Prenatal dexamethasone exposure and developmental programming of the rat offspring ovary: a structural study

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Abstract

Overexposure to glucocorticoids during fetal development alters fetal organ growth and maturation patterns, which can result in adverse programming outcomes in adulthood. The aim of this study was to determine whether exposure to dexamethasone (Dx) during the fetal period programmed ovarian development and function in infantile and peripubertal female offspring. Pregnant Wistar rats were separated into control and Dx-treated (0.5 mg/kg body mass) group, and were injected on gestational days 16, 17 and 18. Ovaries from 16- and 38-day-old female offspring were prepared for histological and stereological examinations. Ovarian volume and the number of primordial and primary follicles were significantly reduced in the infantile and peripubertal female offspring prenataly exposed to Dx in comparison with control values. The number of multilaminar follicles was decreased in infantile females. In peripubertal females, prenatal exposure to Dx increased the number of multilaminar and large follicles of all classes. Since the vaginal opening did not occur up to the 38th day, the absence of ovulations and *corpora lutea* was a confirmation that the onset of puberty had been delayed. We can conclude that overexposure to glucocorticoids early in life programs ovarian development, which may affect fertility in adulthood.

Keywords: development, histology, stereology

Abridged title: Programming of ovary development by dexamethasone

Introduction

Normal fetal development in mammals is characterized by a series of sensitive – critical periods during which intensive cell divisions occur, leading to the formation and maturation of tissues and organs. During these periods, an adverse prenatal environment may shape the offspring phenotype *via* permanent morphological, physiologic and metabolic changes, often accompanied by reduced fetal growth. Responses to environmental challenges may have short-term adaptive benefits for the fetus but increase the risk of later disease (Cottrell and Seckl, 2009). This concept is well known as developmental programming (Rabadán-Diehl and Nathanielsz, 2013). Intrauterine growth retardation (IUGR) and low birth weight are often used as predictors of later disease risk (Barker et al. 1993). Research in the field of developmental programming is generally focused on cardiovascular issues (especially hypertension), predisposition to metabolic dysfunction (mainly obesity and diabetes) and neuroendocrine disorders (Manojlović-Stojanoski et al., 2014; O'Regan et al., 2008); however, the reproductive system has recently been recognized as a new target of fetal programming (Dupont et al., 2012).

The most commonly used experimental approaches employed to investigate the mechanisms by which IUGR induces disease development are maternal undernutrition or stress, placental insufficiency and overexposure to glucocorticoids (Nathanielsz, 2006). Although different experimental setups lead to IUGR, glucocorticoid excess probably represents a common pathway by which adverse conditions are transferred from the mother to the fetus, triggering changes in offspring growth and permanently affecting tissue and organ structure and function. Dexamethasone is often used as a drug of choice in maternal and neonatal human

and veterinary health care for the treatment of pregnancy-related diseases such as ketosis, pregnancy toxemia, mastitis, prenatal fetal lung malformations and management of neonatal diseases (Crowley, 1995; de Steenwinkel et al., 2017; Gregersen and Ulrik, 2013; Yahi et al., 2017). In both humans and animals, dexamethasone reduces the incidence of respiratory distress syndrome (RDS) in the newborn (Crowley, 1995). Overexposure to dexamethasone, utilized in numerous experimental protocols to induce developmental programming (Iwasa et al., 2014; O'Regan et al., 2008; Ortiz et al., 2001), represents maternal chronic stress or a therapeutic dexamethasone-induced IUGR model (Iwasa et al., 2011).

The reproductive system begins to form early in gestation, but structural and functional maturation is not completed until puberty. During oogenesis, three processes can be observed – germ cell proliferation and differentiation and germ cell death (apoptosis). Balance between the proliferation and apoptosis of germ cells is a major phenomenon in normal ovarian development and enables the establishment of the maximal reproductive potential of adult females (Poulain et al., 2012). Exposure to dexamethasone during the most vulnerable period of ovarian development decreases the proliferation rate of germ cells, while increasing their apoptotic rate (Ristić et al., 2019). Disturbed balance between the proliferation and loss of germ cells by apoptosis leads to a reduction of total germ cell number in the fetal ovary (Ristić et al., 2019). Fewer oocytes in the fetal rat ovary lead to a significant decrease of the overall follicle number in the ovaries of neonatal rats (Ristić et al., 2008). Herein, we investigated whether the quantitative changes established during fetal development and confirmed in the neonatal period were sustained until the later stages of ovarian development and whether they were accompanied by some functional changes.

