

Induction of castration by immunization of male dogs with recombinant gonadotropin-releasing hormone (GnRH)-canine distemper virus (CDV) T helper cell epitope p35

Mi-Jeong Jung¹, Young-Chan Moon¹, Ik-Hyun Cho², Jung-Yong Yeh¹, Sun-Eui Kim¹, Wha-Seok Chang¹, Seung-Young Park¹, Chang-Seon Song¹, Hwi-Yool Kim³, Keun-Kyu Park¹, Steven McOrist⁴, In-Soo Choi¹, Joong-Bok Lee^{1,*}

¹Department of Infectious Diseases, College of Veterinary Medicine, Konkuk University, Seoul 143-701, Korea

²Department of Physiology College of Dentistry, Seoul National University, Seoul 110-749, Korea

³Department of Veterinary Surgery, College of Veterinary Medicine, Konkuk University, Seoul 143-701, Korea

⁴Tufts University School of Veterinary Medicine, Westboro Road, North Grafton, Massachusetts 01536, USA

Immunocastration is a considerable alternative to a surgical castration method especially in male animal species for alleviating unwanted male behaviors and characteristics. Induction of high titer of antibody specific for gonadotropin-releasing hormone (GnRH) correlates with the regression of testes. Fusion proteins composed of canine GnRH and T helper (Th) cell epitope p35 originated from canine distemper virus (CDV) F protein and goat rotavirus VP6 protein were produced in *E. coli*. When these fusion proteins were injected to male dogs which were previously immunized with CDV vaccine, the fusion protein of GnRH-CDV Th cell epitope p35 induced much higher antibody than that of GnRH-rotavirus VP6 protein or GnRH alone. The degeneration of spermatogenesis was also verified in the male dogs immunized with the fusion protein of GnRH-CDV Th cell epitope p35. These results indicate that canine GnRH conjugated to CDV Th cell epitope p35 acted as a strong immunogen and the antibody to GnRH specifically neutralized GnRH in the testes. This study also implies a potential application of GnRH-based vaccines for immunocastration of male pets.

Key words: Immunocastration, GnRH, canine distemper virus, T helper cell epitope, dogs

Introduction

Gonadotropin-releasing hormone (GnRH), a very small protein composed of 10 amino acids, is produced from hypothalamic neurons. Its main function is to control the

reproductive system in both male and female animals [4]. Studies to sterilize the male animals reproductive ability have been attempted by using GnRH as an immunogen [7, 14]. The immunocastration was demonstrated only in the GnRH-immunized animals showing the high titer of antibody specific for GnRH [8]. The method of immunocastration has been used in practice for several reasons, such as relieving aggressive behavior of male animals, eliminating boar tints, and enhancing growth rates of domestic animals [6,12]. In addition, it was proved that surgically castrated dogs are prone to accelerate prostate carcinoma [13]. Therefore, the immunocastration by inducing neutralizing antibody to GnRH is considered as a better and safer way than the surgical removal of testes in male animals.

In order to induce production of neutralizing antibody against GnRH, it should be coupled with carrier materials because of its too small size as an antigen [2]. GnRH conjugated with typical immunostimulating materials, such as keyhole-limpet hemocyanin (KLH) or tetanus toxoid, elicited immunocastration effects [13], but with some variation in the different animal species [7]. A few kinds of T helper (Th) cell epitope have been identified in canine distemper virus (CDV) F protein, influenza virus HA protein, and rotavirus VP6 protein [1,8,9]. These virus-originated Th cell epitopes played an important role for enhancing the production of GnRH-specific antibody when injected as complexes coupled with GnRH.

The objective of this study was to identify castration effects in male dogs immunized with fusion proteins composed of canine GnRH-CDV Th cell epitope p35 and rotavirus VP6 protein. We observed considerably elevated levels of GnRH-specific antibody in the blood and a reduced spermatogenesis in the testicular tissues in immunized male dogs with GnRH-CDV Th cell epitope p35 indicating a successful performance of immunocastration.

