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Inhibitory effects of long-term repeated treatments of a sustainable GnRH antagonist, degarelix acetate, on caprine testicular functions

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Abstract. We investigated the effects of long-term repeated treatments with a sustainable gonadotropin-releasing hormone (GnRH) antagonist, degarelix acetate, on testicular hormonal secretion, size, ultrasound images, histology and spermatogenesis in goats to assess its efficacy as a chemical castration method. Male Shiba goats (3–6 months of age) were treated subcutaneously with degarelix acetate every 4 weeks for 24 weeks. Plasma testosterone and insulin-like peptide 3 concentrations decreased ($P < 0.05$) within 2 days after the first treatment and remained low until 29 weeks ($P < 0.05$). Scrotal circumference and testicular pixel intensity were lower from 2–6 months and from 1–6 months, respectively, compared to the pretreatment values ($P < 0.05$). The testis and epididymis weights were lower at 24 weeks compared to those in untreated goats ($P < 0.05$). There were no sperm in the seminiferous tubules of testicular tissue sections or in homogenates of the epididymis at 24 weeks. These results suggest that repeated treatment with degarelix acetate is an effective chemical castration method for goats.

Key words: Chemical castration, Male goat, Sperm, Sustainable GnRH antagonist, Testicular hormones

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Surgical castration of male food animals is performed to make them docile, to improve meat quality or to prevent the meat from damage [1]. In companion animals, it is done to avoid pregnancy or to suppress problem behaviors [1]. Chemical castration has been introduced as an alternative method [2, 3], since the surgical castration has drawbacks such as the necessity of anesthesia and possibility of post-operative infection [4]. It has been demonstrated that long-acting gonadotropin-releasing hormone (GnRH) agonists and antagonists, and GnRH vaccines can be used for the chemical castration [5, 6].

The GnRH antagonists inhibit luteinizing hormone (LH) and testosterone secretions by inhibiting the binding of endogenous GnRH to its receptor. The GnRH antagonists can rapidly suppress LH and testosterone secretions, which constitutes an important advantage over GnRH agonists and GnRH vaccines [7, 8]. A long-acting GnRH antagonist, degarelix acetate, has been used as a drug to treat prostate cancer in men, because it suppresses testosterone secretion more than one month [9].

We have reported previously that after a single injection of degarelix acetate in goats, the blood concentrations of insulin-like peptide 3 (INSL3) and testosterone were reduced significantly for two months, followed by a recovery to basal level [10]. However, the effects of repeated treatment of degarelix acetate on testicular hormonal secretion and morphology remain unknown.

We designed the present study to investigate the effects of repeated treatments with degarelix acetate on testicular hormonal secretion, size, ultrasound images, histology and spermatogenesis. The goal was to examine whether administration of a sustained GnRH antagonist can be used for chemical castration in goats.

There was a significant effect of time on plasma testosterone concentrations in the male goats (Fig. 1A, $P < 0.0001$). The plasma testosterone concentrations were lower from Day 1 until the end of blood sampling compared to the pretreatment value (Day 0; just before the first treatment with the GnRH antagonist) ($P < 0.05$).

A significant effect of time on plasma INSL3 concentrations was observed in the male goats (Fig. 1B, $P < 0.0001$). The plasma INSL3 concentrations were lower from Day 2 until the end of blood sampling compared to Day 0 ($P < 0.05$).

There was a significant effect of time on scrotal circumference (Fig. 2A, $P < 0.0001$). The scrotal circumference was lower from 2 months to 6 months from the start of treatment compared to pretreatment ($P < 0.05$), and treatment was found to have a significant effect on scrotal circumference ($P < 0.05$). The values for scrotal circumference were lower at 6 months after the first GnRH antagonist treatment than those in the control group ($P < 0.05$).

