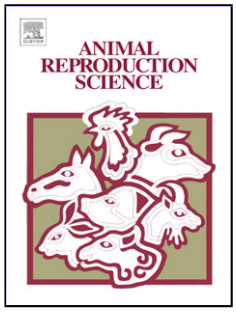

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PII: S0378-4320(16)30250-0
DOI: http://dx.doi.org/doi:10.1016/j.anireprosci.2016.05.015
Reference: ANIREP 5430

To appear in: *Animal Reproduction Science*

Received date: 21-3-2016
Revised date: 26-5-2016
Accepted date: 31-5-2016

Please cite this article as: Demaldé, Lucía, Merlo, Mariana Lopez, Vercellini, Rosario, Barbeito, Claudio G., Fernandez, Patricia, Gobello, Cristina, Disrupting effect of androgens in postnatal female domestic cats.Animal Reproduction Science http://dx.doi.org/10.1016/j.anireprosci.2016.05.015

Disrupting effect of androgens in postnatal female domestic cats

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ABSTRACT

To test the hypothesis that in domestic cats, postnatal androgens induce sterility, the aims of this study were to describe the reproductive effects and the clinical safety of a postnatal administration of a long term release androgen in this species. Thirteen newborn littermate female kittens were randomly assigned to one of the following treatment groups within the first 24 hours of birth: testosterone enanthate 12.5 mg sc (TE; $n = 8$) or Placebo (PL; $n = 5$). The animals were subsequently assessed for fecal sexual hormones until puberty was attained and subsequently when matings occurred. After 21 days, ovulation and gestation were diagnosed. All queens were subsequently ovario-hysterectomized. Fecal testosterone concentrations differed between the treatment groups throughout the study period ($P < 0.05$) being greater during the first 2 postnatal weeks in those of the TE group ($P < 0.01$). Fecal estradiol was not affected by treatment ($P > 0.1$). While all the females were receptive during the pubertal estrus ($P > 0.1$), two TE (2/8) compared with all (5/5) females of the PL group had ovulations ($P < 0.05$). Only one (1/2) compared with three (3/5) of the queens of the TE and PL groups, respectively became pregnant. All kittens of the TE group had transient clitoral enlargement. Anovulatory TE-treated cats had no corpus luteum, and a significant diminution of the endometrial glands as well as of the height of the uterine epithelium. It is concluded that, in domestic cats, a single postnatal supra-physiological dose of testosterone caused a large proportion of queens to be anovulatory and there were also histological endometrial abnormalities that also occurred with this treatment that were accompanied by mild and transient side effects.

Keywords: Postnatal; Testosterone; Anovulation; Endocrine disruption; Felid

1. Introduction

Domestic cat (*Felis catus*) overpopulation is an insurmountable problem in almost all the countries of the world. Female cats are extremely prolific breeders and can reproduce from early puberty (e.g. 4 month) until death (Johnston et al., 2001). Furthermore, from the equator to areas with temperate photoperiods domestic felids breed throughout the year (Faya et al., 2011). In developing countries, unwanted kittens are simply abandoned to suffer and die on the streets being problematic to both the animals themselves and humans. Safe, efficient and practical pharmaceutical protocols are still needed to manage the feline overpopulation problem.

Sex steroids have organizational actions during late gestation and early postnatal life in both altricial and precocial species such as mice, rats and sheep (Jackson et al., 2013). Specifically, during the early postnatal period the developing central nervous system is sensitive to the organizing effects of androgens. In this respect, both genders normally differ in pattern of pituitary gonadotropin secretion and the male non-cyclic pattern of gonadotropin secretion is induced by the action of testicular androgens on the anterior hypothalamus during the postnatal period (Jackson et al. 2013).

In female rats, there exists a critical postnatal period when there is greater tissue differentiation sensitivity during which a single androgen administration permanently alters both the hypothalamic mechanisms which underlie “cyclic” gonadotropin release patterns and the target tissue response to estrogens (Gorski, 1971; Harris, 1970; Lobl et at., 1975, Lobl and Gorski, 1974, Mena et al., 1992). Androgenization of developmental tissues in neonatal pigs also resulted in anovulatory infertility (Ventanas et at., 1992). As a result, androgenized females are anovulatory, anestrous, and have altered uterine morphology and

function as well as greater male behavioral expression than untreated females (Barraclough and Gorski, 1961; Lobl and Maenza, 1977). When female dogs were injected postnatally with an androgen for 3 months, there was delayed puberty, anovulatory syndrome and complete lack of receptivity to males (Beach et al., 1983). Furthermore, rats injected on the first days of life with testosterone had ovarian atrophy (Pinilla et al., 1993).

Thus, androgen treatment early in life prevents ovulation as well as normal uterine function and sexual behavior and may have implications for development of this methodology as a contraceptive strategy for felids. To the best of the authors´ knowledge the effect of postnatal androgenization has never been assessed in domestic cats.

To test the hypothesis that in domestic cats, similar to other mammals (Barraclough and Gorski, 1961; Beach et al., 1983; Ventanas et at., 1992) exogenous androgens induce sterility if administered during the postnatal critical reproductive developmental period, the aims of the present study were to describe the clinical and histological reproductive effects of the postnatal administration of an androgen. Secondly, the clinical safety of the treatment was also assessed. For this purpose, testosterone enanthate, a potent, long term effective androgen (Plumb, 2015) which is inexpensive and available worldwide was selected as the endocrine disruptor for this experiment.

