AKADÉMIAI KIADÓ

Acta Veterinaria Hungarica

70 (2022) 1, 44–50

DOI:
10.1556/004.2021.00052

RESEARCH ARTICLE

Testicular effects of a postnatal GnRH antagonist in domestic cats

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Received: 12 April 2021 • Accepted: 16 November 2021
Published online: 15 December 2021

ABSTRACT

The aim of this study was to describe the histological effects of two high postnatal doses of the potent third-generation GnRH antagonist, acyline in the domestic cat testicle. Secondly, the physical, endocrine, and steroidogenic findings of this pharmaceutical protocol are also reported. Twelve postnatal littermate male kittens were administered acyline in a dose of 2.2 mg/100 g SC weekly for 2 weeks (ACY; $n = 6$), or placebo (PL; $n = 6$). All the animals were followed up until puberty when they were castrated. Serial faecal samples were collected until the age of 10 weeks for testosterone (T) measurement. The kittens achieved puberty without either age (236.5 ± 19.7 vs. 221.7 ± 23.7 days) or body weight (3.05 ± 0.15 vs. 2.78 ± 0.28 kg, $P > 0.05$) differences between ACY and PL, respectively. Acyline suppressed faecal T concentrations for 3 weeks ($P < 0.01$). From the fourth week on, both groups had low concentrations up to the end of the follow-up period ($P > 0.05$). Histological assessment of the testes showed that ACY cats presented a reduced height of the epithelium ($P < 0.01$) due to the diminished number of germinal cells accompanied by an enlarged luminal area ($P < 0.01$) with cellular debris ($P < 0.01$). The immunostaining of P450c17 also appeared partially diminished in ACY testes.

KEYWORDS

feline, testicle, contraception, GnRH analogue, gonad

INTRODUCTION

Domestic cats are highly efficient at reproduction, and feline overpopulation has become a serious problem in many cities all over the world. Surgical methods are one of the first choices as a tool for population control. However, surgery is expensive and labour intensive for stray animals (Mehl et al., 2017). Other permanent alternatives to surgical sterilisation (i.e., hormonal, immunologic, chemical, and so forth) are being investigated including postnatal endocrine disruption (Faya et al., 2013; Carranza et al., 2015). In this respect, it should be known that in mammals the pituitary–gonadal function during the postnatal period is essential for normal sexual maturation and for the proper development of adult sexual functions (Kolho and Huhtaniemi, 1989). Thus, the early neonatal period is a well-recognised period of reproductive vulnerability in most mammals (Mann and Fraser, 1996; Pryor et al., 2000). Postnatal gonads are very active in cats. Thus, birth is followed by the presence of elevated faecal sexual steroids which are followed by low 'infantile' concentrations after the first postnatal weeks (Faya et al., 2013).

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GnRH analogues, which include agonists and antagonists, have been produced by amino acid substitutions within the native GnRH molecule, resulting in greater potency and a longer duration of effectiveness. Differently to GnRH agonists, antagonists competitively block and inhibit GnRH-induced GnRH receptor gene expression leading to an immediate, dose-dependent, pituitary suppression (Gobello, 2012). Neonatal administration of GnRH agonists and antagonists has delayed puberty in domestic cats and laboratory rodents, respectively (Carranza et al., 2014; Kolho et al., 1988). When the potent third-generation GnRH antagonist acyline (33 µg/100 g) was administered postnatally for 3 months, it diminished faecal testosterone (T) concentrations during the first postnatal weeks but this antagonist did not cause low 'infantile' concentrations (Carranza et al., 2014). Therefore, higher postnatal doses seemed to be necessary to deprive the cats of the physiological postnatal androgen effect.

Cytochrome P450 side chain cleavage (P450scc) is a mitochondrial enzyme that catalyses the conversion of cholesterol to pregnenolone, the first reaction in the process of steroidogenesis. Cytochrome P450 17α-hydroxylase (P450c17) is a key enzyme in the androgenic pathway. Both P450scc and P450c17 are expressed in Leydig cells of the testes (Heinrich and De Falco, 2020).