There was also a significant effect of time on the testicular pixel intensity (Fig. 2B, $P < 0.0001$). The testicular pixel intensity was lower from 1 to 6 months after the first GnRH antagonist treatment compared to that at Day 0 ($P < 0.05$), and the effect of treatment on testicular pixel intensity was significant ($P < 0.05$). The values for testicular pixel intensity were lower at 6 months after the first GnRH

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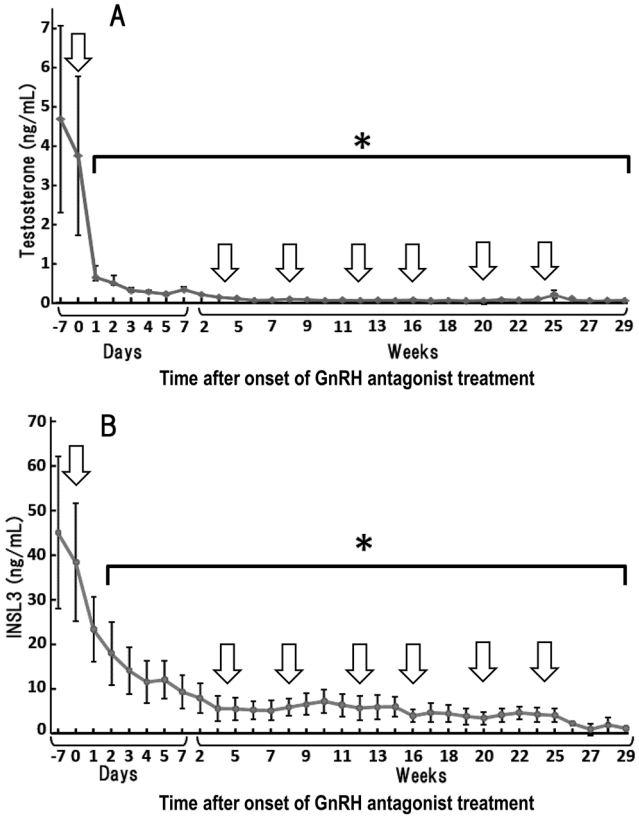

Fig. 1. Plasma testosterone (A) and insulin-like peptide 3 (INSL3) (B) concentrations in goats treated with a sustained gonadotropin-releasing hormone (GnRH) antagonist (4 mg/kg; subcutaneously). Data are expressed as mean ± SEM (n = 4). Asterisks indicate significant differences ($P < 0.05$) compared to the pretreatment (day 0). The arrow shows the days of GnRH antagonist treatment.

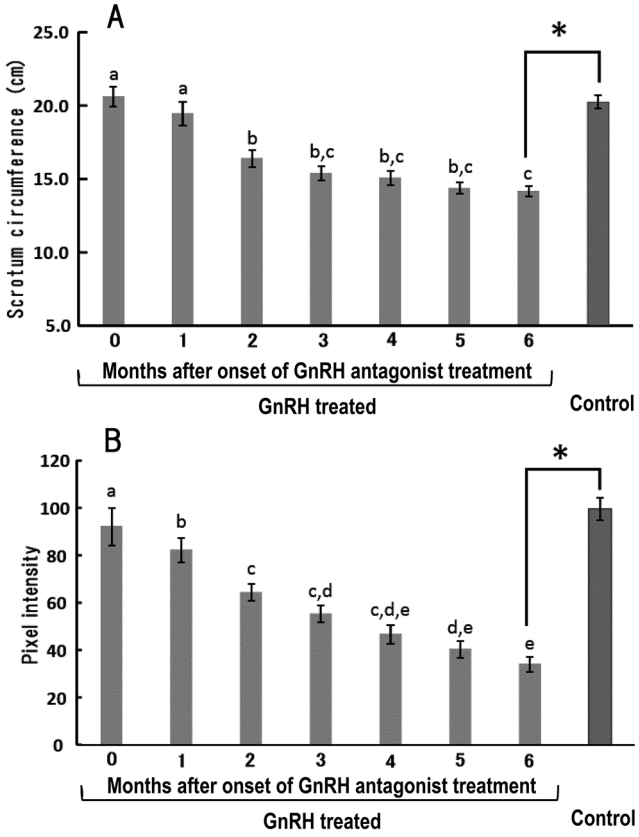

Fig. 2. Scrotal circumference (A) and testicular pixel intensity (B) in goats treated with a sustained gonadotropin-releasing hormone (GnRH) antagonist (4 mg/kg; subcutaneously) or without treatments (control). Control animals are non-treated males at corresponding age to the GnRH antagonist-treated males. Data are expressed as mean ± SEM (n = 4). The different letters represent significantly different results (a, b, c, d, e; $P < 0.05$). The asterisks indicate significant differences compared with the control group (* $P < 0.05$).

antagonist treatment than those in the control group ($P < 0.05$).