2. Materials and methods

2.2. Animals and pharmacological protocols

Thirteen (five litters) newborn littermate female 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 hours

of light per day, weaned at the age of 40 days and fed premium commercial 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 hours of birth: Testosterone enanthate 12.5 mg total dose (Testoviron Depot 250, Bayer, Argentina) subcutaneously (TE; n = 8) or Placebo: 0.05 ml corn oil subcutaneously injection (PL; n = 5). The dose was selected according to previous pilot studies in cats based on a classical animal model (Barraclough and Gorski, 1961).

2.3. Physiological and anatomical assessments

All the animals were subsequently assessed for specific anatomical, behavioral, and physiological characteristics until the first indications of puberty occurred. During this assessment period, the animals were observed 1.5 hours twice a day for evaluating sexual behavior and were physically examined and weighed once a week. Eventual appearance of clinical side effects was also recoded. Vaginal cytology (Mills et al., 1979) assessments were conducted three times per week after the third month of age. Puberty was defined by the finding of more than 80% superficial keratinized cells and a clean background in the vaginal smears accompanied by the typical estrous behavior (Johnston et al., 2001).

2.4. Fecal collection, extraction and hormone determinations

Fecal samples were collected weekly until the time of puberty and frozen for testosterone (T) and estradiol-17β (E2) determinations. Fecal collection was initiated at the end of the first postnatal week. For this purpose, each cat was confined in an individual cage with clean sanitary litter one night per week. 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. Fecal steroids were extracted using the methods described by Brown et al. (2008) and T (ng/ml) and E2 (pg/ml) were determined using electrochemiluminescence immunoassays (Elecsys Testo II and Estradiol II, Roche Diagnostics, Mannheim, Germany). Inter- and intra-assay coefficients of variation of the assays were <10% and sensitivity were 0.025 ng/mL and 12 pg/mL for T and E_2 kits, respectively. All fecal data were expressed on a wet-weight basis (Faya et al., 2013).

2.5. In vivo fertility test, ovulation and pregnancy diagnosis

As the female cats attained puberty, there was exposure to a fertile tomcat during the estrous period. Matings were observed and/or diagnosed by the presence of spermatozoa in the vaginal smears. Twenty-one days after the end of estrus, blood samples were taken for ovulation assessment by electrochemiluminescence immunoassay determination of serum progesterone (Elecsys Progesterone II, Roche Diagnostics, Mannheim, Germany; P_4 > 5 ng/mL) and gestation was diagnosed by ultrasonic examination in all the females (Mattoon and Nyland, 1995).

2.6. Ovariohysterectomies

Following assessments for pregnancy status, all queens were ovariohysterectomized and the ovaries and uteri were subjected to histological study. For the surgery, the animals were

pre-medicated with atropine sulfate, (Atropine Sulfate, John Martin; 0.04 mg/kg, subcutaneously), acepromazine maleate (Acedan, Holiday; 0.03 mg/ kg subcutaneously), and butorphanol (Torbutol Plus, Fort Dodge; 0.2 mg/kg, intramuscularly). Anesthesia was induced with sodium thiopental (Pentovet TM, Richmond; 8 mg/kg, intravenous). After the females were endotracheally intubated, anesthesia was maintained with isoflurane and oxygen in a closed system. A mid-line laparotomy was performed to excise the ovaries and uteri (Arnold, 2002). After surgery ketoprofen (Ketofen®, Fort Dodge; 1 mg/kg) was injected subcutaneously (once) and then orally every 24 hours for 4 additional days. All the queens were then placed for adoption.

2.7.Gross and histomorphometrical examination

Immediately after surgery genital tracks were excised and ovaries and uteri were macroscopically examined and weighed. The ovaries were sectioned longitudinally, placed in Bouin's fixative for 12 h and placed in 70% alcohol 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.

Follicles were classified as primordial (small oocyte surrounded by a single layer of squamous granulosa cells), primary (oocyte surrounded by a single layer of cuboidal cells), secondary (two or more layers of granulosa cells and a theca cell layer), antral (fluid-filled antrum, mural and cumulus granulosa cells and two or more layers of thecal cells) or atretic (degenerated granulosa cells and follicular fluid containing cellular debris as previously described; Bristol-Gould and Woodruff, 2006). The number of primordial, primary,

secondary and antral follicles, corpora lutea, and atretic follicles per square millimeter was determined on a computer screen using 20 captured images (X20) per animal obtained from two, 3 mm- sections.

Uteri were sectioned longitudinally for internal inspection. Cross-sections (approximately 0.5-1 cm) were collected from a point midway between the external bifurcation and the tip of each uterine horn and processed as described for the ovaries. The uteri were examined for the presence or absence of endometrial glands. The area occupied by uterine glands per mm^2 of endometrium over the total area of each microscope field was measured by planimetry. The height of the glandular and luminal uterine epithelium was assessed counting 100 cells in a total of 10 images per uterus taken with a 10X objective while myometrial thickness was examined in a total of four images per uterus taken with a 4X objective.

All histological images were obtained from a microscope (Olympus BX50, Tokyo, Japan; 10X 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 analyzed using Image Pro Plus v6.0-Media Cybernetics (Silver Spring, MA, USA).

2.8. Statistical analysis

Quantitative and qualitative differences between TE and PL groups were conducted by Fisher Exact and Student´s t tests, respectively. Fecal T and E_2 concentrations were analyzed by repeated measures ANOVA followed by Tukey comparison test. Data were expressed as (mean ±SEM) and the level of significance was set at $P < 0.05$ (SPSS 17.0, SPSS, Chicago, IL, USA).

