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Normal Gonadal Functions and Fertility After 23 Months of Treatment of Prepubertal Male and Female Dogs with the GnRh Agonist [D-Trp^6, des-Gly-NH$_2$^{10}]GnRH Ethylamide

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The GnRH agonist [D-Trp^6, des-Gly-NH$_2$^{10}]GnRH ethylamide was administered to prepubertal male and female dogs daily for 23 months by subcutaneous injection. During GnRH agonist treatment, plasma steroid levels, namely dehydroepiandrosterone, androst-5-ene-3β-17β-diol, androstenedione, testosterone, dihydrotestosterone, 5α-androstane-3α,17β-diol, 5α-androstane, 3β,17β-diol were markedly inhibited in male animals, whereas in female animals, the plasma concentrations of DHEA and Δ^5-diol were decreased. Within 2 months following cessation of therapy, all steroids increased to normal adult levels. Morphological studies reveal that treatment of male animals with the GnRH agonist is accompanied by a small volume of seminiferous tubules, Leydig cells, and prostate gland, whereas in the ovaries of female animals, there is a large number of primordial follicles, a few primary follicles, but no secondary follicles. In the pituitary gland of animals of both sexes, LH-secreting cells have high levels of glycogen particles in their cytoplasm and tend to be either of normal appearance with dilated rough endoplasmic reticulum (RER) or strongly atrophied with a dark-stained cytoplasm, a contraction of RER, and a decrease in the number of secretory granules. Reticular cells of the connective tissue also show high levels of glycogen particles. After the 14 month recovery period, spermatogenesis has a normal adult appearance, the prostate gland shows a normal secretory epithelium, and secondary follicles are easily observed in the ovary. Gonadotrophs are free of glycogen accumulation, but reticular cells continue to show an accumulation of glycogen particles in their cytoplasm. Two male and two female animals were mated after the recovery period and produced normal offspring with normal fertility. The present results indicate that GnRH agonist treatment achieves a blockade of sexual maturation and that following cessation of treatment, normal pituitary-gonadal functions resume, with appararently normal fertility and normal offspring.

Key words: LHRH agonist, [D-Trp^6, des-Gly-NH$_2$^{10}]LHRH ethylamide, testes, ovaries, prostate, fertility, steroids, contraception, precocious puberty

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Since the first experiments demonstrating the antigonadal effects of GnRH agonist administration in the male rat (Auclair et al, 1977; Labrie et al, 1978) and in adult men (Labrie et al, 1980) and women (Bergquist et al, 1979), GnRH agonists have been extensively used in clinical medicine for the treatment of androgen- and estrogen-sensitive diseases, especially prostate cancer (Labrie et al,

1980, 1982, 1985; Tolis et al, 1982), breast cancer (Crowley, 1985), precocious puberty (Crowley et al, 1980; Comite et al, 1981; Pescovitz et al, 1986), polycystic ovarian syndrome (Chang et al, 1983), endometriosis (Meldrum et al, 1982), induction of luteolysis (Sheehan et al, 1982; Lemay et al, 1979), and male contraception (Linde et al, 1981). GnRH agonist administration also decreases plasma androgens and estrogens to castration levels in men and women (Labrie et al, 1980; Bergquist et al, 1979; Meldrum et al, 1982) throught the suppression of bioactive LH secretion (St-Arnaud et al, 1986).

Although the original studies demonstrating the antigonadal action of GnRH agonists were performed in the rat (Auclair et al, 1977; Labrie et al, 1978; Pelletier et al, 1978), the dog appears to be the best available model to study both the mechanism of action and the reversibilty of action of GnRH agonists. The changes in testicular steroidogenesis and spermatogenesis induced by chronic treatment with GnRH agonists are similar in man and dogs (Labrie et al, 1980; Vickery et al, 1982; Vickery and McRae, 1984; Linde et al, 1981; Bélanger et al, 1984; Tremblay and Bélanger, 1984; Tremblay et al, 1984a b, 1985); the main mechanism of action of GnRH agonists is a loss of LH bioactivity causing a secondary atrophy of the gonads and accessory sex organs (St-Arnaud et al, 1986; Lacoste et al, 1988). In the rat, however, a compensatory increase in 5α-reductase activity delays or prevents the complete blockage of testicular steroidogenesis (Carmichael et al, 1980).

Since GnRH agonists are widely used for the treatment of precocious puberty in both boys and girls, we studied the effects of long-term (23 months) GnRH agonist administration on the reproductive functions and sexual maturation of male and female prepubertal dogs, investigated the reversibilty of the effects following cessation of therapy. In addition to performing a histological evaluation of the gonads, accessory sex organs, and the pituitary gland at the end of chronic treatment and after the recovery period, we have measured a series of steroids in the plasma before, during, and after GnRH agonist administration and have studied fertility during the recovery period.

Materials and Methods

Chemicals

The GnRH agonist [D-Trp^6, des-Gly-$NH_2{}^{10}$]GnRH ethylamide (GnRH-A) was obtained from Bachem, California. All other chemicals and steroids were of analytical grade and were obtained from Anachemia and BDH (Montreal, Canada).