*Corresponding author

Tel: 82-2-450-3714; Fax: 82-2-458-5113

E-mail: virus@konkuk.ac.kr

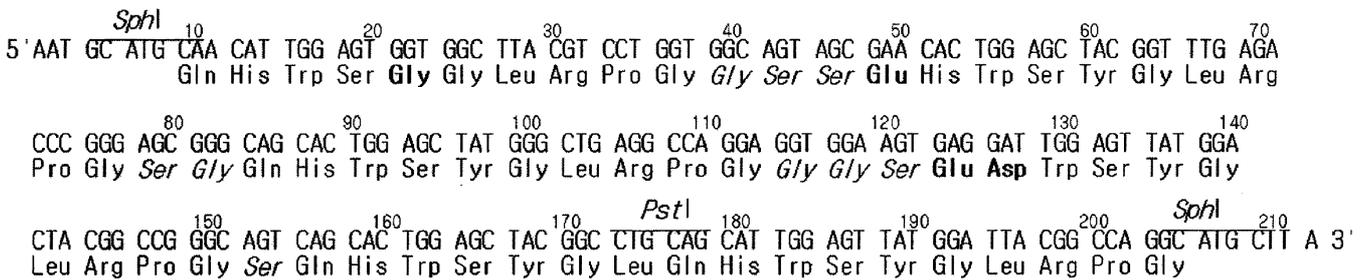


Fig. 1. DNA and amino acid sequences of canine GnRH hexamer. Six tandem repeats of canine GnRH cDNA showing restriction enzyme sites for *Sph* I at both 5' and 3' ends and minor modifications on its sequence. Bold amino acids denote mutated residues to increase antigenicity. Italic amino acids were used as linkers to connect each GnRH gene.

Materials and Methods

Construction of GnRH-conjugated vectors

A tandem repeated GnRH hexamer cDNA with minor amino acid substitutions (Fig. 1) was subcloned into pGEX-4T1 vector (Pharmacia, USA) from the plasmid pUC19 in a previous study. cDNA sequences of CDV Th cell epitope p35 (GenBank accession number, M21849) [9] and goat rotavirus VP6 (personally obtained from Korean isolate of goat rotavirus, but not reported to GenBank) were fused to the GnRH hexamer as described in the followings. To amplify CDV p35 gene artificially, single-stranded CDV p35 cDNA, 5'-GAA TTC ACT GCT GCT CAG ATC ACT GCT GGT ATC GCT CTA CAT CAG TCA AAT CTA AAT GAG CTC TGA GTC GAC-3', was synthesized (Bionex, Korea) and then the single-stranded template was amplified with the forward primer 5'-CGG AAT TCA CTG CTG CTC AG-3 and the backward primer 5'-GCG TCG ACT CAG AGC TCA TT-3. The PCR product digested with *Eco*RI and *Sal*I was inserted into the *Eco*RI and *Sal*I-digested pGEX-4T1 to obtain pGST-p35. The GnRH hexamer was amplified by PCR with primers harboring appropriate linker sequences (the forward primer 5'-GCG AGC TCC AAC ATT GGA GTG GTG GC-3 and the backward primer 5'-GCG TCG ACG CCT GGC CGT AAT CCA TA-3). The PCR product after digestion with restriction enzyme, *Sac*I and *Sal*I, was subcloned into *Sac*I- and *Sal*I-treated pGST-p35 to construct pGST-p35-GnRH. The pGST-VP6-GnRH was constructed by insertion of a PCR-amplified GnRH hexamer fragment after digestion with a restriction enzyme *Sph*I into the goat rotavirus VP6 gene that was previously treated with *Sph*I. The pGST-GnRH was constructed by insertion of the GnRH fragment digested with *Bam*HI and *Eco*RI into the same enzyme-treated pGEX-4T1 plasmid.

Expression and purification of recombinant proteins

Fusion proteins, such as GST-p35-GnRH, GST-VP6-GnRH, and GST-GnRH, were expressed in *E. coli* and purified in denaturing conditions by following the manufacturer's instructions (Pharmacia, USA). Briefly, protein expression was induced by addition of IPTG into bacterial culture at the

log phase to a final concentration of 1 mM. Fusion proteins were recovered from inclusion bodies in denaturing conditions by lysis of bacteria with 8 M urea. Each fusion protein was concentrated in polyethylene glycol and its identity was confirmed on SDS-PAGE.