Furthermore, the histological and steroidogenic effects of a postnatal GnRH antagonist on pubertal male gonads have not been investigated in this species. Thus, the aim of this study was to describe the histological effects of high doses of the potent third-generation GnRH antagonist acyline in the domestic cat testicle. Secondly, the physical, endocrine, and steroidogenic findings of this pharmaceutical protocol are also reported.

MATERIALS AND METHODS

Animals and pharmacological protocols

Twelve (5 litters) newborn littermate male kittens from the National University of La Plata institutional cat colony were included in this study. The animals were sexed according to anogenital distance and identified at birth, reared under 14 h of light per day, weaned at the age of 40 days and fed a premium commercial kitten food and water *ad libitum*. This study was reviewed and approved by the Animal Care and Use Committee of the Veterinary School of the National University of La Plata, and all experiments were conducted under the guidelines established in The Guide for The Care and Use of Laboratory Animals, USA. The kittens of the same litters were randomly assigned to one of the following treatment groups within the first 24 h of birth: acyline (Contraception & Reproductive Health Branch Center for Population Research, NIH, Bethesda, MD, USA) 2.2 mg/100 g SC weekly for 2 weeks [ACY]; $n = 6$), or placebo (sterile distilled water, PL; $n = 6$). Acyline [acetyl-D2Nal-D4ClPhe-D3PalSer-Aph(ac)-DAph(Ac)-Leu-Lys(lpr)-Pro-D-Ala-Nh2] was provided as a lyophilised powder which was suspended in sterile distilled water to a final concentration of 2 mg mL^{-1}. The dose was selected on the basis of pilot trials in cats.

Follow-up

All the animals were followed up until puberty occurred. The felids were observed twice daily, looking for their behaviour. The cats were also physically examined including body weight (kg), balano-preputial separation, and penile spines once a week. Puberty was defined as complete balano-preputial separation and the appearance of penile spines (Carranza et al., 2014). The cats were castrated immediately after puberty was attained (Boothe, 1993) and placed for adoption.

Faecal sample collection, extraction and hormone determinations

Faecal samples were collected and frozen weekly during the first month and then in weeks 7 and 10 for T analysis (ng g^{-1}). During the first 4 weeks of age, the neonates had to be rectally stimulated by a thin plastic suppository attached to a string to obtain the sample. Then, each cat was confined in an individual cage with clean sanitary litter during one night. Testosterone was extracted using the methods described by Brown et al. (2008) and, then, determined using electrochemiluminescence immunoassays (Elecsys Testo II, Roche Diagnostics, Mannheim, Germany). Inter- and intra-assay coefficients of variation of the assays were <10% and sensitivity was 0.025 ng mL^{-1}. All faecal data were expressed on a wet-weight basis (Faya et al., 2013).

Gross, seminal and histological examination of the testes

Immediately after surgery, the testes were measured (length, width and depth; cm) using a Vernier calliper and weighed (*g*). Gonadal volume (cm^3; Lin et al., 2009) and gonadosomatic index (%; França and Godinho, 2003) were also calculated.

Spermatozoa were obtained by manual slicing of the cauda epididymides. The presence of sperm progressive motility was subjectively assessed at × 400 magnification on a warmed glass slide. Then, the samples were centrifuged at 4 °C for 1,400 *g* for 8 min. The supernatant was removed and an aliquot of the sperm pellet was used to perform a smear for evaluation of spermatozoa morphology following Giemsa staining at × 1,000 magnification using bright field microscopy (Valiente et al., 2014). One testis was sectioned longitudinally, placed in Bouin's fixative for 24 h and then changed to alcohol 70% and processed routinely with paraffin embedding. After processing, 5-µm serial sections were cut, mounted on slides, dried, deparaffinised in xylene, rehydrated in graded ethanol solutions and stained with haematoxylin and eosin (Bancroft et al., 1990). Histological images were obtained from a microscope (Olympus BX50, Tokyo, Japan; 10 × or 40 × through an attached digital RGB video camera (Evolution VF Colour, Q Imaging, USA) and digitalised in a 24 bit true colour TIFF format. Thirty round

tubular profiles per testis were evaluated for mean tubular diameter (µm), mean germinal epithelium height (µm) as well as the mean number of each cellular component, i.e. spermatogonia, primary and secondary spermatocytes, round spermatids, elongated spermatids, spermatozoa and Sertoli cells. The number of Leydig cells in 20 complete intertubular spaces was recorded and their nucleus areas (μm^2) measured. The number of spermatids per Sertoli cell was also calculated. Images were analysed by planimetry (Image Pro Plus v6.0-Media Cybernetics, Silver Spring, MA, USA).