Animals

Twenty-three prepubertal beagle dogs (obtained from Laval University Animal Resources, Quebec, Canada) weighing between 1.5 and 3.0 kg were divided into two groups. The animals of each group were maintained in colony under controlled environmental conditions with lights on from 0600–1800 h. The animals were fed Purina dog chow *ad libitum* and had free access to tap water. The health status of the animals was checked monthly by a veterinarian.

Study Design

For 23 months, six males numbered 105, 161, 163, 004, 007 and 008 and nine females numbered 101, 102, 103, 104, 162, 164, 001, 002, 003, aged 140 to 160 days (prepubertal dogs) received daily subcutaneous injections of 100 μg of the GnRH agonist [D-Trp^6, des-Gly-$NH_2{}^{10}$]GnRH ethylamide dissolved in 0.9 NaCl-1% gelatin. During the same period, four males and four females (control dogs) received the vehicle alone. After 23 months of treatment, three male and four female treated dogs along with four female and four male control dogs were euthanized. The other dogs were kept alive to study the reversibility of gonadal function. A recovery period of 14 months was allowed before the other animals were euthanized. Blood samples were taken before the beginning of treatment and once a week thereafter. Reproductive functions were assessed as previously described (Tremblay et al, 1984b).

Steroid Radioimmunoassays

Plasma steroid concentrations were measured by radioimmunoassay following diethyl ether extraction and separation on LH-20 columns as described elsewhere (Bélanger et al, 1980).

The steroids measured included: progesterone, 17-OH-progesterone, dehydroepiandrosterone (DHEA), androst-5-ene-3β-17β-diol (Δ^5-diol), androstenedione, testosterone, dihydrotestosterone (DHT), 5α-androstane-3α,17β-diol (3α-diol), 5α-androstane-3β,17β-diol (3β-diol), and 17β-estradiol (E_2).

Histology

For light microscopic studies, the testes, prostate, ovaries, and pituitary glands were fixed by immersion in Bouin's fluid for 3 days. The tissues were then dehydrated in graded ethanol, cleared with toluene, and embedded in paraffin. Cross sections were cut at 7 μm thickness and studied after staining with hematoxylin and eosin. For the electron microscopic studies, small pieces of pituitary, testicular, and ovarian tissue were immediately immersed in 2.5% glutaraldehyde in a 0.1 M phosphate buffer, pH 7.4, for 2 h, washed in 5% sucrose in a 0.1 M phosphate buffer, post-fixed in 2% osmium tetroxide

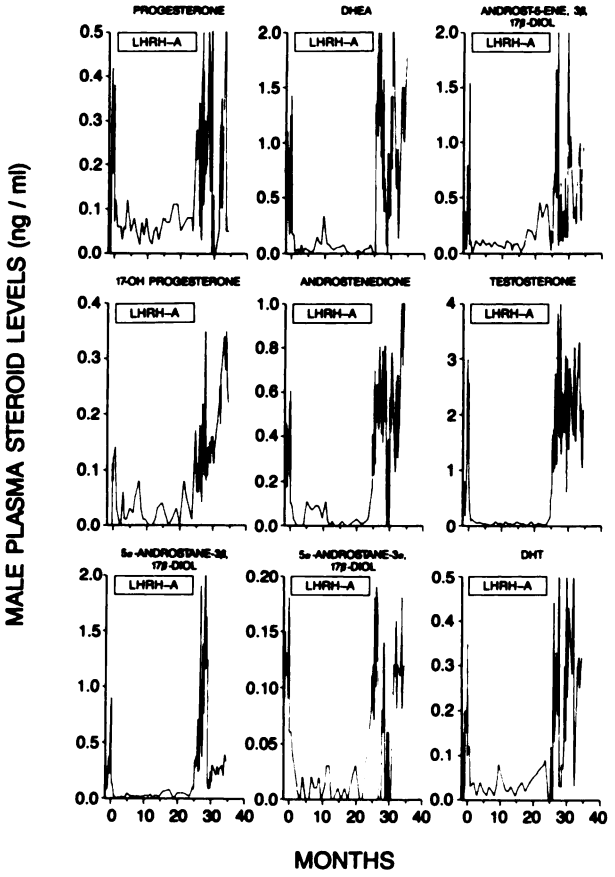

Fig. 1. Pattern of plasma steroid levels before, during, and after long-term GnRH agonist administration to prepubertal male dogs. The limit of detection for all steroids is 0.05 ng/ml.

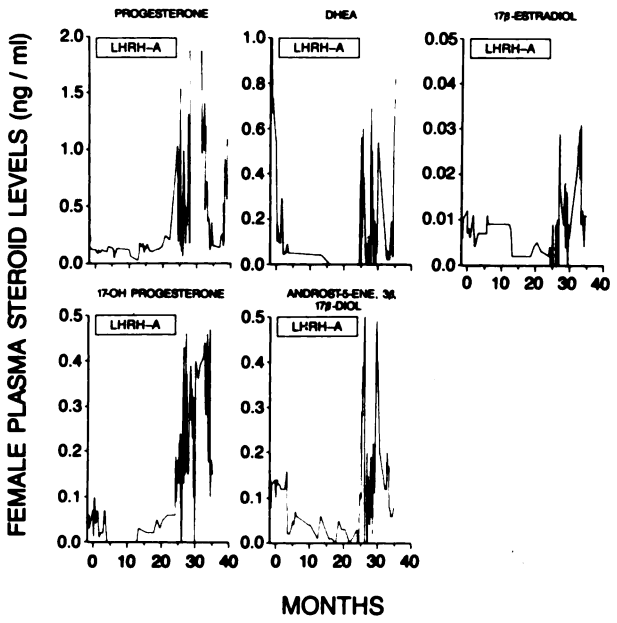

Fig. 2. Pattern of plasma steroid levels before, during, and after long-term GnRH administration to prepubertal female dogs. The limit of detection for all the steroids is 0.05 ng/ml except for 17β-estradiol, which is 0.10 ng/ml.

