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Postponement of canine puberty by neonatal administration of a long term release GnRH superagonist

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ABSTRACT

The objective of this study was to assess the efficiency and clinical safety of postnatal administration of a GnRH agonist on canine puberty postponement. Sexual steroids and histological gonadal changes were also described. Twenty-four littermate puppies were randomly assigned to: Deslorelin acetate 18.8 mg sc (DESLO; n = 12) or Placebo: sc (PLACE; n = 12) postnatally. The dogs were clinically and endocrinologically followed up until puberty when they were gonadectomized and their gonads histomorphometrically studied. Deslorelin postponed the age of puberty (72.7 ± 4.8 vs. 35.8 ± 1.9 weeks; $P < 0.01$) in these dogs. At the time of this submission, 3 DESLO dogs (108 weeks old) remain non-pubertal. All dogs concluded growing at a similar age (29.75 ± 2.44 vs. 29.25 ± 0.90 weeks; $P > 0.1$) independently of their group and pubertal status. None of the females had side effects while the 2 non pubertal DESLO males presented bilateral cryptorchydism. All the bitches ovulated at puberty ($P > 0.1$) and the 2 DESLO that were mated became pregnant. Deslorelin postponed basal serum sexual steroids up to puberty in both genders ($P < 0.01$). The histomorphometrical study of the testes revealed that the tubular diameter ($P < 0.05$), germinal epithelium height and composition ($P < 0.01$) were decreased in DESLO group. Ovarian structures did not differ between treatments ($P > 0.05$). It was concluded that postnatal deslorelin decreased sexual steroids reversibly postponing puberty in both genders without side effects in bitches and causing 2/6 of cryptorchydism and impairment of testicular histomorphometry in male dogs.

1. Introduction

Reversible pubertal postponement is usually required for genetically valuable pure-bred dogs intended for working and/or breeding purposes. Progestins, the most widely used hormones for canine temporal prevention of reproduction, have been traditionally excluded from prepubertal animals because of the eventual permanent consequences they could provoke on the immature gonadal axis and uterus [1,2].

Gonadotropin-releasing hormone (GnRH) agonists have been produced by amino acid substitutions of the native GnRH molecule to create greater potency and longer half-life leading to longer duration of effectiveness. Long-term administration of agonists functions through desensitization and down-regulation of GnRH pituitary receptors inhibiting gonadotropin production and release after an initial stimulation. This initial "flare up" of the gonadal axis, which is manifested as an estrus cycle, is the main drawback of these compounds limiting their widespread use as contraceptives in females [3]. With the purpose of avoiding this disadvantage, a few studies have been conducted to test the efficacy of prepubertal GnRH agonists to postpone puberty [4–6]. Thus, an initial investigation in female dogs showed that puberty can be delayed without "flare up effect" if treated with the long term release GnRH agonist, deslorelin acetate (6-D-tryptophan-9-[N-Ethyl-LProlinamide]-10-desglycinamide), at the age of 4 months but not later [4]. Coincident results were obtained using both 4.7 and 9.4 mg deslorelin implants in nine, 4 to 5- months old bitches presenting a dose related prolongation of the prepubertal state until the age of 20 months or older. Importantly, six of these bitches had an increase in serum estradiol (E2) concentrations after implant insertion [6].

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Similarly, when the same protocols were used in eight, 4- months old male dogs, puberty was delayed in a dose dependent manner becoming pubertal after the second year of age [5]. In spite of these initial promising results, care should be taken when using chronological age as a threshold for efficiency and safety for these treatments. Four months of age may represent quite different proportions of body and reproductive development in different breeds and body sizes [7] severely limiting the reliability of these protocols. The finding of a safer period of treatment could maximize the utility of GnRH agonists to postpone puberty.