Immunohistochemistry

Sections from Bouin-fixed paraffin blocks were deparaffinised and antigens were demasked by pretreatment with citrate buffer (pH = 6). Endogenous peroxidase activity was inhibited using 3% hydrogen peroxide in methanol. Goat serum (10%) and bovine serum albumin (BSA, 3%) in phosphate-buffered saline (PBS) buffer was used to block unspecific binding sites followed by incubation with the primary antibodies diluted in PBS with 3% BSA over night at 4 °C. Antibodies used were a rabbit polyclonal anti-rat antibody against cytochrome P450 side chain cleavage (P450scc), (dilution 1:200; US Biological, distributed by Biomol, Hamburg, Germany; Roby et al., 1991; Gentil et al., 2012) and a rabbit polyclonal antibody against recombinant bovine cytochrome P450 17α-hydroxylase (P450c17) (dilution 1:500; provided by Prof. A. J. Conley, Davis, USA; Peterson et al., 2001; Gentil et al., 2012). On the second day, the samples were first incubated with a Polymer-Enhancer (SuperVision-2 HRP Enhancer, DCS Innovative Diagnostic-System, Hamburg, Germany) followed by incubation with the HRP-polymer-reagent (SuperVision-2 HRP-Polymer, DCS Innovative Diagnostic-System). Each step of the immunohistochemistry was followed by a washing step with PBST (PBS containing 0.2% Tween 20) buffer (2 ×) and PBS buffer (1 ×). Immunopositive signals were visualised with DAB (DAB 2-komponenten kit, DCS ChromoLine, DCS Innovative Diagnostic-System) according to the manufacturer's protocol. Negative controls were set up using PBS buffer and irrelevant isotype controls (I-1000, Rabbit IgG, Control Antibody, Vector Laboratories) in the respective dilution instead of the primary antibodies. In the last steps, samples were washed, dehydrated in ascending alcohol dilutions and mounted with Eukitt® (Sigma-Aldrich Chemie GmbH, Munich, Germany). Evaluation of the immunohistochemistry was performed using computer-assisted image analysis with GIMP 2.8 (https://www.gimp.org/) and ImageJ FIJI win-64 (https://imagej.net/Fiji) to quantify the percentage of the immunopositive area (PIA) and the staining intensity (mean grayscale; MGS) of the interstitial tissue as described before (Körber and Goericke-Pesch, 2019a). The MGS and PIA of the interstitial tissue were assessed in twenty randomly selected images at 200-fold magnification per sample. To allow the analysis of the interstitial tissue, the seminiferous tubules, blood vessels and artefacts were cropped. Then, the images were transferred into grayscale pictures and an individual threshold was chosen subjectively for each antibody. Positive signals above the threshold were used for the determination of the staining intensity (MGS). For the grayscale, 0 was defined as white and 255 as black. The PIA was assessed using the binarised grayscale image in which the black area represents the immunopositive stained area (Gentil et al., 2012; Körber and Goericke-Pesch, 2019a, b).

Statistical analysis

Normality of the distribution of results was tested using a Shapiro–Wilk normality test. Faecal T concentrations in the different treatments (ACY vs. PL) were compared throughout the weeks by repeated-measures ANOVA followed by Tukey's comparison *post hoc* test. Physical, histological and immunohistochemical differences in the treatment group were analysed by Student's *t*-test. Results were expressed as mean ± SEM. A $P < 0.05$ was considered to be significant (Microsoft Excel; Windows XP; Microsoft and Graph Pad Prism7 software; GraphPad Software, Inc., La Jolla, USA).