3. Results

Age (34 ± 2.6 compared with 33.0 ± 2.3 weeks; $P>0.1$) and body weight (2.92 ± 0.7 compared with 2.76 ± 0.9 kg; $P>0.1$) at puberty attainment did not differ between TE and PL groups. Fecal T concentrations differed between the treatments throughout the weeks of the study ($P < 0.05$) being greater in the TE than in PL groups during the first 2 postnatal weeks ($P < 0.01$; **Fig. 1A**). Conversely, fecal E_2-17β concentrations varied during the study without differences between treatments ($P > 0.1$). Thus, in both groups, E_2 was elevated during the first 5 postnatal weeks ($P < 0.01$). The E_2 remained basal until 3 to 4 weeks before puberty when concentrations began to increase (Fig. 1B).

All TE-treated kittens had vulva and clitoris enlargement during postnatal weeks 3 to 13 and were of typical developmental size, thereafter. There was also one case (1/8) of transient mammary nodules in an animal of the TE-treatment group. In this case two 1.8 cm diameter nodules appeared in the pectoral and inguinal left mammary glands on week 27 and gradually decreased in size to week 39 at the time of spaying.

One kitten of the TE group displayed mounting behavior preceding puberty. All the females had normal estrous behavior, were receptive to males ($P > 0.1$) and were, therefore, repeatedly mated during the pubertal estrus. While all the cats of the PL group (5/5) had ovulations and three were pregnant when assessments occurred, ovulation occurred in two of eight females of the TE group ($P < 0.05$) and one of the females was pregnant at the time of assessment. In both groups, gestations progressed normally until the time of ovariohysterectomies when the examination of the excised uteri revealed four to five conceptuses.

Ovarian weight did not differ between animals in the TE and PL (0.17 ± 0.0 compared with 0.16 ± 0.0 g; $P > 0.1$) groups. Gross ovarian examination revealed follicles and

corpora lutea in the animals that failed to and those that had ovulations, respectively. No further gross nor microscopical differences were detected between treatment groups for the ovarian variables that were studied (Table 1).

Gross external and internal uterine observations were normal in all but one queen in the TE group which was in estrus again at the time of surgery and had generalized abdominal hyperemia and a slightly increased bleeding. In this queen the entirety of the uterine horns and endometrium were also thicker than in the other cats.

Microscopic assessment of the uteri revealed that the area occupied by uterine glands per mm^2 of endometrium was smaller in the TE than in the PL group (0.34 ± 0.01 compared with 0.49 ± 0.03; $P < 0.01$). Conversely, the area occupied by stroma per mm^2 was larger in the TE queens (0.64 ± 0.01 compared with 0.50 ±0.03; $P < 0.01$). The height of the glandular (μm; 13.89 ± 0.29 compared with 21.43 ±1.10; $P < 0.01$) and luminal (μm; 8.09 ± 0.39 compared with 9.44 ± 0.42; $P < 0.05$) uterine epithelium were shorter in the TE than in the PL group (Fig. 2). Myometrial thickness did not differ between the two groups (μm; 286.43 ± 8.23 vs. 298.36+8.62; $P < 0.05$).

4. Discussion

Although, postnatal androgenization effects have been reported in several mammalian species including dogs (Barraclough and Gorski, 1961; Beach et al., 1983; Jackson et al., 2013; Lobl and Gorski, 1974; Ventanas et al., 1992), to the authors' knowledge, this is the first study that describes the effect of a single postnatal supra-physiological dose of a potent, time-released androgen on female cat reproduction. In the kittens of the present study, androgen treatment did not alter growth rate, age at puberty attainment nor estrous

239 behavior and receptivity. Similarly, in postnatally androgenized rats, body weight did not
240 differ from that of control animals (Almirón et at., 1984).

241 It is generally accepted that testosterone, when present for a period after birth, can
242 permanently suppress development of female reproductive behavior but this premise is not
243 consistent with findings in a previous study where sexual receptivity was not affected by
244 testosterone treatment of female pigs early in life (Ford and Christenson, 1987).
245 Conversely, normal sexual behavior was abolished in neonatally-androgenized rodents and
246 bitches (Beach et al. 1983, Gogan et al. 1980, Thomas et al. 1983). These inconsistencies in
247 results may be due to dose and species differences in the behavioral response to postnatal
248 testosterone.

249 The increased fecal T during the first 2 postnatal weeks in the kittens of the TE group in
250 the present study clearly indicates the exogenous effects of the hormonal treatment on
251 anatomical and behavioral characteristics in cats and defines the time period of the "the
252 critical period" to permanently differentiate sexual reproductive function in this species.
253 Conversely, fecal E_2 does not seem to be affected by androgen treatment. Furthermore, the
254 augmented E_2 concentrations during the first 5 postnatal weeks could be due to an
255 endogenous origin (Faya et al., 2013).

256 The transient vulvar and clitoris abnormalities and the mounting behavior observed in
257 all and one the TE-treated cats, respectively, are evidence of the peripheral effects of
258 testosterone. Furthermore, these changes were expected according to previous reports in
259 other postnatally androgenized species (Brown et al., 1999, Morali et al., 1985).

260 Consistent with findings in the present study with what has been extensively described
261 for other mammals (Jackson et al., 2013), the hypothalamic-pituitary-ovarian axis
262 functionality was probably affected in most of these queens as 75% of the TE-treated

263 animals were anovulatory. It is assumed that anovulation resulted from an androgen-
264 induced disorder of the postnatal hypothalamus and the subsequent change in the release
265 pattern of luteinizing hormone from the pituitary gland (Barraclough, 1961; Wagner et al.,
266 1966). This supports the hypothesis that the hypothalamus is sensitive, at least in part, to
267 programming by postnatal steroids in this altricial species. Both the absence of luteal tissue
268 and ovarian abnormalities in the testosterone-treated cats that were anovulatory suggest the
269 central origin of the infertility in these cats. It could also be possible that postnatal
270 testosterone might alter target tissue receptor sensitivity and, therefore, response to
271 hormones.