The early neonatal period is a well recognized period of reproductive vulnerability in most mammals [8,9]. Neonatal administration of GnRH analogs has delayed puberty in laboratory rodents and cats [10–12]. Importantly, GnRH postnatal immunized sheep presented a long term impairment of reproduction which exceeded the presence of serum antibodies [13]. Neonatal GnRH agonists may have a safe potential for contraceptive purposes in this species. The objective of this study was to assess the efficiency and clinical safety of the early neonatal administration of a high dose of deslorelin acetate on canine puberty postponement. Secondly, sexual steroids and histological gonadal changes were also described.

2. Materials and methods

2.1. Animals and pharmacological protocols

Twenty-four (5 litters) newborn, littermate puppies born in our Institutional Dog Colony were included in this study. Their progenitors were cross-bred, 13 ± 1.9 kg dogs. The puppies were sexed, weighed and identified at birth, weaned at the age of 50 days and fed with premium commercial puppy food and water *ad libitum*. This study was reviewed and approved by the Animal Care and Use Committee of the Veterinary School of the NULP and all experiments were conducted under the guidelines established in The Guide for The Care and Use of laboratory Animals, USA.

The dogs (male: n = 12 and female: n = 12) of the same litters were randomly assigned to one of the following treatment groups within the first 24 h after birth: Deslorelin acetate 18.8 mg (Suprelorin, Virbac, Carros, France) sc (DESLO; n = 12) or Placebo: placebo implants sc (PLACE; n = 12) in a way that each dog received one implant at each shoulder blade. Deslorelin acetate was supplied in the form of biocompatible, 9.4 mg implants (0.23 × 15.2 mm) in preloaded disposable syringes for sc administration.

2.2. Follow up

All the dogs were followed up until the first pubertal signs appeared. During the follow up period, the animals were physically examined (including body weight, withers height and scrotal volume [14]) once a week and observed 1 h twice a day looking for sexual behavior. The eventual appearance of clinical side effects were also recorded. Vaginal cytology and manual semen collection [15] were carried twice a week from the fourth month of age onwards.

Puberty was defined as the appearance of both the typical sexual behavior of each gender [15] in addition to > 80% superficial keratinized vaginal cells on a clean smear background and spermatozoa at semen collection in females and males, respectively [7,15].

Whenever possible (see below), the female puppies which attained puberty were exposed to a fertile male dog during the whole estrus period. Matings were observed and/or diagnosed by the presence of spermatozoa in the vaginal smears. Then, gestation was tested by ultrasound examination in these mated females [16].

2.3. Blood sampling and hormone determinations

Blood samples were collected by peripheral venipucture every week, every other week and every 4 weeks, until 1 month, 7 months and puberty, respectively. Serum testosterone (T; ng/ml) or estradiol-17β (E2; pg/ml) were measured by electrochemiluminescence immunoassay (Elecsys Testo II and Estradiol II, Roche Diagnostics, Mannheim, Germany) in the male and female dogs, respectively. Inter and intra coefficients of variation of the assays were <10% and sensitivity were 0.025 ng/mL and 5 pg/mL for T and E_2 kits, respectively.

In the females, 21 days after the end of estrus, blood samples were also taken for ovulation diagnosis by electrochemiluminescence immunoassay determination of serum progesterone (Elecsys Progesterone II, Roche Diagnostics, Mannheim, Germany; $P_4 > 5$ ng/mL; [15]).

2.4. Gonadectomies and histomorphometrical examination of the gonads

After puberty (or pregnancy diagnosis) all the experimental animals were gonadectomized [17,18]. Immediately after surgery the gonads were excised and sectioned longitudinally, placed in Bouin's fixative for 12 h and then changed to alcohol 70 and processed routinely with paraffin embedding. After processing 5 μm serial sections were cut, mounted on slides, dyed, deparaffinized in xylene, rehydrated in graded ethanol solutions and stained with hematoxylin and eosin.

All histological images were obtained from a microscope (Olympus BX50, Tokyo, Japan; 10X or 40X through an attached digital RGB video camera (Evolution VF Color, Q Imaging, USA) and digitalized in a 24 bit true color TIFF format. These images were measured by planimetry (Image Pro Plus v6.0-Media Cybernetics, Silver Spring, MA, USA).