RESULTS

The kittens achieved puberty without differences between groups (236.5 ± 19.7 vs. 221.7 ± 23.7 days for ACY and PL, respectively; $P > 0.05$). Body weight (3.05 ± 0.15 vs. 2.78 ± 0.28 kg, $P > 0.05$) did not differ between the treatments either. One ACY cat presented unilateral inguinal cryptorchidism. None of the other cats presented clinical side effects ($P > 0.05$) nor behavioural alterations throughout the study period. Gross testicular parameters including weight, volume as well as the gonadosomatic index also did not vary between the groups ($P > 0.05$, Table 1).

Faecal T concentration differed between treatments during the first 3 postnatal weeks ($P < 0.01$), being higher in the PL group. From the fourth week on, both groups achieved low 'infantile' concentrations to the end of the follow-up period in week 10 ($P > 0.05$). The T values of the acyline group did not differ between before and after the third week ($P > 0.05$; Fig. 1).

Microscopic evaluation revealed that all the males presented epididymal spermatozoa with >90% of normal motility and morphology. Histological assessment showed that the ACY group presented a reduced height of the epithelium ($P < 0.01$) due to the diminished number of some of the germinal cells such as spermatogonia ($P < 0.01$),

Table 1. Morphometric data (mean ± SEM) of male cats which had been postnatally administered acyline ($n = 6$) or a placebo ($n = 6$). No significant differences were found

	Acyline	Placebo	*P*
Testis weight (g)	1.70 ± 0.11	1.65 ± 0.01	0.331
Testicular volume (cm^3)	1.92 ± 0.09	1.33 ± 0.65	0.378
Gonadosomatic index (%)	0.08 ± 0.00	0.11 ± 0.00	0.461

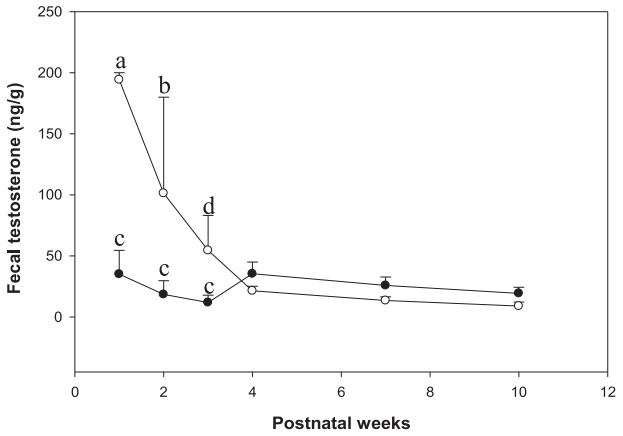

Fig. 1. Faecal testosterone (mean ± SEM) of the male kittens of Table 1: Acyline-treated (solid circles) and placebo-treated (empty circles) cats. Different letters above time points represent differences at $P < 0.01$

round and elongated spermatids ($P < 0.05$), and spermatozoa ($P = 0.025$) accompanied with an enlarged luminal area ($P < 0.01$) with more detritus ($P < 0.01$) inside when compared to PL testes which appeared completely normal (Table 2; Fig. 2A,B; França and Godinho, 2003). The spermatids/Sertoli cells ratio was also reduced in ACY cats ($P < 0.05$). Immunohistochemistry against P450SCC and P450c17 revealed a specific staining in the Leydig cells of both groups (Fig. 3). Although no differences in PIA were found for either P450scc ($P > 0.05$) or P450c17 ($P > 0.05$) between the groups, the MGS was lower for P450c17 ($P < 0.01$) and had a tendency to be decreased for P450scc ($P < 0.1$) in ACY cats (Fig. 4).