272 Similarly to what has been described for other endocrine disruptors in postnatal cats
273 (Carranza et al., 2014), differences in individual response may have also accounted for the
274 findings in the present study. It is worth noting that a dose effect has been previously
275 described for aromatizable and non-aromatizable androgens in the induction of anovulatory
276 syndrome in rats (Arai et al., 1981, Gorski and Barraclough, 1963). Although a larger
277 testosterone dose may have decreased or eliminated the variability of anovulation in the
278 present study, the possibility of use of a larger dose for this purpose does not appear to be
279 feasible as there would likely be an increase in the prevalence and severity of side effects.

280 The follicles from which ovulation did not occur might have functioned to increase
281 estrogens in the bloodstream and may have led to the notable inflammation, hemorrhages,
282 and hyperplasia in the endometrium in the testosterone-treated queen of the present study
283 which was spayed while in estrus. These abdominal findings were similar, in some aspects
284 to those described for human ovarian hyper-stimulation syndrome which is characterized
285 by multiple ovarian cysts and subsequent hyper-esterogenemia (Jones et al., 1987).

The mammary nodules in the TE-group of the present study were not histologically examined and the transient existence suggests a non-neoplastic etiology. Similarly, postnatal dihydrotestosterone treatment resulted in the stimulation of mammary development at advanced ages in female mice (Yanai et al., 1981). Importantly, it should also be taken into account that the eventual long term undesired effects should not be discarded for the treatments used in the present study as these were not studied in the time frame of present trial.

Postnatal testosterone treatment, in the felids of the present study, reduced the area occupied by endometrial glands and cell development in both glandular and luminal epithelial tissues. Similar findings have been described in postnatal androgenized rats (Arutiunian et al., 1987) and also in progesterone-treated postnatal dogs (Wilborn et al., 2014). As normal endometrial glands are required for establishment and maintenance of pregnancy the question about a concomitant uterine infertility arises and remains to be unveiled in most of these anovulated testosterone treated females. Normal uterine functionality could only be ascertained in the pregnant androgenized female of the present study. Inconsistent with what has been found in postnatal androgenized rats (Lobl and Maenza, 1977), no variations in myometrium thickness was found in the present feline study.

It is concluded that a single postnatal supra-physiological dose of testosterone enanthate caused a large proportion of queens to be infertile due to anovulation and histological endometrial abnormalities were prevalent in these cats. Furthermore, this

pharmacological protocol seemed to predispose queens to mild reversible physical side effects. More knowledge is necessary before these pharmacological protocols could be widely recommended as a contraceptive strategy in female cats.

Acknowledgments

This study was partially funded by the National Agency of Research and Technology, Argentina (PICT 2014 0426), Argentina. MLP and RV are Research Fellows and CB, and CG are Career Scientists of CONICET, Argentina.

Conflict of interest

None of the authors of this article has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Figure legends

Fig. 1. Fecal testosterone (A; $P < 0.05$) and estradiol-17β (B; $P > 0.1$) concentrations (mean ± SEM) of 13 female kittens treated postnatally with testosterone enanthate 12.5mg (solid symbols) or a placebo (open symbols) and subsequently assessed until the first pubertal signs appeared for anatomical and behaviorally characteristics; Asteriks over the symbols indicate selected differences ($P < 0.01$) between groups

Fig. 2. Ovaries (hematoxylin and eosin, 4X) of female cats; Animals treated with testosterone enanthate (A) or placebo (B); No significant differences ($P > 0.05$) were detected between groups for any ovarian structure except for the presence of luteal cells (LC) in the testosterone- treated cats

Fig 3. Uteri (hematoxylin and eosin, 4X and 20X) of 13 female cats; Animals treated with testosterone enanthate (A and A´) or placebo (B and B´); Notice fewer ($P < 0.01$) endometrial uterine glands in the testosterone-treated cats (A and A´)

436

437 **Table 1.** Ovarian gross and histologic variables (mean ± SD) of female cats; No

438 differences ($P > 0.05$) were detected between groups

Parameter	TE	Placebo
Length (cm)	1.02 ± 0.0	0.95± 0.0
Height (cm)	0.58 ± 0.0	0.65 ± 0.0
Weight (g)	0.16 ± 0.0	0.17 ± 0.0
Volume (cm^3)	0.17 ± 0.0	0.20 ± 0.0
Primordial follicles/mm^2	35.13 ± 15.0	38.67 ± 7.7
Primary follicles/mm^2	6.80 ± 1.4	10.62 ± 1.8
Secondary follicles/mm^2	0.95 ± 0.2	1.91 ± 0.7
Antral follicles/mm^2	0.24 ± 0.0	0.08 ± 0.0
Atretic follicles/mm^2	3.80 ± 0.6	3.64 ± 0.7