in water for 1 h, dehydrated in ethanol, embedded in Araldite, and cut in a Reichert Ultramicrotome. Thin sections were stained with uranyl acetate and lead citrate, and examined in a Siemens Elmiskop 102 electron microscope. To identify glycogen at the electron microscopic level, small pieces of tissue were enzymatically digested before inclusion as previously described (Dubé et al, 1987).

Calculations and Statistical Analyses

Radioimmunoassay data were analyzed using a program based on model II of Rodbard and Lewald (Rodbard et al, 1970). Statistical significance was measured according to the multiple-range test of Duncan-Kramer (Kramer, 1966). The data are presented as means ± SEM. When no bar is indicated, SEM is smaller than the symbol used.

Results

As illustrated in Fig. 1, the plasma levels of DHEA, Δ^5-diol, androstenedione, testosterone, DHT, 3α-diol, 3β-diol, progesterone, and 17-OH progesterone are reduced to the limit of detection in male dogs during the 23 months of GnRH agonist administration. After cessation of treatment, a rapid increase in the plasma concentration of all the steroids mentioned above is observed. In fact, within the first month after cessation of GnRH agonist administration, the plasma concentration of all the steroids measured (except 3α-diol) had returned to values above those measured at the start of treatment and typical of those found in adult animals.

The plasma steroid levels in female animals who received the same treatment are illustrated in Fig. 2. While there is no significant change in the plasma levels of progesterone and 17-OH progesterone during the treatment period, the concentrations of DHEA and Δ^5-diol are significantly decreased. The plasma levels of the four above-mentioned steroids increased rapidly following cessation of treatment. Although plasma 17β-estradiol levels were at the limit of detection of the assay before and during GnRH agonist treatment, a significant increase was observed after cessation of therapy. The serum levels of testosterone, androstenedione, DHT, 3α-diol, and 3β-diol were at the lower limit of the radioimmunoassays used and could not be measured adequately

in the female animals prior to or during GnRH agonist treatment.

Histology of the testes revealed that chronic treatment caused a retarded development of the seminiferous tubules; germ cells were almost absent, leaving only Sertoli cells, reserve spermatogonia, and an occasional primary spermatocyte in the epithelium of the tubules (Fig. 3A). The Leydig cells are scarce and small, and there is a predominance of fibroblasts in the intertubular space. At the electron microscope level, Sertoli cells are small and show an accumulation of glycogen in their cytoplasm (Fig. 4A). Spermatocytes also show a large amount of glycogen in their cytoplasm. Whorl bodies that are not normally present in the Sertoli cells are frequently seen. There is an accumulation of glycogen in some fibroblasts surrounding the tubules and in some pericytes of the blood vessels. Reserve spermatogonia appear to be normal.

As illustrated in Fig. 5A, the Leydig cells of treated animals have a small nucleus with a large amount of heterochromatin. There are large amounts of lipid droplets with the appearance of dispersed saccules of the smooth endoplasmic reticulum. Mitochondrial cristae are predominantly tubular in appearance. Following long-term treatment with the GnRH

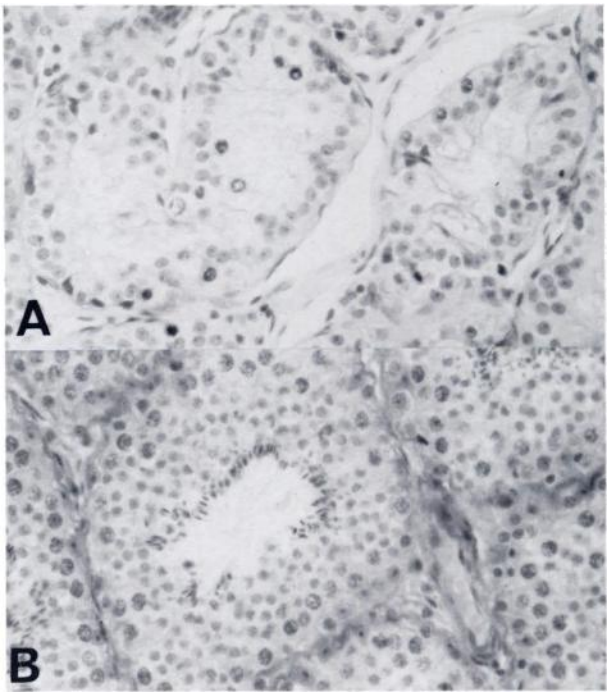

Fig. 3. (A) Section through dog testis showing the severe atrophy following 23 months of GnRH agonist treatment. (B) After the recovery period of 14 months, spermatogenesis has returned to control levels.