To our knowledge, there are no reports of a systematic study of folliculogenesis and the stereological properties of ovaries of infantile and peripubertal rats prenatally exposed to dexamethasone. Design-based stereology is considered the gold standard for estimating follicular numbers in the ovary (Charlston et al., 2007; Mayers et al., 2004; Medigović et al., 2012; Ristić et al., 2008) and provides important information about the function of the ovary and the factors that regulate it. Application of these approaches in a structural study of the ovary may contribute to understanding whether prenatal exposure to Dx affects ovarian development and function and indicate its implications for offspring fertility.

Materials and Methods

Animals and experimental groups

Adult female Wistar rats (average body mass 200–250 g; bred at the Institute for Biological Research "Siniša Stanković" - National Institute of Republic of Serbia, University of Belgrade, Belgrade, Serbia) were maintained under controlled conditions (12 h:12 h light–dark cycle at 22°C) with *ad libitum* access to food (standard rat chow) and tap water. Two nulliparous females in estrus were mated with one fertile male. The day when sperm was detected in the vaginal smear was designated as day 1 of gestation. Gravid females were randomized into a control and an experimental group, each consisting of 10 animals. On days 16, 17 and 18 of pregnancy, experimental dams *s.c.*received 0.5 mg Dx (dexamethasone phosphate dissolved in 0.9% saline; injected volume was 0.4 ml; Krka FARMA, Belgrade, Serbia) *per* kg body mass (BM). The dosing used in the present study has been previously adopted and designated to fall within the range of clinical application in woman (0.1– 0.5

mg/kg), (Carbone et al., 2012). Control gravid females received the same volume of saline. On postnatal days 16 (infantile period) and 38 (peripubertal period), female pups were randomly chosen from each of the ten control litters and ten litters of Dx-treated mothers and sacrificed under ether narcosis. These periods correlate with similar developmental stages in humans (Picut et al., 2015). Randomization obviated any potential litter bias. In brief, one ovary from each female was excised and immediately fixed in Bouin's solution for 48 h, dehydrated in a series of increasing concentrations of ethanol (30%–100%), enlightened in xylol and embedded in Histowax® (Histolab Product AB, Göteborg,Sweden). After embedding, each tissue block was serially sectioned at 3 μm thickness on a rotary microtome (RM 2125RT Leica Microsystems Wetzlar, Germany). The slices were processed for routine hematoxylin–eosin (H&E) staining. The experimental protocols were approved by the Local Animal Care Committee and conformed to the recommendations given in *Guide for the Care and Use of Laboratory Animals* (National Academy Press, Washington, DC, 1996).

Morphometry and classification of the ovarian follicles

Follicles were classified as small follicles measuring <275 μm in the largest cross-section (LCS) and large follicles measuring >275 μm in the LCS. According to Gaytan et al. (1998), the small follicles were divided into six classes: primordial (non-growing) follicles, which consist of an oocyte partially or completely surrounded by a layer of three to six flattened pregranulosa cells; primary follicles, identified by a single layer of cuboidal pregranulosa cells around the oocyte; small growing follicles (secondary follicles) that include multilaminar class A follicles (Ma), which have one to two layers of granulosa cells measuring up to 75 μm

in diameter; multilaminar class B follicles (Mb) measuring from 76 to 150 μm; multilaminar class C follicles (Mc) measuring from 151 to 200 μm; and multilaminar class D follicles (Md) measuring from 201 to 274 μm. The large (antral) follicles were divided into five classes, as suggested by Osman (1985): class 1, from 276 to 350 μm in diameter; class 2, from 351 to 400 μm; class 3, from 401 to 450 μm; class 4, from 451 to 575 μm; and class 5, more than 575 μm in diameter. The diameters of the follicles were measured (two diameters at right angles) in the LCS containing the oocyte nucleolus.