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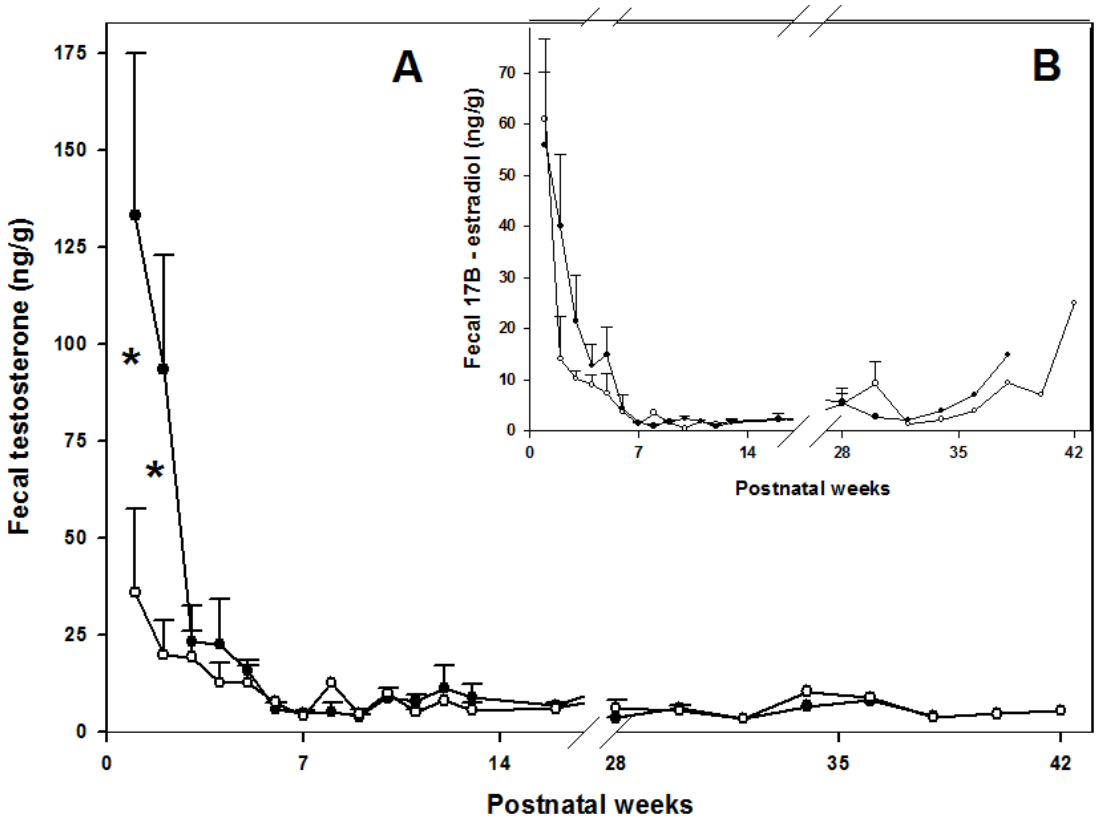

