this was the first study to investigate the effects of anti-GnRH immunization on serum inhibin B in males. Inhibin is produced in males by the Sertoli cells, and secreted as heterodimers of a common α subunit with either a β_A subunit forming inhinbin A, or β_B subunit forming inhibin B.[21] In nearly all of the male mammalian species including rats, testes only secrete inhibin B and there is no evidence of the presence of inhibin A in peripheral blood.[21] In the present study, we confirmed the presence of inhibin α, β_A and β_B subunit mRNA in testes of rats by real-time quantitative PCR. Furthermore, we found that the mRNA expressions of inhibin α, β_A and β_B were all significantly decreased in immunocastrated rats compared to intact controls. Based on our findings, although not investigated, we speculated that male mammals (at least for rats) probably could produce inhibin A within testes, which may play a role in manipulating reproduction and spematogenesis through paracrine and autocrine signaling pathways. Our previous studies suggested that inhibin A could be a useful parameter for estimating the success of immunocastration in female pigs[22] and rams.[10] Although testosterone was usually considered as a good parameter for estimating the success of immunocastration in males, changes in serum inhibin B were tightly parallel with testosterone in the present study, suggesting that inhibin B can completely replace testosterone as a new useful marker for estimating the success of immunocastration in males. Furthermore, measurement of circulating inhibin B was shown to accurately predict the level of spermatogenesis in males.[23] Indeed, in the present study, the severely arrested spermatogenesis was accompanied by the significantly decreased mRNA expressions of inhibin α and β_B in testes. In that regard, using inhibin B as a maker for estimating the success of immunocastration in males appears to be superior to testosterone, as it covers both testosterone and spermatogenesis.

Testicular produced sex steroids are responsible for the efficient feed conversation and lean meat production.[7] In the present study, the decrease of serum testosterone concentration in immunocastrated rats did not occur until after the booster immunization. Therefore, compared to surgical castrated animals, serum testosterone in immunocastrated animals was retained high during most of their productive life. As a consequence, ADG of immunocastrated rats

was similar to the intact controls, and higher than that of their surgical castrated counterparts before the booster immunization. Thus, though not investigated, immunocastrated rats presumably had more lean meat production. Therefore, if applied to animals used for meat production, active immunization against GnRH using TDK probably leads to superior fattening performance as compared with surgical castrated. Indeed, Turkstra *et al.* (2002) reported that growth performance of GnRH-immunized boars was related to the time of the onset of biological response; the late responding animals had better growth performance in relative to the early responding animals and surgical castrates.[24]

Although all immunized rats developed antibody titers, 3 of the 12 immunized rats shown substantially lower immune responses than other immunized rats. In these nonresponding animals, serum concentrations of LH, FSH, testosterone and inhibin B were only slightly decreased after immunization, and there were no signs of testicular dysfunction. Variable individual-animal responses after anti-GnRH immunization were observed in various animal species, such as rats,[5] heifers,[25] sheep,[26] pigs,[22,27] cats[28] and elk.[29] The reason for the lower immune response after immunization in these nonresponding animals was unknown. However, our previous study in pigs indicated that the antibody production and consequently the biological responses of GnRH-immunized animals were dependent on the dose of the antigen.[27] Given that the rats in the present study were only immunized with 100 μg TDK, we thus suspected that the relative more numbers of nonresponders in the present study were (at least in part) attributed to the low-dose usage of antigen. Further dose-effect of this GnRH vaccine in target animals should be conducted to confirm finally its efficacy before the practical applications.

In conclusion, active immunization of young male rats with G6K-GnRH-tandem-dimer, nonconjugated to carrier protein, in Specol caused dysfunction of the pituitary-testicular axis, reduced serum reproductive hormone levels and blocked spermatogenesis. Using G6K-GnRH-tandem-dimer as a GnRH sterilization antigen can effectively avoid drawbacks caused by conjugation GnRH molecule to carrier protein, and potentially improve growth performance of immunized animals. Thus, it could replace GnRH-carrier conjugates to be a promising veterinary sterilization medicine.

AUTHOR CONTRIBUTIONS

XYZ and XFH designed the study; XFH, JLL, QYZ, XHR, GCL and XHC performed the study; XFH drafted the manuscript; XGD performed the statistical analysis. All authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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Supplementary information is linked to the online version of the paper on the *Asian Journal of Andrology* website

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Supplementary table 1: Primer sequences for tissue genes

Gene	*Genbank accession No.*	*Primer sequence (5'-3')*	*Amplification length (bp)*	*Annealing temperature (^{0}C)*
GnRH-R	NM_031038	F: TCTGCAATGCCAAAATCATC R: GTAGGGAGTCCAGCAGATGAC	164	61
LH-β	NM_012858	F: CTTCTCCTCTTCTGATGC R: TTTATTGGGAGGGATGGT	80	58
FSH-β	NM_001007597	F: CATCCTACTCTGGTGCTT R: CACTCTTCCTTCTCTACTGA	84	60.5
LH-R	NM_012978	F: TTACACATAACCACCATACC R: TCCAGCGAGATTAGAGTC	134	60
FSH-R	NM_199237	F: GAATGATGTCTTGGAAGTAATAG R: CTTAATGCCTGTGTTGGA	163	59.5
INH-α	BC083564	F: GCCTTGGTCTCCTGCA R: ACGCGTAGGGACCTCATGCTCC	62	300
INH-β_A	NM_017128	F: TGGAGTGTGATGGCAAGGTC R: AGCCACACTCCTCCACAATC	60	339
INH-β_B	NM_080771	F: TCTTCATCGACTTTCGGCTCAT R: TGTCAGGCGCAGCCACACTCCT	60	304
β-actin	NM_031144	F: CACAGCTGAGAGGGA AAT R: TCAGCA ATGCCTGGGTAC	155	60

GnRH-R: gonadotropin-releasing hormone receptor; *LH-β*: luteinizing hormone β subunit; *FSH-β*: follicle stimulating hormone β subunit; *LH-R*: luteinizing hormone receptor; *FSH-R*: follicle stimulating releasing hormone receptor; *INH-α*: inhibin alpha subunit; *INH-β_A*: inhinhin beta A subunit; *INH-β_B*: inhinbin beta B subunit