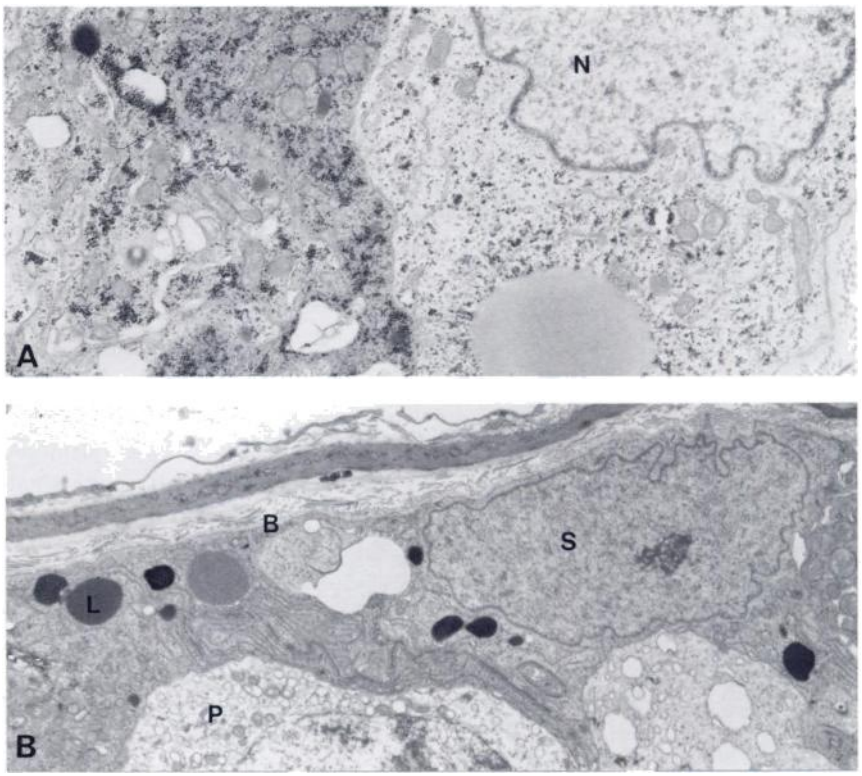

Fig. 4. (A) Two representative Sertoli cells in a GnRH agonist-treated animal can be seen with various degrees of accumulation of glycogen particles in their cytoplasm. N: nucleus. (B) A normal seminiferous tubule is apparent after the recovery period. L: lysosome. P: primary spermatocyte. B: basement membrane.

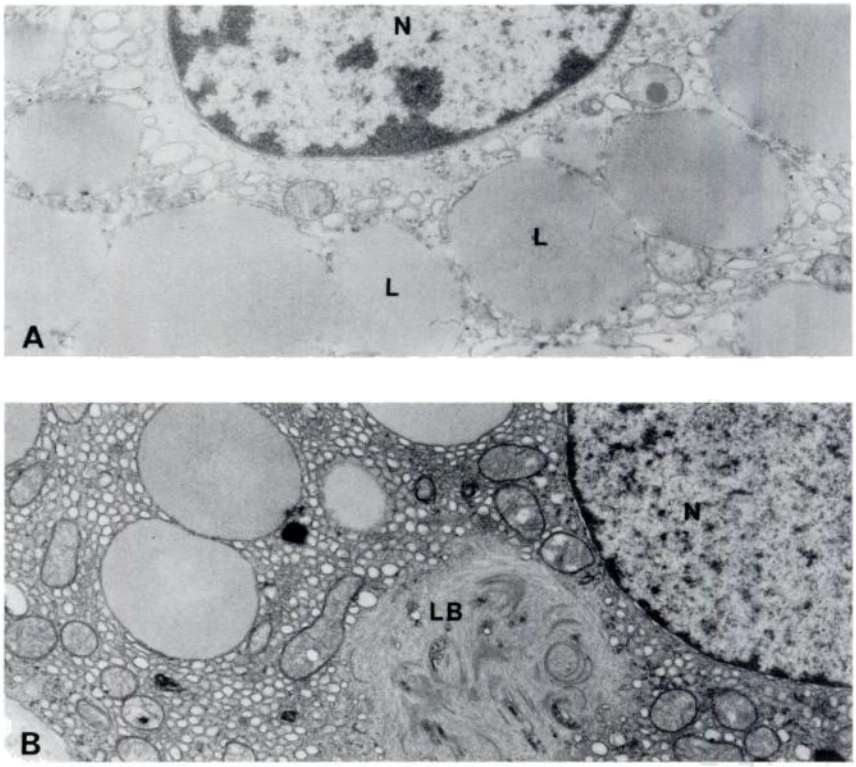

Fig. 5. Sections through Leydig cells. (A) Appearance of a Leydig cell present in the testis of a GnRH agonist-treated dog. This cell is relatively rare in the intertubular space, which is largely occupied by fibroblasts. The nucleus (N) is small, and a large amount of heterochromatin is present. There is an accumulation of lipid droplets (L) and the smooth endoplasmic reticulum is scarce. (B) A Leydig cell from a recovery animal can be seen. This cell is normal, with its nucleus (N) showing predominantly euchromatic, mitochondria with vesicular cristae, and regular saccules of the smooth endoplasmic reticulum; but the presence of a lamellar body (LB) is unusual. This body is often associated with lysosomes.