Experimental animals and immunization

Experimental animals used in this study were housed at the laboratory animal research facility, Konkuk University, Korea. Eight healthy male beagle puppies were vaccinated with attenuated CDV (Fort Dodge, USA) prior to immunization and their sera were analyzed for identification of CDV-specific immune response. Twenty nM of each fusion protein mixed with Iscomatrix adjuvant was used for a single immunization dose. Eight of 12 week-old vaccinated dogs were divided into four groups and two dogs in each group were intramuscularly immunized with one of the fusion proteins, GST-p35-GnRH, GST-VP6-GnRH, GST-GnRH, and GST. Four weeks later, a boosting injection was conducted to dogs with the same dose and route.

ELISA for detection of anti-GnRH antibody

Serum samples were obtained from dogs in 2 weeks after the second injection of recombinant proteins. The titers of antibody specific for GnRH were determined by ELISA. Briefly, 400-fold diluted serum samples were added to an each well of ELISA microplate that was coated with KLH-conjugated GnRH. The plate was incubated for 60 min at room temperature. The plate was incubated for 60 min with 500-fold diluted biotinylated anti-dog IgG antibody. The streptavidin-HRP solution was added to the plate and that was incubated for 30 min. Color was developed by adding OPD and the reaction was stopped in 30 min by adding 2 M H₂SO₄. Optical density values were determined at 492 nm.

Histological study

Testes were surgically removed from both control and vaccinated dogs 18 weeks after vaccination. Their weights were measured before fixation with 10% buffered formalin. Five mm-thick sections of testicular tissues were prepared and they were stained by the hematoxylin and eosin (HE).

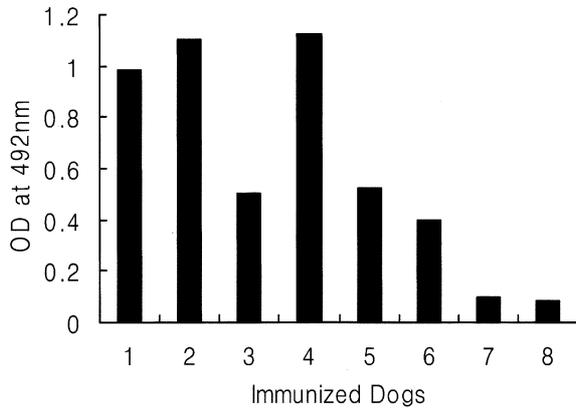


Fig. 2. Titers of antibody specific for canine GnRH in dogs immunized with recombinant GnRH vaccines. Twenty nmol of each fusion protein of GST-CDV p35-GnRH, GST-rotavirus VP6-GnRH, GST-GnRH, and GST was injected intramuscularly to male dogs. Boosting injection of the fusion proteins was conducted with the same dose four weeks later. Serum samples were collected from the dogs 6 weeks post 1st immunization (18 week-old) for evaluation of anti-GnRH antibody titer with ELISA. Dogs of number 1 and 2, 3 and 4, 5 and 6, and 7 and 8 are immunized with the fusion proteins of GST-CDV p35-GnRH, GST-rotavirus VP6-GnRH, GST-GnRH, and GST, respectively.

Results

Serum samples were collected from each dog 6 weeks post 1st immunization (18 week-old) for evaluation of anti-GnRH antibody titer with ELISA. Considerably high antibody titers were demonstrated in the two dogs immunized with GST-p35-GnRH (Fig. 2). Their average antibody levels were almost two-fold higher than those of dogs immunized with GST-GnRH. In one dog immunized with GST-VP6-GnRH also demonstrated a higher antibody titer than that of GST-GnRH-immunized dogs or control dogs. However, the other dog did not show any enhancement of antibody production compared to GST-GnRH-immunized ones (Fig. 2). On the aspect of average titer of GnRH-specific antibody, the antibody titer of dogs immunized with GST-p35-GnRH was much higher than that of dogs immunized with GST-VP6-GnRH. These data indicate that the intrinsic ability of CDV Th cell epitope p35 to assist for production of GnRH-specific antibody is superior to that of rotavirus VP6. The antibody titer of dogs immunized with control GST was negligible.

Testes of all 8 dogs were surgically removed 18 weeks after the 1st immunization (30 week-old) and the weights of them were measured. The testis weight of three dogs showing considerably high antibody titers, including two immunized with GST-p35-GnRH and one immunized with GST-VP6-GnRH, was about 2.0 g on average. However, other dogs, including controls, did not demonstrate production of GnRH-specific antibody and the average weight of their testes was about 5.0 g (data not shown).