Twenty round tubular profiles per testis were evaluated for tubular diameter (μm), mean germinal epithelium height (μm) as well as their components i.e. spermatogonia, primary and secondary spermatocytes, round spermatids, elongated spermatids, spermatozoa and Sertoli cells. The number of Leydig cells in 20 intertubular spaces was also recorded and their nucleus areas (μm^2) measured.

Ovarian follicles were divided into five classes on the basis of morphology and number of follicular cells in the widest cross-section, as previous described [19]: 1) primordial (an oocyte without a zona pellucida (ZP) surrounded by a single layer of flattened granulosa cells, 2) primary (oocyte with distinctive ZP surrounded by a single layer of cuboidal granulosa cells); 3) secondary (oocyte surrounded by several layers of granulosa cells); 4) early antral (space among granulosa cells or a segmented cavity with two or more compartments); 5) antral (one large, continuous cavity) or atretic (degenerated granulosa cells and follicular fluid containing cellular debris). The number of primordial, primary, secondary and antral follicles, corpora lutea, and atretic follicles was determined on a computer screen using 20 captured images (X20) per female dog.

2.5. Statistical analysis

All the calculations were carried out including the pubertal dogs. Discrete and continuous studied variables were compared between groups (DESLO vs. PLACE) by Fisher's Exact and Student's comparison tests, respectively. To characterize the dogs' growth rate, withers height and body weight were contrasted between treatments up to cessation of growth (defined as the maximum withers height attained) by ANOVA for repeated measures. In all

the cases descriptive data were expressed as mean ± SEM and P values < 0.05 were considered significant (SPSS 17.0, SPSS, Chicago, IL, USA).

3. Results

Postnatal deslorelin significantly postponed puberty in these dogs. Nine DESLO (72.7 ± 4.8 weeks old) and all PLACE (11/12; 35.8 ± 1.9 weeks old) dogs have attained puberty (P < 0.01). At the time of writing, the 3 remaining DESLO non pubertal dogs (2 males and 1 female) are 108 weeks old. One PLACE (1/12) dog died before puberty due to an accident not related to the trial. Puberty postponement occurred in both males (70.75 ± 35.5 vs. 35.2 ± 2.5 weeks; P < 0.05) and females (72.00 ± 8.7 vs. 39.3 ± 1.2 weeks; P < 0.05) when compared with PLACE group. In DESLO animals, postponement ranged from 40 to >104.3 weeks. Importantly, only one DESLO female achieved puberty at the age 40 weeks, all the other animals of this group became pubertal from 64.6 weeks of age on with a mean of 76.8 ± 2.9 weeks.

When withers height (P > 0.1; Fig. 1) and body weight (P > 0.1; Fig. 1 **Inset**) were serially compared up to mean age of cessation of growth they did not differ between treatments. Both groups (DESLO vs. PLACE) of animals concluded growing, defined as the maximum withers height, at a similar age (29.75 ± 2.44 vs. 29.25 ± 0.90 weeks; P > 0.1), respectively and independently of their pubertal status. Furthermore, neither withers height (47.56 ± 2.66 vs. 48.50 ± 3.25 cm; P > 0.1) nor body weight (12.07 ± 1.05 vs. 13.85 ± 1.75 kg; P > 0.1) differed between the same groups at puberty. While none of the females presented clinical side effects related to the treatments including post GnRH flare up (neither overt or covert endocrine; P > 0.1), the 2 non pubertal DESLO males suffered from bilateral cryptorchydism.