DISCUSSION

In spite of the high antagonist doses used in the present study, neither the age nor the body weight at puberty of the

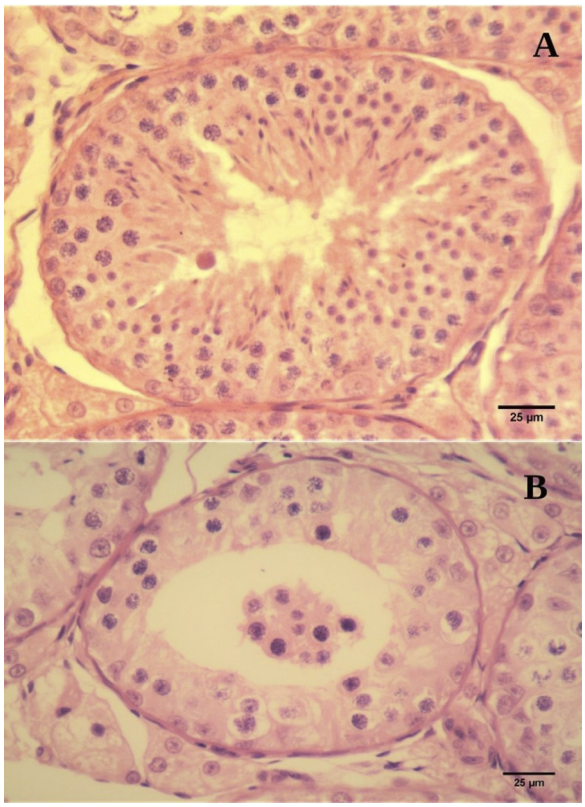

Fig. 2. Testicular tissue of the cats of Table 1. Haematoxylin and eosin (HE), × 25. A) Placebo: Normal germinal epithelium. B) Acyline: Thin seminiferous epithelium containing only spermatogonia, primary spermatocytes and Sertoli cells. A cumulus of cellular detritus in the tubular lumen

acyline-treated cats were different from those of the placebo animals. Furthermore, both parameters were within the normal range for the species (Feldman and Nelson, 1996). As expected for GnRH analogues (Gobello, 2012), no clinical side effects, except a case of unilateral inguinal cryptorchidism, appeared. Similarly, two dogs which were treated postnatally with a GnRH agonist presented bilateral

Table 2. Testicular histological components (mean ± SEM) of the male cats of Table 1. Different superscript letters in each line represent: a vs. b ($P < 0.01$) and c vs. b ($P < 0.05$)

	Acyline	Placebo	*P*
Tubular diameter (µm)	205.54 ± 2.38^a	194.54 ± 2.97^b	0.0010
Germinal epithelium height (µm)	19.42 ± 0.5^a	38.35 ± 1.28^b	<0.0001
Luminal area (µm^2)	6689.55 ± 319.12^a	4564.56 ± 252.33^b	<0.0001
Detritus (µm^2)	1737.68 ± 225.05^a	111.98 ± 18.55^b	<0.0001
Spermatogonia/tubule	2.93 ± 0.15^a	5.14 ± 0.21^b	<0.0001
Primary spermatocytes/tubule	61.36 ± 1.78^a	67.68 ± 2.70^a	0.3113
Secondary spermatocytes/tubule	0.00^a	0.01 ± 0.01^a	0.3173
Round spermatids/tubule	35.29 ± 3.21^c	49.27 ± 4.17^d	0.0395
Elongated spermatids/tubule	8.98 ± 1.85^c	11.94 ± 2.34^d	0.0496
Spermatozoa/tubule	16.97 ± 2.92^c	28.56 ± 2.92^d	0.0252
Sertoli cells/tubule	24.44 ± 0.63^a	23.91 ± 0.58^a	0.6765
Spermatids/Sertoli ratio	2.43 ± 0.34^c	3.19 ± 0.37^d	0.0263
Leydig cells/intertubular space	5.08 ± 0.56^a	5.14 ± 0.25^a	0.4461
Leydig cell nuclear area (µm^2)	32.38 ± 0.37^a	30.15 + 0.42^a	0.3351