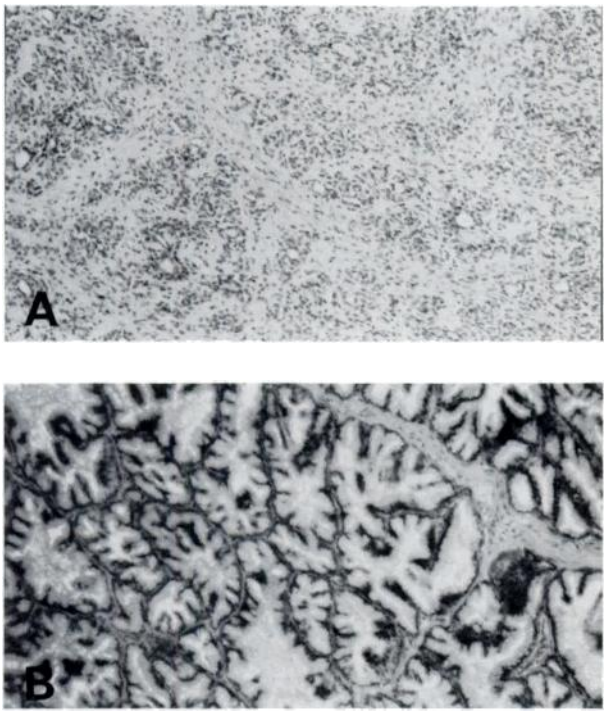

Fig. 6. (A) Section through the prostate gland of a GnRH agonist-treated animal can be seen. The glands are small. (B) The glandular epithelium of a recovery animal is of normal adult appearance.

agonist, the prostate gland contains precursor cells of the glandular epithelium with the presence of some secretory ductules but the secretory material as well as the luminal space within the glands are absent (Fig. 6A). Complete appearance of all normal adult prostatic morphology can be seen in Fig. 6B.

In treated female animals, the ovarian cortex contains a large number of primordial follicles, a few primary follicles, but no secondary follicles (Fig. 7A). Occasionally, atretic follicles are observed. At the electron microscope level, the stroma and the follicles have normal adult appearance except for the presence of glycogen accumulation in a fibroblast-like cell occasionally observed in the stroma (Fig. 8).

The pituitary gonadotrophs have been described in the normal dog using immunohistochemistry (Dubé et al, 1987). More detailed observations have permitted identification of LH-secreting cells that have no dilatation of the RER, probably representing cells with lower secretory activity than those with dilated RER. In the GnRH agonist-treated prepubertal male and female dogs, LH-secreting cells show two morphological types. One is a normal-looking cell with dilated RER and granules distributed in patches in the cytoplasm. The other demonstrates

a darkly stained cytoplasm with contracted RER and a few secretory granules. The second cell type is seen more frequently, although depots of glycogen particles are characteristic of both cell types (Fig. 9A). Both cell types contain LH as observed after identification of the gonadotropin by immunohistochemistry at the electron microscopic level (data not shown). A third type of cell, containing an accumulation of glycogen in the cytoplasm, is identified as reticular cells of the connective tissue (Fig. 10A).

After the 14 month recovery period, the testis shows a completely normal adult seminiferous epithelium with all germ cells present in the tubules (Fig. 3B). Leydig cells have increased in size and show an ovoid form with a round nucleus containing a well-developed nucleolus. At the electron microscopic level, Sertoli cells and spermatocytes (Fig. 4B), as well as pericytes and fibroblasts, have a normal level of glycogen in their cytoplasm. Leydig cells have a decrease in lipid droplet content, an increase in smooth endoplasmic reticulum, and a round nucleus with a well-developed nucleolus. A round lamellar structure is occasionally seen in the cytoplasm (Fig. 5B). This structure is sometimes associated with lysosomes. Following the 14 month recovery period after cessation of treatment mentioned above, the prostate gland shows a fully developed secretory epithelium with primary and secondary projections of glandular papillae in the lumen of the acini (Fig. 6B).

Following the 14 month recovery period after cessation of treatment, the ovary of female dogs contains primordial and primary follicles as well as fully developed secondary follicles (Fig. 7B). In one ovary, a corpus luteum also was observed. The ultrastructure of the ovary has normal adult appearance. The gonadotrophs in recovery animals show a normal looking appearance with an increase