Fig 2 A

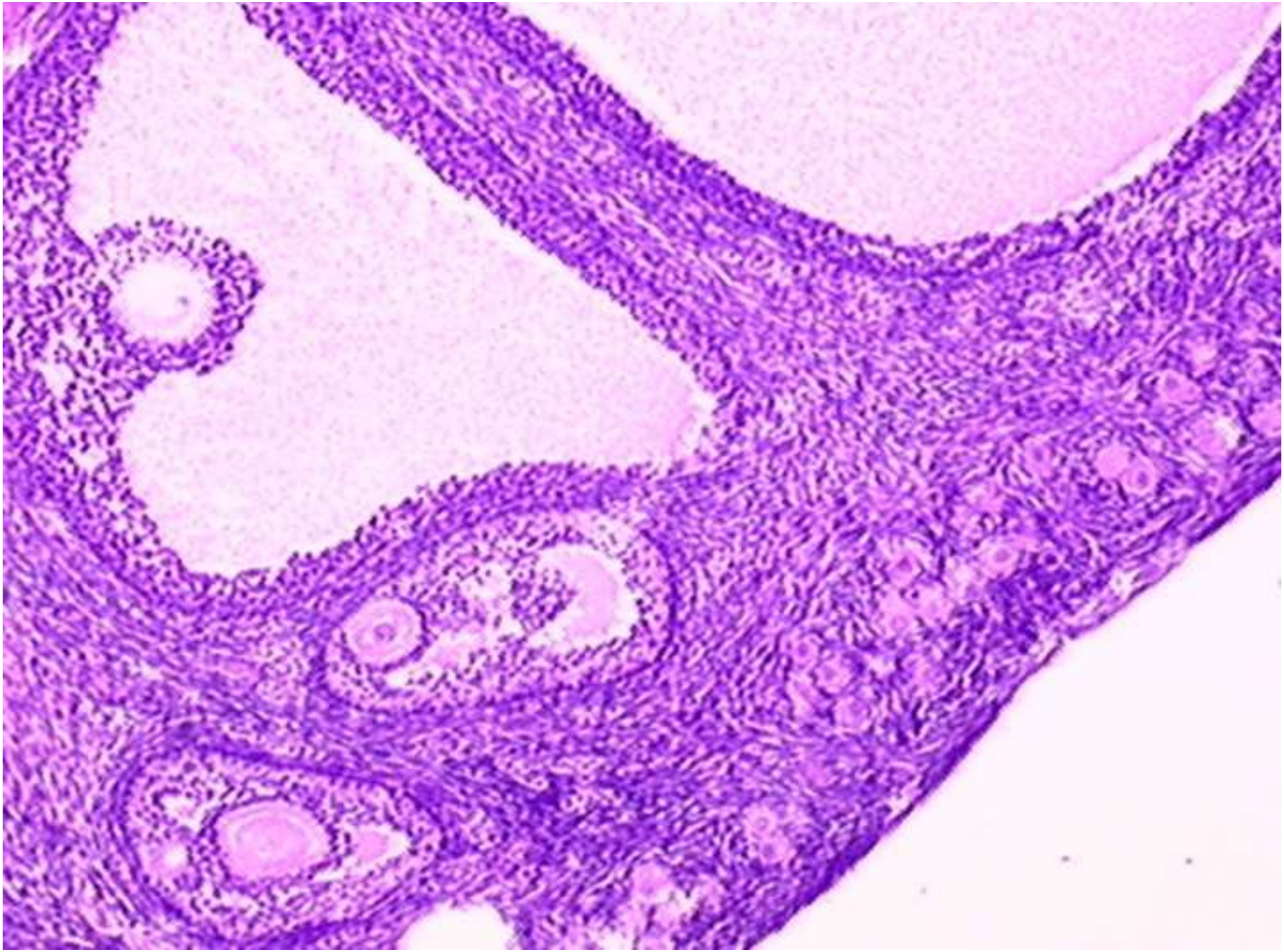

Fig 3A

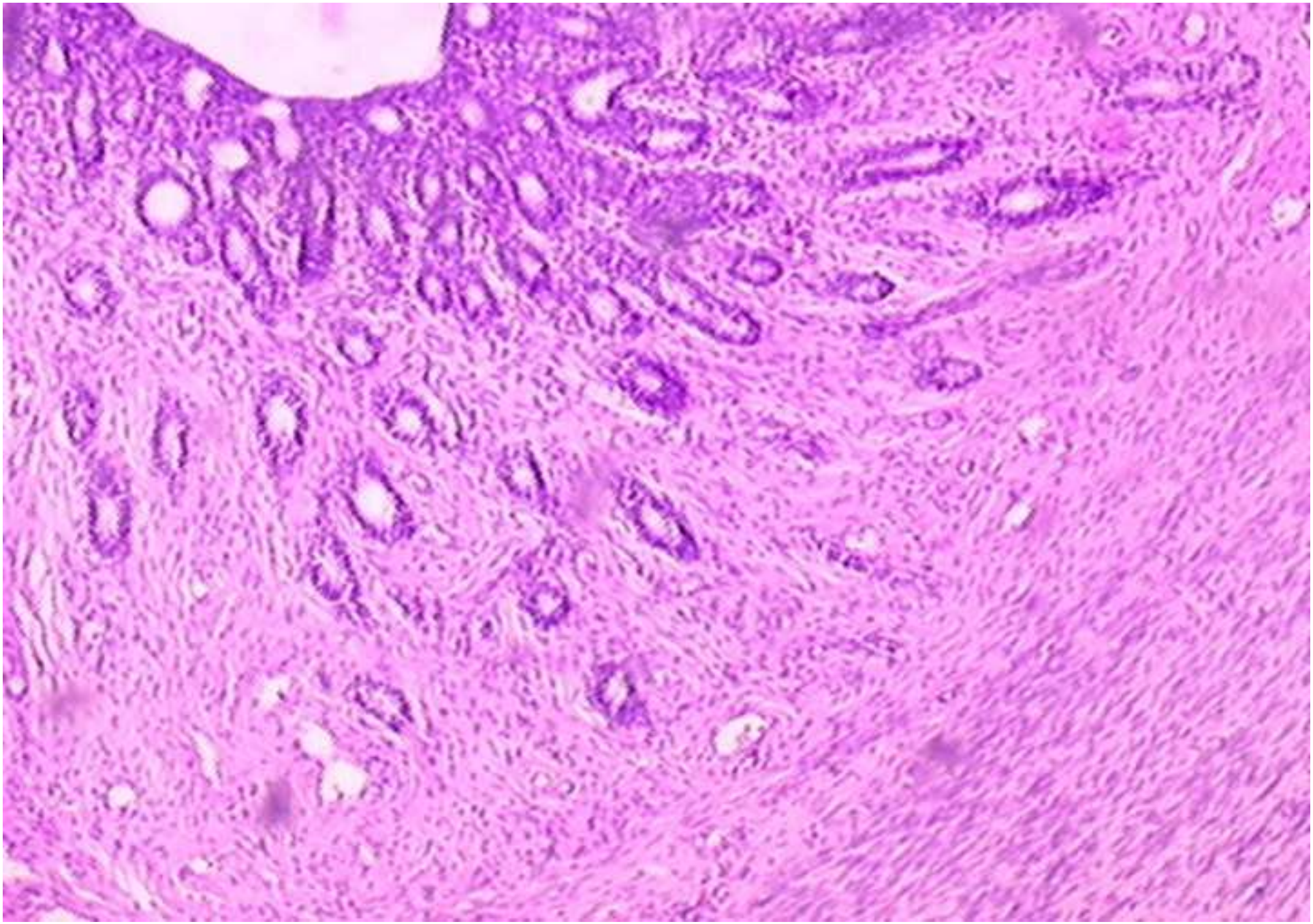