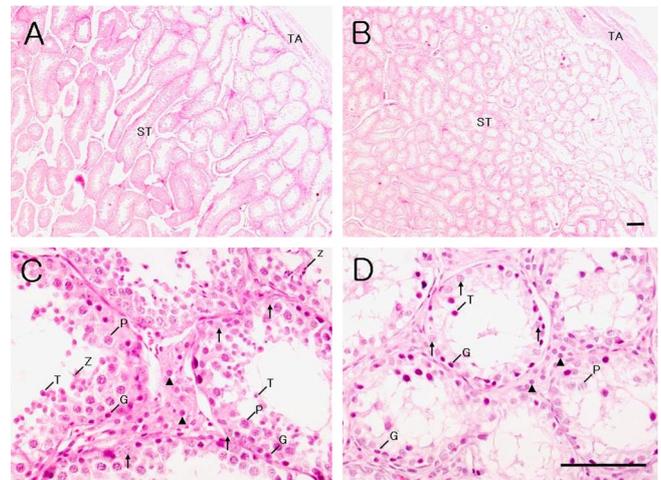


Fig. 3. Testicular tissues of dogs immunized with (A) GST as a control and (B) GST-CDV p35-GnRH fusion protein. Figures (C) and (D) show 4X magnified field of (A) and (B), respectively. Testicular tissues were stained by H&E stain. Spermatogenesis is denoted by spermatogonia (G) or primary (P) spermatocyte or spermatid. Black arrow and arrowhead represent Sertoli's cells and Leydig cells, respectively. Abbreviations ST, Z, and TA represent seminiferous tubules, spermatozoa and tunica albuginea, respectively. The size of black bar in the panel D indicates 200 μ m.

Histologically, the three dogs demonstrating high titer of GnRH-specific antibody had small seminiferous tubules, containing swollen and degenerated spermatocytes or spermatids, with the arrest of spermatogenesis at the developing stage of spermatogonia or the primary and secondary spermatocyte (Fig. 3). Active spermatozoa were not observed and there was a marked atrophy in the Sertoli and interstitial Leydig cells present in the testicular tissues of the three actively immunized dogs (Fig. 3). Other dogs except for the above mentioned three dogs had normal testes and spermatocytes. These data collectively indicate that antibodies specific for canine GnRH were induced in dogs immunized by GnRH conjugated with CDV Th cell epitope p35 or rotavirus VP6, and these antibodies neutralized GnRH resulting in degeneration of spermatogenesis in testicular tissues. Therefore, this study implies that the vaccination strategy of male dogs with GST-CDV p35-GnRH fusion protein is a very effective alternative method for performing immunocastration.

Discussion

In this study, we demonstrated the castration effects in male dogs by vaccinating them with CDV p35-conjugated GnRH. The incorporation of viral B- and T-cell epitopes in vaccine preparations has been proved to be efficient for production of protective antibodies [3,11]. The method of immunization against GnRH was generally applied to male animals for several reasons, such as improving growth rates

and reducing aggressive behavior. However, in these days the same methodology has been used for female animals to suppress ovarian activity [5,10]. Therefore, it is possible that GnRH vaccination can be used in practice as an alternative means for ovary degeneration in female dogs. The induction of castration in male or female pet by utilizing GnRH-specific immune response seems to be better than a traditional castration method.

This study was focused only on the effect of anti-GnRH immune response for regression of spermatogenesis in a short period. Although the anti-GnRH antibody induced degeneration of sperm genesis in this study, we did not examine how many sperms ejected have the fertilizing capacity. We also need to determine how long the GnRH-specific antibody can be produced in immunized animals. Several questions including those mentioned above will be solved in the future study. In summary, vaccination with a canine GnRH fusion protein conjugated with CDV Th cell epitope p35 induced high levels of GnRH-specific antibodies in the vaccinated male dogs. The vaccination also caused a regression of testicular functions in the dogs. These results indicate that the immunocastration in male dogs can be meaningfully accomplished by using a GnRH fusion protein vaccine in the presence of concomitant help of CDV Th cell epitope p35.

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