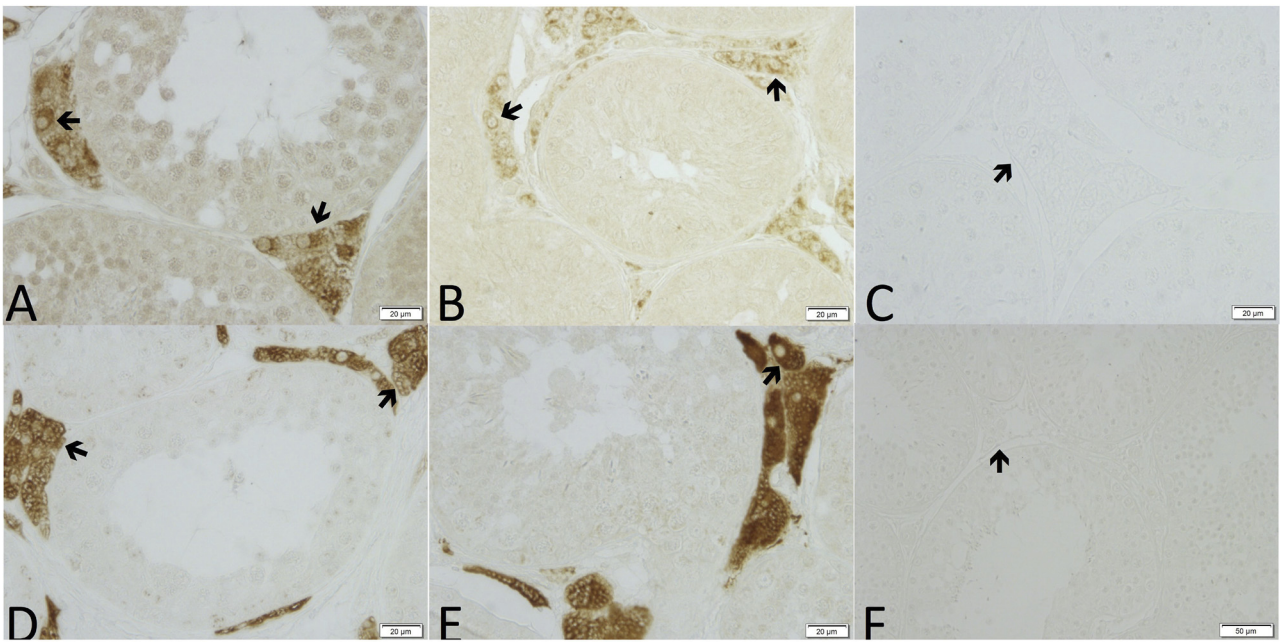

Fig. 3. Immunostaining against P450SCC (A–C) and P450c17 (D–F). Testicular tissue of a placebo-treated animal (PL) given in A and D, testicular tissue of an acyline-treated animal (ACY) given in B and E. Images C and F represent isotype controls. Leydig cells stained immunopositive against P450SCC (A, B) and P450c17 (D, E), whereas no staining was visible in isotype controls. Examples of Leydig cells indicated by arrows. Magnification: × 400 (A–E), × 200 (F)

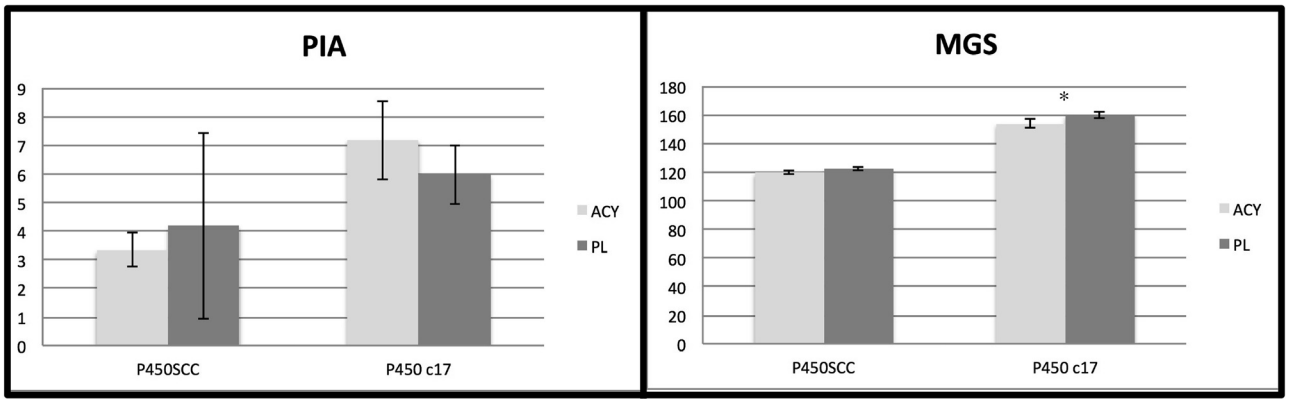

Fig. 4. Percentage of immunopositive area (PIA) and mean grayscale (MGS) for P450SCC and P450c17 in acyline-treated animals (ACY, $n = 6$) and controls (PL, $n = 4$). Results are given as mean ± SD