Fig 3B

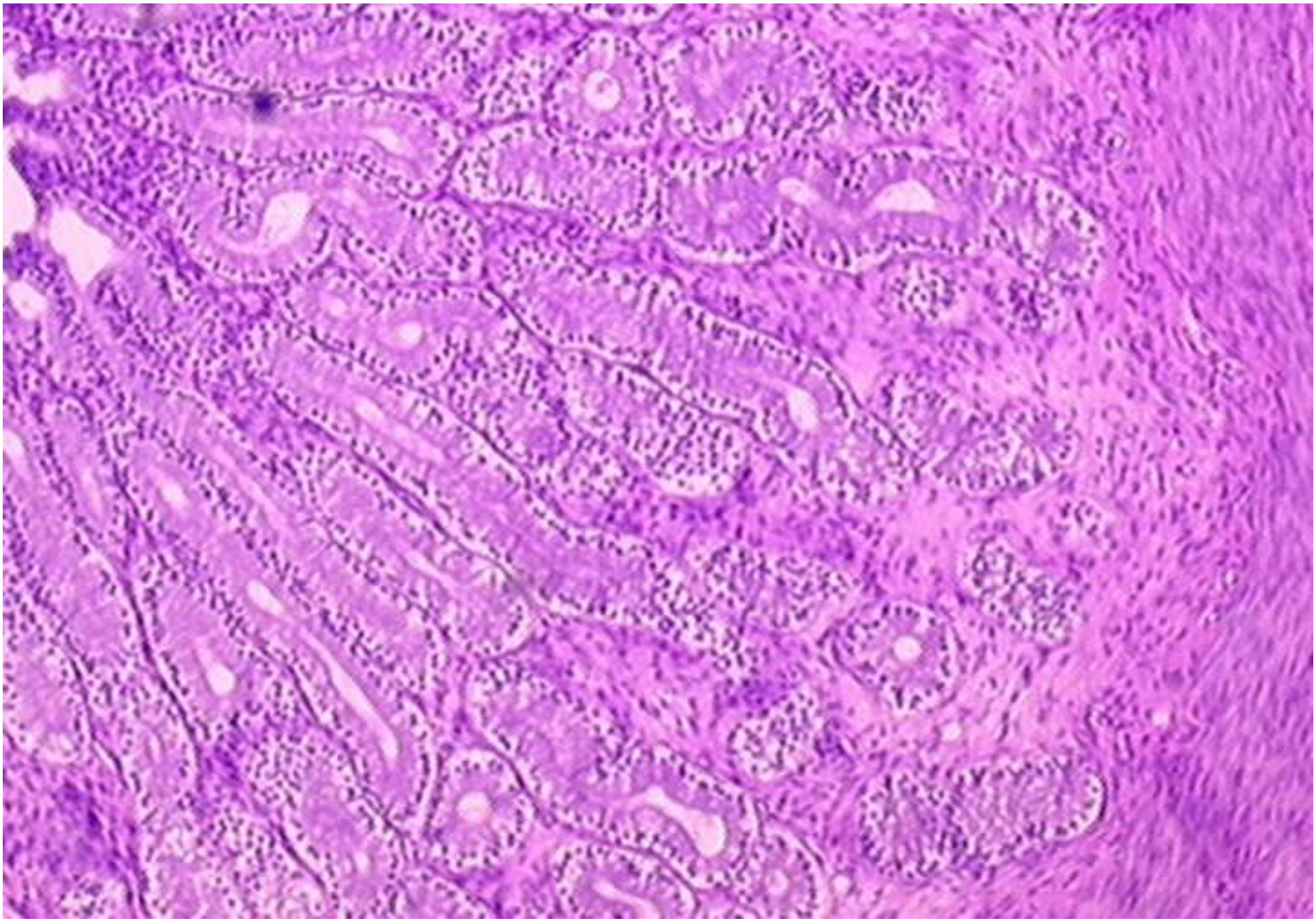

Fig A´