The testicular weight, epididymal weight, cross sectional area of seminiferous tubules and luminal area of seminiferous tubules were lower in the GnRH antagonist treatment group than the control group (Table 1, $P < 0.05$). The sperm count per epididymis was 16.5×10^9 in the control group, whereas the sperm count was nil in the goats of the GnRH antagonist treatment group.

The seminiferous tubules in the testis of the control group animals were well-developed and there were plenty of spermatocytes, spermatids and spermatozoa in addition to Sertoli cells (Fig. 3A). On the other hand, the seminiferous tubules in the GnRH antagonist treatment group were atrophied, and there were some spermatocytes but no spermatids or spermatozoa (Fig. 3B). Leydig cells in the GnRH antagonist treatment group were atrophied (Fig. 3D) as compared with those in the control group (Fig. 3C).

The present study revealed that plasma testosterone concentrations dropped on day after the first GnRH antagonist treatment, while the INSL3 concentrations fell at 2 days after the first treatment, and both hormonal concentrations were maintained at low levels throughout the period of repeated treatments. Our results demonstrated for the first time in goats that monthly treatments with a sustained GnRH antagonist over 6 months suppressed not only testosterone but also INSL3 secretions from the testes throughout the entire treatment period. In men with prostate cancer, monthly treatments with degarelix acetate for 12 months were reported to achieve a 90% reduction in plasma testosterone concentrations throughout the treatment period compared to the pretreatment value [11]. Our current study confirmed the previous findings in goats that plasma testosterone concentrations immediately declined after degarelix acetate treatment and INSL3 also decreased within a few days after the treatment [10], suggesting that these two testicular hormones are regulated by different mechanisms.

In previous reports in which GnRH vaccines were given to stallions [12] and rams [13], the seminiferous tubules and their lumens diminished in size and spermatogenesis deteriorated. Similar findings were observed in male rats: atrophy of seminiferous tubules and suppression of spermatogenesis occurred at 2 months after administration of a GnRH vaccine [14]. Here we observed a reduction of

Table 1. Weights of the testes and epididymides, cross sectional area of the seminiferous tubule and lumen area of the seminiferous tubule, and numbers of sperm per epididymis in goats with long-acting gonadotropin-releasing hormone (GnRH) antagonist treatment or without treatments (control)

	Control (n = 4)	GnRH antagonist (n = 4)
Weight of testes (g)	42.7 ± 4.2	11.3 ± 1.1 *
Weight of epididymides (g)	7.1 ± 0.9	1.7 ± 0.1 *
Area of seminiferous tubule (μm^2)	23575 ± 577	9983 ± 505 *
Lumen area of seminiferous tubule (μm^2)	3364 ± 160	103 ± 49 *
Number of sperm per epididymis ($\times 10^9$)	16.5 ± 3.1	0

Control animals are non-treated males at corresponding age to the GnRH antagonist-treated males. Data are expressed as mean ± SEM. An asterisk indicates a significant difference compared with the control (* $P < 0.05$).