cryptorchidism at puberty (Faya et al., 2013). The insufficient perinatal androgen concentrations might prevent or delay testicular descent in these animals.

The acyline doses used in this study suppressed the initial faecal T concentrations that appeared in the control cats. Furthermore, in males treated with this antagonist, faecal T values did not vary and remained low throughout the whole study period. Thus, the acyline suppression of faecal T obtained in this trial was deeper than that obtained previously using 33 mg/100 g where no low concentrations could be caused (Carranza et al., 2014). This finding further corroborates the expected dose effect of the antagonist (Gobello, 2012). Gross testicular parameters as well as their relationship with body weight also were not altered by the antagonist treatment. Conversely, the postnatal GnRH antagonist Ac-D2Nal1, D4-Cl-Phe2, D-Trp3, D-Arg6, D-Ala10]-GnRH*HOAc (1 mg kg^{-1}) reduced testicular weight in male piglets (Ziecik et al., 1989). These differences could be attributed to species, drug and methodological differences among reports. In our previous study with low postnatal doses of acyline in cats, a diminution of scrotal volume could be detected at puberty (Carranza et al., 2014). This disparity could have been caused by the fact that those males achieved puberty earlier and, therefore, the effect of the antagonist was closer.

In coincidence with the present histological testicular findings a severe impairment of spermatogenesis was found in rats treated postnatally with a GnRH antagonist

(Huhtaniemi et al., 1986). Neonatal felids treated with a GnRH agonist also evidenced diminished germinal epithelial height at puberty (Carranza et al., 2015). Furthermore, in this study, the spermatids/Sertoli cells ratio, which is considered an expression of spermatogenic efficiency (Russell and Peterson, 1984), was reduced in the acyline-treated animals.

In another study in neonatal rats using a GnRH antagonist, at reproductive maturation, final Leydig cell number and nuclear volume were comparable with the control volume (Sharpe et al., 2003), suggesting that adult steroid production is not affected by early androgen deprivation. Similarly, Leydig cell number and nuclear area were not affected in these cats. Furthermore, immunohistochemical findings demonstrated that only the mean staining intensity of P450c17 expression was reduced in these neonatal treated cats, whereas the percentage immunopositive area did not differ for P450SCC and P450c17 in acyline- and placebo-treated animals. The previously mentioned results suggest that, in cats, early-in-life T concentrations may not have a significant role in adult androgen production. To the best of the authors' knowledge, the steroidogenic evaluation of postnatally endocrine disrupted cats had not been performed before.

Although in this study the fertility of the treated cats was not tested, rats (Huhtaniemi et al., 1986; Kolho et al., 1988; Simon et al., 2012) treated in a similar way proved to be infertile. Further work is still necessary to determine if this outcome is also valid for felids. It was concluded that two high postnatal doses of the GnRH antagonist acyline suppressed faecal T concentrations for three weeks and caused a severe impairment of the germinal epithelium accompanied by a low presentation of unilateral cryptorchidism at puberty. These findings support further investigations on the use of postnatal GnRH antagonists for feline contraception/sterilisation.

ACKNOWLEDGEMENTS

This work was partially supported by the Teaching Incentive Program of UNLP (V269) and the Minister of Science & Technology Cordoba (45/16), Argentina. The authors thank the Contraception & Reproductive Health Branch Center for Population Research, National Institute of Child Health & Human Development, National Institutes of Health, USA, for providing acyline. MG and CM are Research Fellows and MF and CG are Career Scientists of CONICET, Argentina.

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