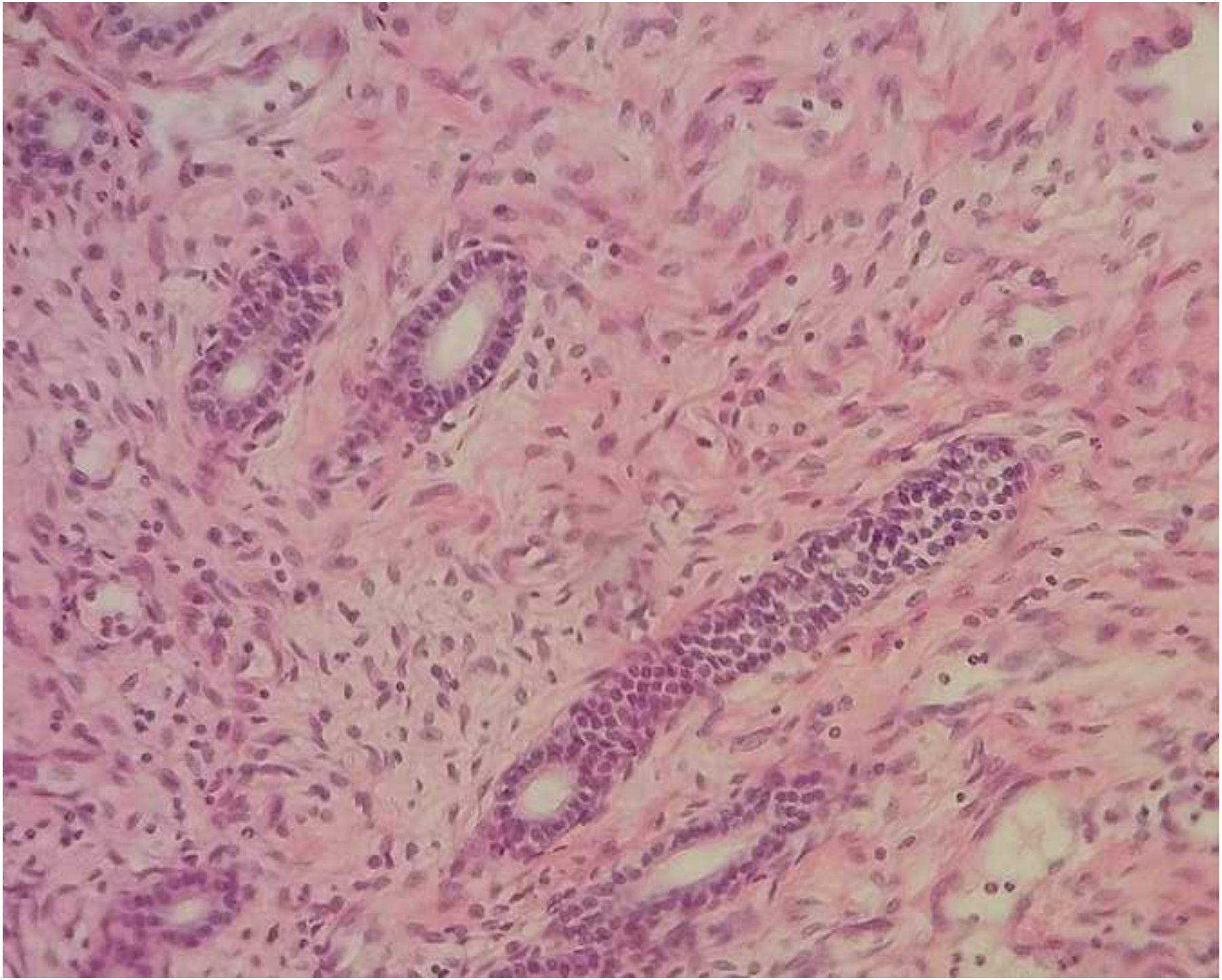

Fig B´

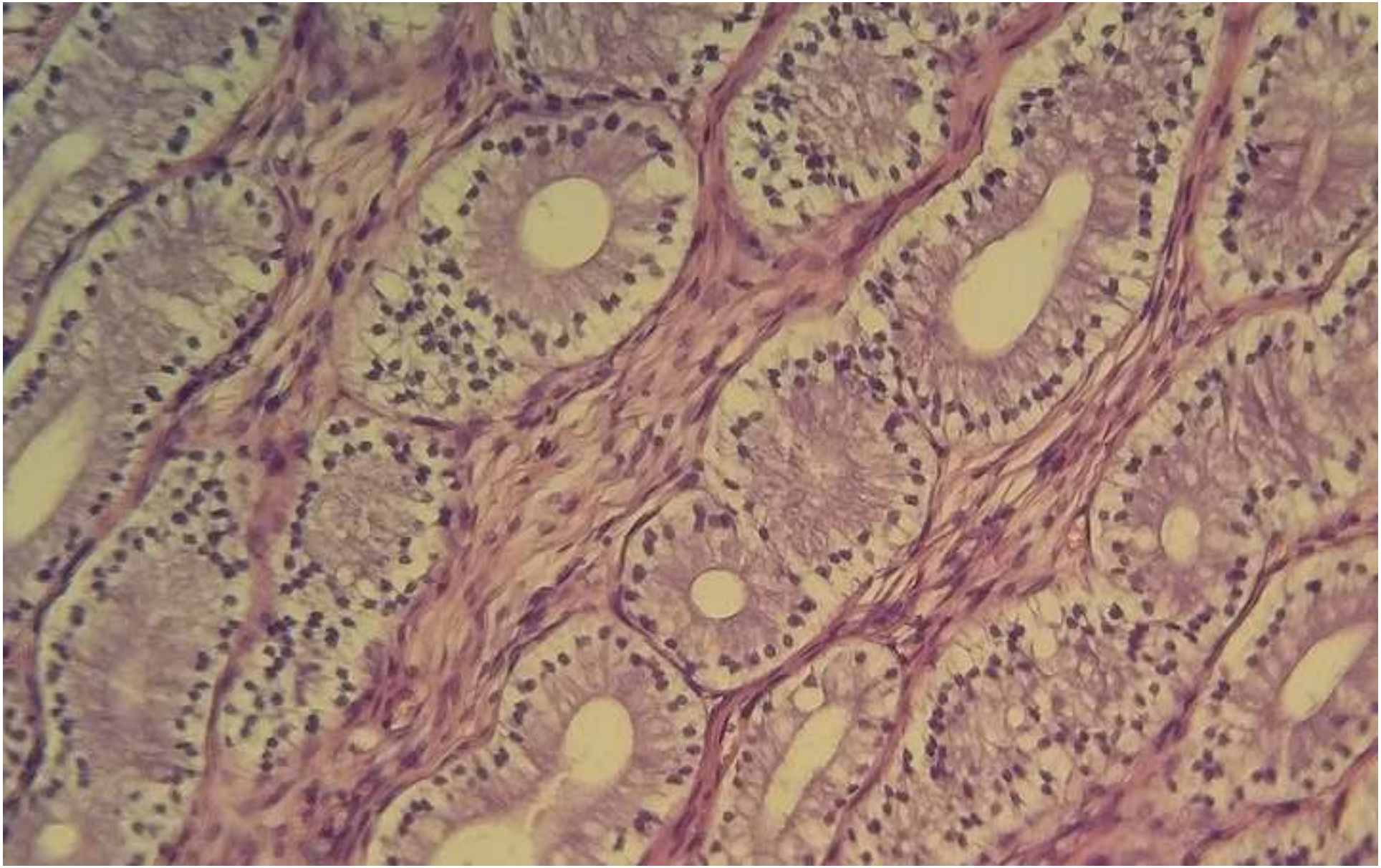

Fig 2B