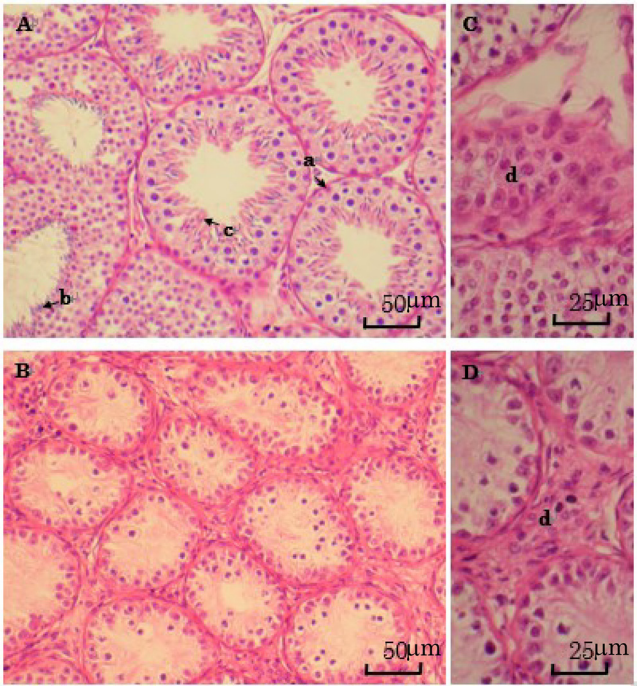

Fig. 3. Representative testicular histology stained with hematoxylin and eosin in goats treated with a sustained gonadotropin-releasing hormone (GnRH) antagonist (B, D) or without treatments (control) (A, C). Control animals are non-treated males at corresponding age to the GnRH antagonist-treated males. a, Sertoli cells; b, spermatozoa; c, spermatid; d, Leydig cells.

tubular diameters and complete suppression of spermatogenesis in the testicular seminiferous tubules, a complete absence of sperm in the epididymis, and atrophy of Leydig cells after repeated treatments with a GnRH antagonist for 6 months. Thus we suggest that a long-term GnRH antagonist can be used as an effective chemical castration agent in goats, since the inhibitory effects of the GnRH antagonist on testicular functions in our present experiments were similar to those previously observed by the GnRH vaccine [12, 13]. GnRH antagonists have an advantage over GnRH vaccines in terms of their rapid inhibition of testicular functions. That is, in the present study the testicular hormonal secretions were suppressed within 2 days after a single treatment of the antagonist, whereas in the previous reports using a vaccine, two shots with an at-least one-month interval were required to raise the antibodies [12–14].

It has been reported that scrotal circumference is correlated with sperm output in young dairy bulls [15, 16] and that, beginning around the time of puberty, early-maturing bulls have a greater scrotal circumference than late-maturing bulls [17]. In the current study using goats, the scrotal circumference started to decrease at 2 months after the first treatment and it was approximately 70% of the pretreatment length at 6 months. We reported previously that the scrotal circumference declined at 1 to 2 months after a single treatment with degarelix acetate in Shiba goats [10]. These findings suggest that the scrotal circumference can be utilized as a simple test for testicular functions in goats. In this context, it is noteworthy that Bongso *et al*. [18] found a correlation between scrotal circumference and body weight or age and predictability for onset of spermatogenesis in goats.

In the present study, we showed that the testicular and epididymal weights were reduced to only about 25% of the values in the control group after repeated treatments with a GnRH antagonist for 6 months in the goats. The long-term antagonist treatment also induced atrophy of both of the testicular seminiferous tubules and interstitial Leydig cells in our study. In stallions, the testicular volume was reported to decline at 5 weeks after treatment with another GnRH antagonist, acyline, every 5 days for 50 days [19]. Testosterone was shown to have a stimulatory effect on both testicular and epididymal development and maintenance in goats [20] and bulls [21]. However, the roles of INSL3 in the epididymal development and functions remain completely unknown, and further studies will be required to elucidate them.

In the present study, we found that the testicular pixel intensities in ultrasound examination were diminished after the degarelix treatment, and they reached nearly 35% of the pretreatment level after 6 months of repeated treatments. The diminution of testicular pixel intensity was clear and lasted throughout the antagonist-treatment period, whereas the reduction of scrotal circumference was slower and more subtle. We infer that ultrasonography of the testicular parenchyma is a useful and sensitive clinical test to check its functions. In support of this idea, the testicular pixel intensities by ultrasonography in

bulls [22] and stallions [23] showed highly positive correlations with the areas of seminiferous tubules in histological examinations.

In conclusion, this study revealed that repeated treatments with a sustainable GnRH antagonist suppressed testicular hormonal secretion in goats. The treatments also induced testicular and epididymal dysfunctions, including the atrophy of seminiferous tubules and interstitial cells, the loss of spermatogenesis and the absence of sperm. Thus, repeated treatment with a GnRH antagonist is an effective chemical castration method for male goats.