At the time of puberty, libido appeared normal in all the dogs without differences between treatments (P > 0.1). In males, scrotal

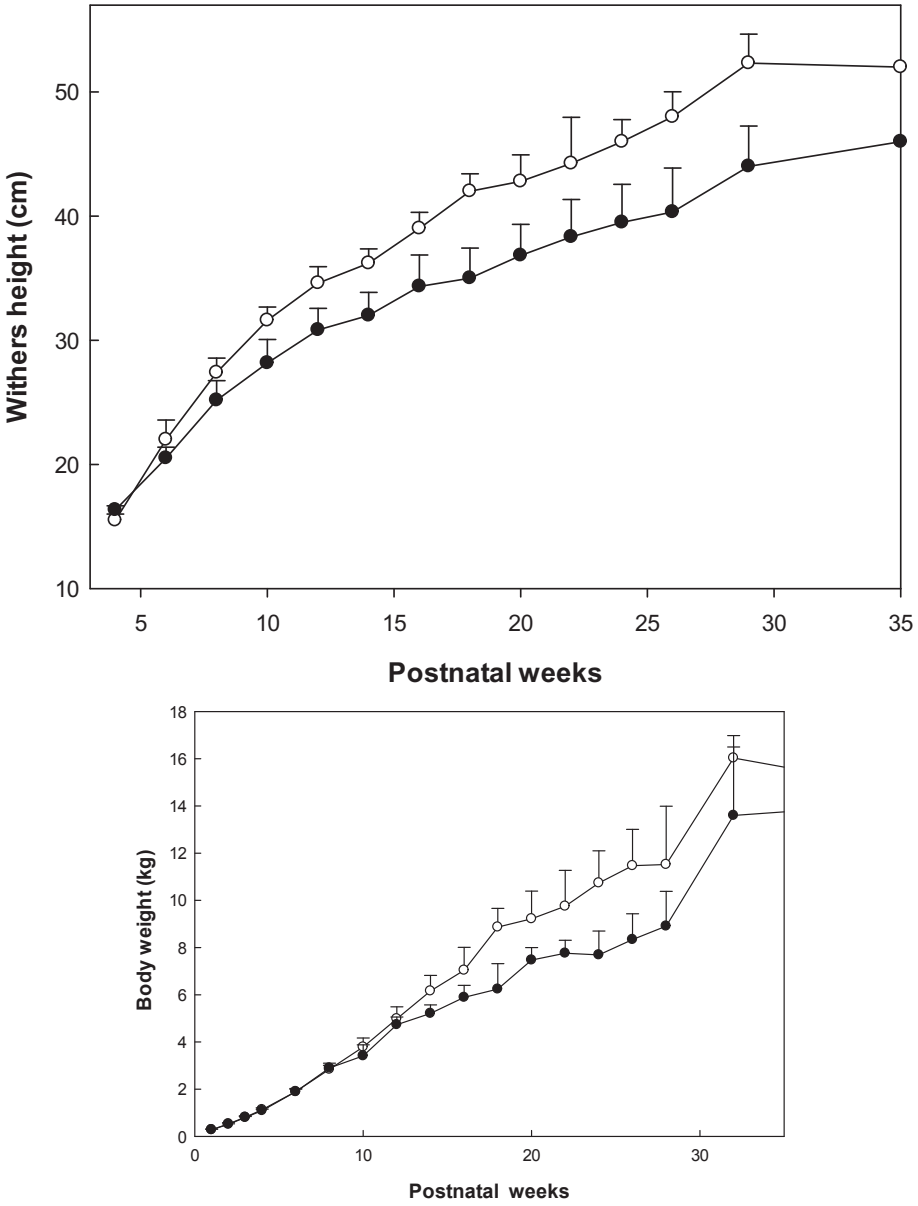

Fig. 1. Withers height (mean ± SEM; p > 0.1; A) and body weight (mean ± SEM; p > 0.1; B) of 24 littermate dogs treated neonatally (Day 0) with deslorelin acetate (18.8 mg; n = 12; solid symbols) or placebo (n = 12; open symbols) and followed up until cessation of growing.

volume at puberty did not also differ between DESLO and PLACE animals (9.91 ± 1.55 vs. 14.57 ± 3.50 cc; P > 0.1).

All the bitches ovulated during their pubertal estrus cycle (P > 0.1) and in the 8 (out of 12) females that were mated, gestation was confirmed in 2/2 and 5/6 (P > 0.1) in the DESLO and PLACE groups, respectively. Both DESLO and PLACE pregnancies appeared normal at the time of ovariohysterectomies.