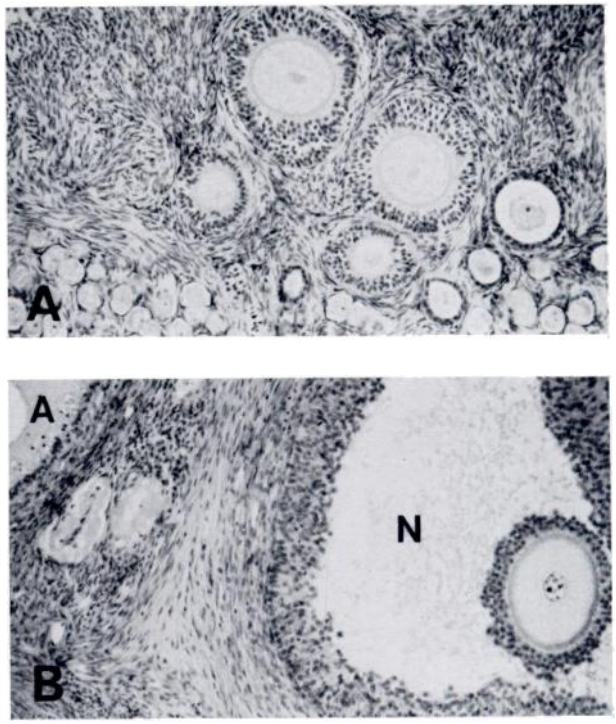

Fig. 7. Sections through the ovarian cortex of female dogs. (A) The primordial and primary follicles are present in a GnRH-agonist-treated animal, but no secondary follicles can be seen. (B) A fully developed secondary follicle is present in the ovary of a recovery animal. A: atretic follicle. N: antrum.

in number of characteristic granules distributed in patches in the cytoplasm, flat or dilated RER, and a nucleus with a well-developed nucleolus. Little glycogen accumulation can be seen in the cytoplasm (Fig. 9B). Reticular cells of the connective tissue still contain an accumulation of glycogen in their cytoplasm (Fig. 10B).

The endocrine and morphological parameters described above indicate a normally functioning pituitary-gonadal activity. Table 1 shows that two female dogs (101, 103) treated for 23 months with

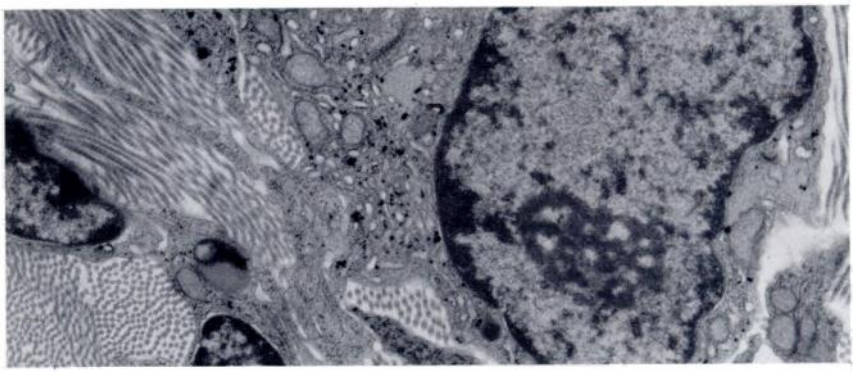

Fig. 8. Section through a dog ovary showing a fibroblast-like cell that contains glycogen particles in its cytoplasm. This type of cell was never seen in the recovery animals.

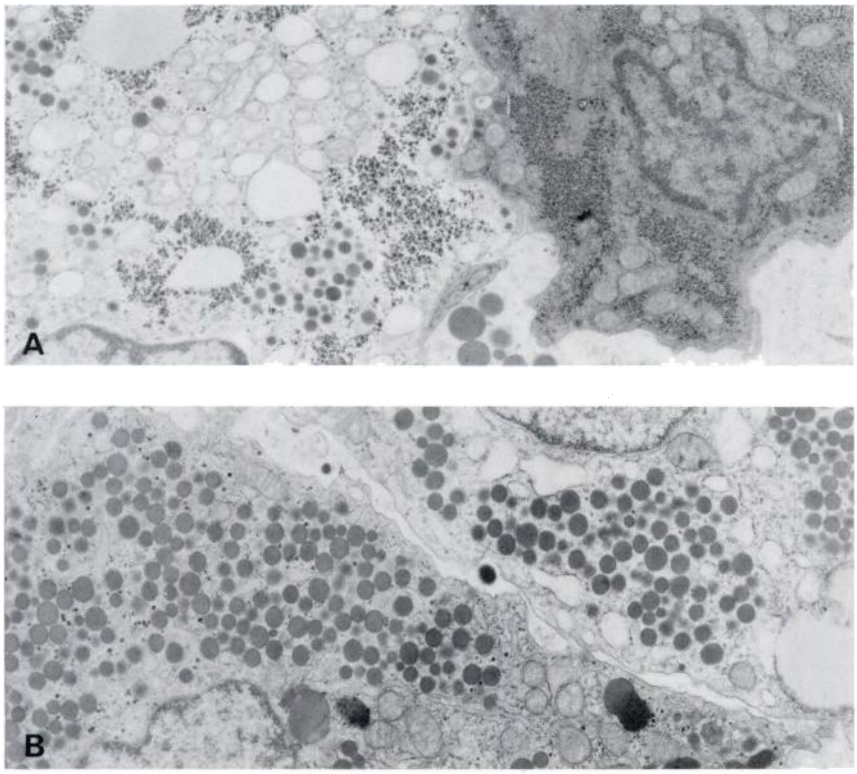

Fig. 9. (A) Section through the pituitary gland of a GnRH agonist-treated male dog showing two LH-secreting cells side-by-side. The cell on the left shows high levels of glycogen in the cytoplasm while also containing dilated saccules of RER and a few secretory granules distributed in patches. The cell on the right shows a highly condensed nucleus with a darkly stained cytoplasm and a large accumulation of glycogen particles. Rare secretory granules are present. (B) Section through the pituitary gland from a recovery animal. These two LH-secreting cells show an increased number of secretory granules. The cell on the left shows an increased number of lysosomes and retracted saccules of the RER. The cell on the right shows dilated saccules of RER.