Methods

Animals

A total of eight male Shiba goats, a Japanese miniature breed, were used for the present experiments, and they were housed in an experimental room at Osaka Prefecture University throughout the study period. Examination of the goats before the start of the experiments revealed no apparent abnormalities in reproductive status; and both testes, checked manually, were located inside the scrotum. Goats were fed a concentrate and hay, with water *ad libitum*. This study was performed in accordance with the Guidelines for Animal Experimentation of Osaka Prefecture University, Japan as approved by the Animal Experiment Committee of Osaka Prefecture University.

GnRH antagonist treatment, blood sampling, scrotal circumference and testicular ultrasonogram

A schematic presentation of the protocol is presented in Fig. 4. Four male goats (3–6 months of age; body weight 12.3 ± 0.8 kg, mean ± SEM) in the GnRH antagonist group were treated subcutaneously with a sustained GnRH antagonist (degarelix acetate; Gonax®, Astellas Pharma, Tokyo, Japan; 4 mg/kg) at Day 0 (week 0) and the same treatment was repeated every 4 weeks until week 24 (7 treatments in total). The long-term (6 months) repeated (monthly) treatments with the GnRH antagonist were designed to test its inhibitory efficacy as a chemical castration method for male goats.

Blood samples were obtained from the goats in the GnRH antagonist group at Days –7, 0 (Day 0, week 0), 1, 2, 3, 4, 5, 7 (week 1) and thereafter weekly until week 29, except at weeks 3, 15 and 23 (Fig. 4). They were collected from the jugular vein into heparinized tubes and immediately placed on ice. The blood was centrifuged at 1700 × *g* for 15 min at 4°C. Then, the separated plasma was stored (−30°C) until assay.

Scrotal circumference measurement and testicular ultrasound examination were performed weekly from week 0 to 25. Scrotal circumference was determined using a measuring tape. A B-mode ultrasound scanner equipped with a 7.5 MHz linear array transducer (HS-2100V and HLV-475M; Honda Electronics, Toyohashi, Japan) was used to image the testes. The same equipment was used throughout the experiment and the settings (focus, gains, brightness, and contrast) were standardized and consistently used for all examinations. The ultrasound transducer was held vertically (parallel to the long axis of the testes) on the caudal surface of the scrotum and aligned so that the mediastinum was readily apparent before an image of each testis was obtained. Ultrasonograms were frozen, downloaded to a computer, and analyzed with image analysis software (Image 1.5; National Institutes of Health, Bethesda, MD, USA). Testicular pixel intensity was determined on a selected area by drawing two circles with 5 mm diameter below the mediastinum of each testis, where the parenchyma appeared homogeneous [24]. Testicular pixel intensity represented the average value attributed to each pixel within the selected area according to the shade of gray on a scale of 1 (black) to 255 (white). Results from the left and right testes were averaged for analysis.

Orchiectomy

Orchiectomies of the goats were carried out at week 25 after the first GnRH antagonist treatment, and the testes and epididymides were collected for further analyses. Four other male goats (9–12 months of age; body weight 12.8 ± 1.1 kg) were used as a control group. They underwent measurement of scrotal circumference and testicular ultrasound examination followed by the orchiectomy, and the testes and epididymides were stored for further analyses.

The goats were sedated with xylazine hydrochloride (Celactal 2% Injection solution, Bayer, Tokyo, Japan; 0.1 mg/kg IV) 10 to