In the male dogs, serum T remained low (<0.2 ng/mL) up to weeks 47.25 ± 5.56 and 12.80 ± 1.85 in DESLO and PLACE groups, respectively (P < 0.01). From those weeks on, T began to increase to non-basal concentrations (>0.5 ng/mL) up to 4–8 weeks before puberty, when the highest concentrations were reached in all the dogs (Fig. 2). It could also be visually observed that the prepubertal T increase seemed to be more rapid in DESLO group. The pubertal T determination in DESLO and PLACE males did not differ between groups (3.06 ± 0.83 vs 2.94 ± 0.56; P > 0.1). In all the females, E2 17-β concentrations remained low (<5 pg/mL) throughout the study except in peripubertal (<4 weeks from puberty) and pubertal determinations in which the values were >12 pg/mL. Estradiol 17-β pubertal concentrations were also normal and non different between groups (>20 pg/mL; P > 0.05; [15]).

The histomorphometrical study of the testes (Fig. 3) revealed that the tubular diameter (P < 0.05), germinal epithelium height (P < 0.01), the number of primary spermatocytes (P < 0.01) and the round spermatids (P < 0.01) were decreased in DESLO group. Conversely, in bitches the number of primordial, primary, secondary, antral and atretic follicles as well as corpora lutea were not different between treatments (P > 0.05).

4. Discussion

In this study it is reported the efficiency and clinical safety - including female fertility preservation in 2 mated bitches - of the neonatal administration of a high dose of deslorelin acetate formulated in slow release implants on canine puberty postponement. Acknowledging the dose related duration of deslorelin acetate effect in both prepubertal and mature animals [5–7], a two-fold regular dose was used in a homogeneous population of puppies during a critical time window of reproductive development i.e. the neonatal period [8,9].

Deslorelin acetate implants significantly postponed the age of puberty in dogs of both genders. Similar results have been reported for neonatal cats treated with a lower dose of deslorelin (1.6 mg/cat; [12]). Importantly and independently of chronological time, the mean percentage of puberty postponement obtained in these DESLO dogs represents more than 200% of that of their littermates PLACE animals.

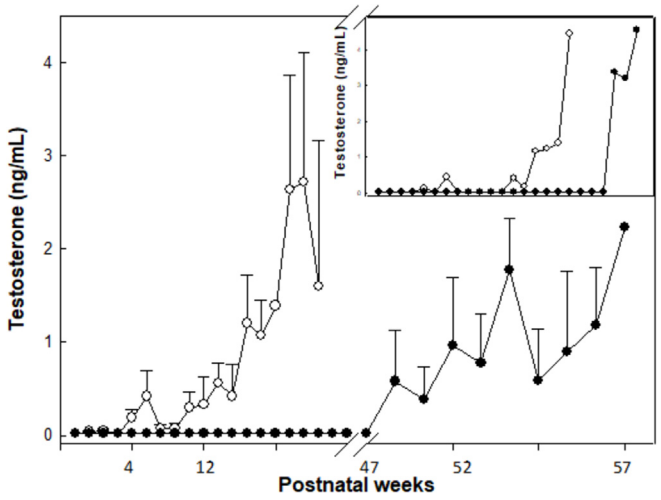

Fig. 2. Serum testosterone (mean ± SEM) of the male dogs (DESLO n = 6; solid symbols and PLACE n = 6; open symbols) of Fig. 1 followed up to puberty. Inset: Serum testosterone of a representative DESLO and PLACE male dogs.

The mean pubertal age for DESLO females (17 months) found in the present study was in line with that described for medium size bitches implanted with either 4.9 or 9.4 mg of deslorelin at the age of 4–5 months [6]. Conversely, puberty postponement in our male dogs was shorter that the one previously reported for male puppies treated with the same doses of deslorelin at the age of 4 months (>24 months of age; [5]). When interpreting this discrepancy it should be considered that comparisons with the latter male trial is of scarce validity as the body weight of those dogs was not reported. Furthermore, there were also differences in the way male puberty was defined in both studies [7]. In spite of these dissimilarities, the wide individual variation in the postponement of the reproduction function obtained in the present study (40 to > 104.3 weeks) is a constant finding in almost all GnRH agonists prepubertal and postpubertal reports [3,5,6]. This wide range could be due not only to individual conditions but also to the variability in the degree and duration of the implants resorption.