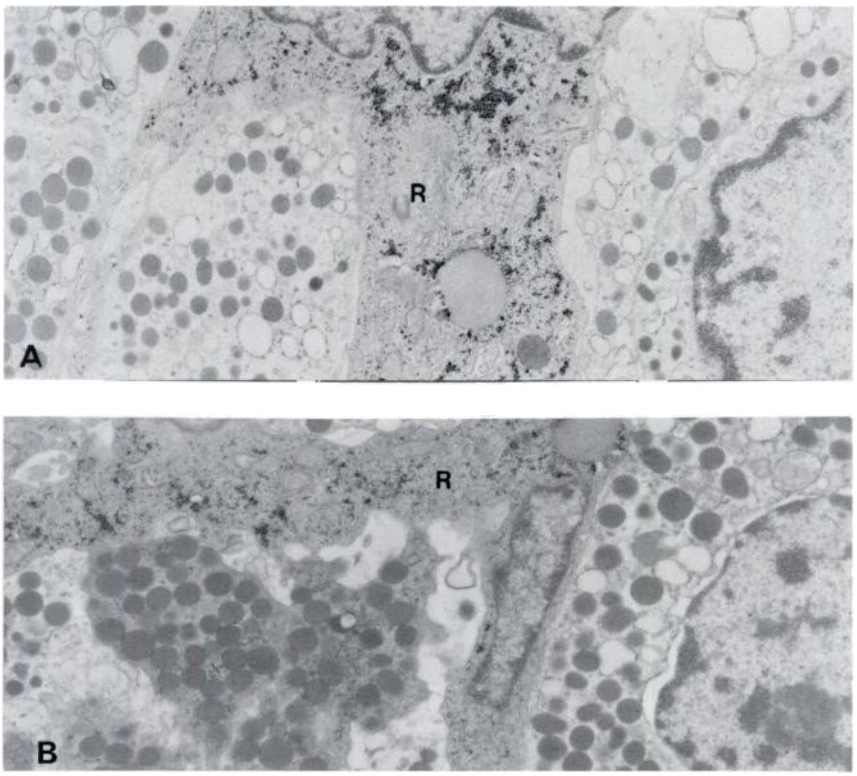

Fig. 10. (A) Thin section through the pituitary gland of a male dog treated with the GnRH agonist. Note the glycogen particles in the cytoplasm of a reticular cell (R). (B) Section through the pituitary gland of a recovery animal. Note that glycogen particles are still present in the cytoplasm of a reticular cell (R).

the GnRH agonist gave birth to nine healthy puppies after 2 months of normal gestation. The two females were fertile in their first cycle, which occurred 4 and 6 months after cessation of GnRH agonist treatment. The other three females showed cyclic bleeding within the year following the cessation of treatment, but were not tested for fertility. Two males showed fertile mating 4 months after cessation of treatment (Table 1). One treated male (004) mated with two control females (145, 147) and gave birth to 10 healthy puppies, whereas the second dog (008) mated with the two treated females (101, 102). The third treated dog (007) had normal testicular volume but apparently had defective libido. Detailed biochemical (SMA-12) and hematological parameters measured after 22 months of treatment showed all values within the normal range, thus indicating an excellent tolerance to treatment. Body weight measured at 6 and 32 weeks of age showed normal values of 1.0–2.5 and 7–12 kg, respectively.

TABLE 1. Fertility of Two Male and Two Female Dogs After 14 Month Recovery Period Which Followed 23 Months of Treatment with the GnRH Agonist [D-Trp^6, Des-Gly-$NH_2{}^{10}$]GnRH Ethylamide

	Animal Number			
Date of Mating	Female		Male	Offspring and Date of Birth
13-04-1987	101 (treated)	+	008 (treated)	17-06-1987 2 females 2 males
30-06-1987	003 (treated)	+	008 (treated)	31-08-1987 2 females 3 males
24-04-1987	145 (control)	+	004 (treated)	26-06-1987 4 females 2 males
15-08-1987	147 (control	+	004 (treated)	26-10-1987 3 females 2 males

Discussion

The present data clearly demonstrate that chronic treatment (23 months) of prepubertal male and female dogs with the GnRH agonist [D-Trp^6, des-Gly-$NH_2{}^{10}$]GnRH ethylamide is followed by a rapid emergence of gonadal function and fertility upon cessation of treatment. Such data support the use of this well-tolerated therapy for the temporary treatment of sex hormone-dependent diseases, especially precocious puberty. The present study complements and extends our previous observation of complete recovery of spermatogenesis and testicular steroidogenesis after a 4 month treatment of adult dogs with the same GnRH agonist (Tremblay and Bélanger, 1984).