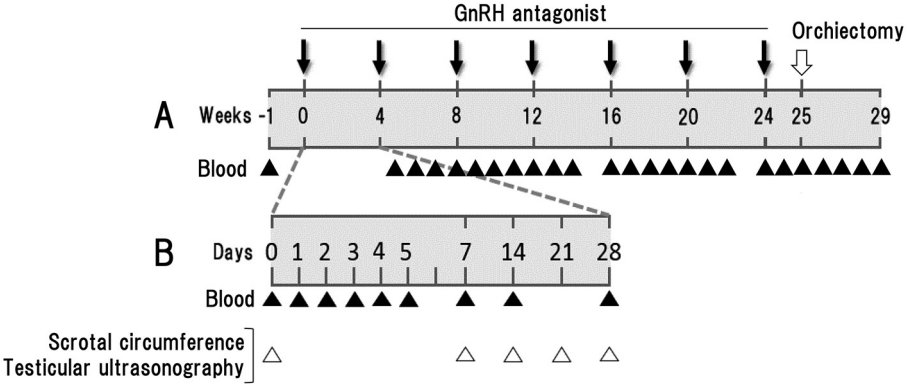

Fig. 4. Diagrams of experimental designs. (A) The black arrows show days of gonadotropin-releasing hormone (GnRH) antagonist treatment. The open arrow indicates day of orchiectomy. (B) The black triangles show days of blood collection, and white triangles indicate days of scrotal circumference measurements and testicular ultrasonography.

20 min before the orchiectomy. Incisions were made vertically on the skin of the anterior scrotum. The exposed spermatic cord was ligated by a surgical silken suture, and severed with a sterile blade at approximately 1 cm distal to the ligation. After the surgery, the testes and epididymides of both sides were separated and weighed.

Epididymal sperm count and testicular histology

Spermatozoa in the caput, corpus and cauda of the epididymis were enumerated as described previously [25]. The left or right side epididymis was removed from the testis, and the caput, corpus and cauda were separated on the basis of external morphology and weighed. Each part of the epididymis was minced and homogenized in saline containing 0.05%TritonX 100 (10 ml/g tissue) for 1 min with a Polytron® (PT-10; Kinematica AG, Littau/Luzern, Switzerland). After a 10-fold dilution for the caput and corpus and a 100-fold dilution for the cauda epididymis, the sperm were counted with a Thoma hemacytometer (Sanlead Glass, Koshigaya, Japan). The sperm numbers for the caput, corpus and cauda were added and expressed as a sperm count per epididymis.

The testis was sectioned in the midsagittal plane, and 1 × 1 × 1 cm samples of the parenchyma were collected for histology. The samples were fixed in Bouin's solution for 24 h, kept in 70% ethanol until embedded in paraffin according to conventional techniques, sectioned 7-μm thick, and stained with hematoxylin-eosin. Histologic evaluation was performed with the aid of image analysis software (ImageJ 1.5; NIH, Bethesda, MD, USA). The cross sectional area and luminal area of the seminiferous tubule were determined from 10 randomly selected tubular cross sections with a round and visible lumen under magnification × 200.

Hormone analysis

Plasma INSL3 concentrations were measured by time-resolved fluorescence immunoassay (TRFIA) without an extraction procedure [26–28]. The minimum detection limit of the INSL3 TRFIA was 0.156 ng/ml, and the detection was reliable in the range from 0.156 to 20 ng/ml. The intra-assay and inter-assay CVs were 2.9% (n = 4) and 19.2% (n = 11), respectively.

Plasma testosterone concentrations were measured by the enzyme immunoassay (EIA) method established in our laboratory [29–31]. The minimum detection limit was 0.156 ng/ml, and the reliable detection limit was 0.156 to 20 ng/ml. The intra-assay and inter-assay CVs were 4.6% (n = 4) and 14.2% (n = 9), respectively.

Data analysis

We investigated the effect of time (day, week or month) on the scrotal circumference, testicular pixel intensity and hormonal concentrations by conducting an analysis of ANOVA using the Generalized Linear Models (GLMs) procedure of SPSS version 24 software (IBM, Somers, NY, USA). Post-hoc pairwise comparisons were made by the Bonferroni correction for differences between two time-points. The weekly data of scrotal circumference and testicular pixel intensity for 4 weeks were averaged, converted to monthly data and subjected to the statistical analyses. We investigated the effect of treatment on the scrotal circumference, testicular pixel intensity, testicular weight, epididymal weight, cross sectional area of the seminiferous tubule, luminal area of the seminiferous tubule and sperm count per epididymis by conducting an analysis of ANOVA using the Generalized Linear Models (GLMs) procedure of SPSS version 24 software. Post-hoc pairwise comparisons were made by the Bonferroni correction for differences between two treatments (GnRH antagonist treatment vs control) and between two time-points. The data are expressed as mean ± standard error of the mean (SEM). Differences were considered significant at $P < 0.05$.

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