Deslorelin implants significantly postponed basal serum sexual steroids in both genders without causing an initial stimulation on the gonadal axis. Conversely, in the previously mentioned female trial, 6 out of 9 bitches presented an increase in serum E2 concentrations (>24 pg/mL) after deslorelin application [6]. In this respect, the advantage of neonatal over "juvenile" deslorelin administration could be explained by the lack of sexual hormonal receptors at the early neonatal period but not later in life [20]. Importantly, in DESLO animals sexual hormones concentrations at puberty were within the normal range described for each gender [15].

Similar to what has been reported for postnatal monkeys [21], neonatal felids [12] and "juvenile" dogs [5] treated with GnRH analogs, in this trial, libido at puberty appeared normal.

In these puppies growth rate was not affected by the neonatal treatment. Interestingly, the age of cessation of growth was similar in all the dogs independently of the group nor the pubertal status. This latter finding is difficult to interpret knowing the role of sexual steroids on growth plates closure [7]. Similarly, the delay in epiphyseal closure despite unaffected body size was proved in prepubertal bitches treated with these same implants [6,22]. Finally, in spite of the significant puberty postponement in DESLO dogs, height and body weight at puberty did not also differ between treatments. These findings are in line with the previous reports in prepubertal dogs treated with the same GnRH agonist [5,6].

Opposite to the findings of Kaya et al. (2015; [6]), none of these bitches developed neither clinical vaginitis nor any other side effect, including covert endocrine stimulation, during the follow up period. Conversely, the 2 non pubertal male dogs presented bilateral cryptorchydism probably due to the insufficient endocrine background at the postanatal time window of physiological testicular descent.

The abnormally long sexual hormone deprivation during the neonatal and "juvenile" periods did not seem to have negative permanent reproductive effects in these dogs as shown by the final attainment of puberty. The reversibility of the contraceptive effect is another constant finding throughout the pre and postpubertal deslorelin trials published so far [3]. Moreover, as shown by P4 serum concentrations, all DESLO bitches ovulated after their pubertal heat indicating that the postnatal sexual differentiation of the hypothalamus had normally occurred. Although *in vivo* fertility could only be tested and probed in part of these females, maintenance of pregnancy cannot be assured after the third week of