In a previous study (Tremblay et al, 1984a), a maximal decrease in serum testosterone levels was observed within 2 wk of daily treatment with the GnRH agonist [D-Ser(TBU^6, des-Gly$NH_2{}^{10}$]GnRH ethylamide; the maximal inhibitory effect was measured at 6 wk. In agreement with the present results, no significant change was observed on the plasma progesterone and 17 OH-progesterone levels in our two previous studies (Tremblay and Bélanger, 1984). The almost complete inhibition of the plasma concentration of DHEA and Δ^5-diol observed in the present study is in agreement with our previous data, obtained during the course of 4 months of daily treatment with the same peptide in adult dogs (Tremblay and Bélanger, 1984. Such data clearly indicate the important contribution of the testes in the plasma concentration of both DHEA and Δ^5-diol. These data are in agreement with our observation that plasma DHEA and Δ^5-diol are markedly increased after acute injection of an GnRH agonist or hCG in the dog (Tremblay and Bélanger, 1985).

While plasma steroid levels in adult male dogs treated with a GnRH agonist have already been reported (Bélanger et al, 1984; Tremblay and Bélanger, 1984, 1985; Tremblay et al, 1984a; Lacoste et al, 1988), no such comparison with other studies is available for plasma steroid levels in female dogs. The fluctuating plasma steroid concentrations measured in both male and female animals during the posttreatment period can be explained, at least partly, by seasonal variations, especially in the females, where such variations could be, at least partially, related to the oestrus cycles.

In agreement with the low levels of androgens in the circulation of animals treated with the GnRH agonist, the development of the prostate during the treatment period remains similar to that observed in the prepubertal animal. After the recovery period, the testes and the prostate rapidly reach their full development and the histologic appearance of both organs does not differ from that of adults. Such complete progression is analogous to the recovery already described for adult male animals treated with the same agonist for 4 months and allowed to recover for a 4 month period (Tremblay and Bélanger, 1984). The ultrastructure of the seminiferous epithelium and of the Leydig cells in the adult dog after a 4 month period of treatment and recovery has already

been described (Dubé et al, 1988). The differences seen in the présent observations pertain to the presence of high levels of glycogen in Sertoli cells, primary spermatocytes, fibroblasts, and pericytes of blood vessels. These high levels of glycogen, however, disappear following cessation of therapy. The functional significance of the microfibrillar structure observed in the Leydig cells in recovery animals is unknown.

The effect of chronic treatment with a GnRH agonist has already been described in the rat and shown to induce no histological alteration in ovarian histology (Séguin et al, 1982). In normal women, treatment with a GnRH agonist is known to cause luteolysis and inhibition of ovulation (Lemay et al, 1979; Bergquist et al, 1979). In monkeys, treatment for 1 yr with a GnRH agonist has been shown to induce various degrees of ovarian function inhibition (Fraser et al, 1980). Normal cyclicity was inhibited in the female animals treated with the agonist. This situation was probably due to the absence of normal development of secondary follicles in the ovary.

The gonadotrophs in the adult dog have already been described at the electron microscopic level (Dubé et al, 1987) and shown to be atrophied and to accumulate glycogen in response to GnRH agonist treatment. In the present study, LH cells were shown to contain high levels of glycogen after the 23 month treatment with the GnRH agonist.

The high levels of glycogen in the cytoplasm of a wide variety of different cells in the present study suggest that the metabolism of glycogen would be altered direclty or indirectly in these cells by GnRH agonist treatment. Defects in glycosylation processes have already been related to the accumulation of glycogen in the cytoplasm of LH-secreting cells after GnRH agonist-induced densitization (Dubé et al, 1987). An alternative to this hypothesis is that glycogen is sometimes present in larger amounts in the cells of younger animals compared with adult cells (Pelletier et al, 1972), and that GnRH agonist treatment maintains an earlier stage of development in some cells.

Other long-term studies of the effect of treatment with GnRH agonists on gonadal functions and fertility parameters have been performed in animals and humans but none exceeds 20 months in duration (Linde et al, 1981; Weinbauer et al, 1987; Comite et al, 1986) except for the treatment of precocious puberty, for which a 4 year therapy has been reported (Comite et al, 1986). In studies performed in the rat, inhibition of spermatogenesis and full recovery of testicular functions are not completely obtained (Pelletier et al, 1980). For this reason, it is believed that the rat is not the appropriate model for studying the effect of chronic GnRH agonist administration. McRae et al (1985) observed the reversible inhibition of oestrus in mature female dogs and later studies showed return of fertility after cessation of therapy (Vickery et al, 1987).

The present study demonstrates that chronic administration of the GnRH agonist [D-Trp^6, des-Gly-$NH_2{}^{10}$]GnRH ethylamide can efficiently delay the onset of puberty without altering somatic growth and subsequent fertility in prepubertal dogs. Normal adult plasma steroid levels and normal histology were found in the ovaries and testes, as well as in the prostate and anterior pituitary gland, 14 months after cessation of long-term treatment with the GnRH agonist. In the mated females, pregnancy occurred in all but one animal. Such data support the use of temporary treatment with GnRH agonists for the treatment of sex steroid-dependent diseases, especially precocious puberty. In addition to full development of gonadal functions following cessation of treatment, GnRH agonists are exceptionally well-tolerated with no other effects than those expected from temporary cessation of gonadal activity.

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