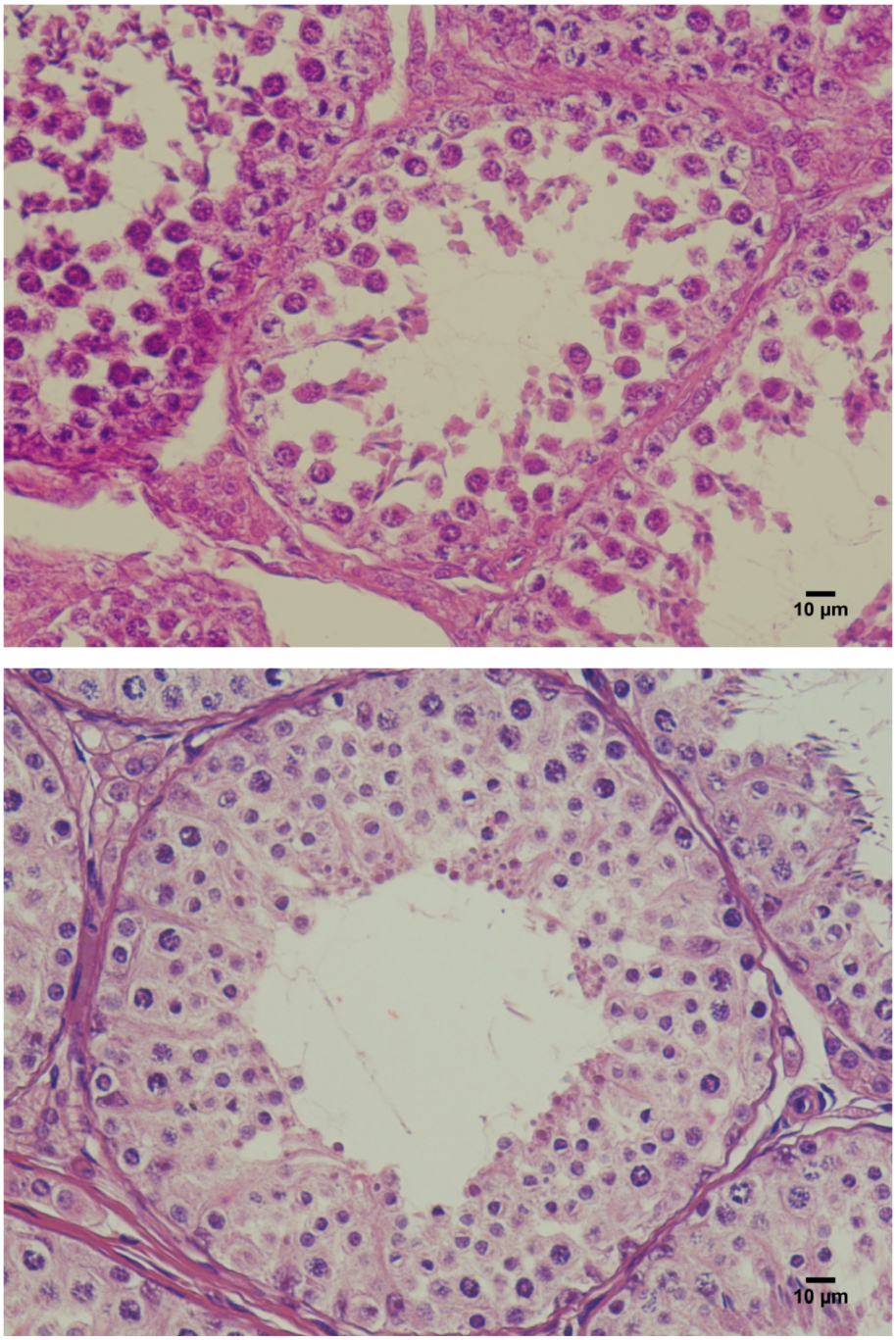

Fig. 3. Testicular tissue of a DESLO (A) and PLACE (B) male dog described. Fig. 2. A: Notice the severe reduction of the germinal epithelium cellularity and the frayed luminal borders. All sections were stained with hematoxylin and eosin (40X).

gestation. In this respect, in a recent study, deslorelin-mediated long-term delay of puberty did not have negative carry-over effects on subsequent ovarian functionality in bitches [22].

The histological testicular findings of these dogs are within those expected for hormonal depletion [23]. Similar results were found in rats [24,25] and cats [26] treated neonatally with GnRH analogs. The functional consequences of the diminution of the germinal epithelium in these GnRH agonist treated dogs remain to be determined. Conversely, neither the follicle pool nor their functionality, as shown by ovulation and pregnancy rate, were affected in these bitches. Similarly, no differences in both primordial and growing follicles pools were described at the date of first estrus in rats treated neonatally with a GnRH antagonist [27]. Although the differences in the histological gonadal impairment between DESLO genders are difficult to interpret, similar differences have been previously described in rats treated with a similar pharmacological protocol [28].

It was concluded that 18.8 mg of deslorelin acetate at the early neonatal period decreased sexual steroids reversibly postponing puberty in both genders without side effects in bitches and causing impairment of testicular histomorphometry and 2/6 of cryptorchydism in male dogs.

Conflicts of interest

The authors do not have any financial nor personal relationships with other people or organizations that could inappropriately influence the study.

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