Atretic follicles contained an abundance of pyknotic granulosa cells and/or degenerated oocytes. Oocyte degeneration was reflected by the highly irregular shape and/or presence of a pyknotic nucleus. Atretic follicles were divided into two groups: primordial atretic follicles, small atretic follicles (<275 μm in the LCS) and antral atretic follicles (>275 μm in the LCS).

Stereological methods were employed to estimate the number of primordial and primary follicles (Mayers et al., 2004; Ristić et al., 2008). Small growing (preantral) and antral follicles were counted exactly in every fourth section encompassing whole cross-sections of the ovary. Oocyte nuclear number was equated to follicular number. The number of follicles was expressed as: total number *per* ovary and number *per* ovary volume, that is, numerical density (Nv).

Stereological measurements

All stereological analyses were carried out using a workstation comprising a microscope (Olympus, BX-51,Olympus, Japan) equipped with a microcator (Heidenhain MT1201, Heidenhain, Traunreut, Germany) to control movements in the z direction (accuracy: 0.2 μm),

a motorized stage (Prior *Scientific Inc., Rockland, USA*) for stepwise displacement in the x/y directions (accuracy: 1 μm) and a CCD video camera (PixeLink, Ottawa, ON, Canada) connected to a 19-inch PC LCD monitor (Dell, 1907FPc, Dell Inc., Round Rock, TX, USA). The whole system was controlled by the new-CAST stereological software package (VIS – Visiopharm Integrator System, version 3.2.7.0; Visiopharm, Horsholm, Denmark). The main objectives were planachromatic 4× dry lens, 10× dry lens, 20× dry lens and 40× dry lens. Control of stage movements and interactive test grids and unbiased dissector frames were provided by the newCAST software package.

Volume estimation

The volume of the ovary was estimated using Cavalieri's principle (Gundersen and Jensen, 1987). Sampling of 3 μm thick ovarian sections was systematically uniform from a random start. Every 10th section from each of the tissue blocks was analyzed. A random number for each block being analyzed was chosen by using the random number table. On the monitor, a final magnification of 182.5× allowed easy and accurate recognition of tissue boundaries.

Primarily, the volumes of follicular parenchyma (follicles in different stages of folliculogenesis) and ovarian stroma (interstitial glands, blood vessels and ovary connective tissue) were estimated. To ensure that 100–200 points were hitting the phase of interest (follicular parenchyma or ovarian stroma), two test grids with different point densities were used.

The volume of follicular parenchyma/ovarian stroma was calculated using the formula:

$$\overline{V} = a(p) \cdot BA \cdot \sum_{i=1}^{n} Pi$$

where *a(p)* is the area associated with each sampling point (1342217.4 μm^2 for follicular parenchyma, and 59652.17 μm^2 for ovarian stroma), BA (block advance) is the mean distance between two consecutively studied sections, *i.e.* real section thickness 3 μm × 10; n is the number of sections studied for each ovary, and ΣPi is the sum of points hitting a given target. The values for volumes of follicular parenchyma and ovarian stroma were summed for the volume of the whole ovary.

Volume density of follicular parenchyma (V_{Vfp}) was calculated using the formula:

$$\overline{V}_{Vfp} = \overline{V}fp \,/\, \overline{V}ov \cdot 100.$$

Volume density (V_{Vos}) of ovarian stroma was expressed as:

$$\overline{V}_{Vos} = 100\text{-}\overline{V}_{Vfp}.$$

Quantification of primordial and primary follicles

The number of healthy and atretic primordial and primary follicles in the ovaries of 16- and 38-day-old female offspring from control and Dx-treated mothers were estimated using a fractionator–physical disector design, with two levels of sampling (Mayers et al. 2004; Ristić et al. 2008). Sampling was systematically uniform from a random start (Gundersen and Jensen, 1987; Miller et al., 1999; Miller et al., 1997; Wreford, 1995). For the first level of sampling, every 10th and 11th section were picked at random all along the serial sections.

Sections designated as disector pairs were first captured into a super-image. After defining and linking the sections into pairs, they were aligned by translation and rotation using the montage option in the newCast software. One section in the pair was designated the reference section and the other the “look-up”section. Subsequently, the analysis was performed in both directions with the reference section also becoming the “look-up”section, duplicating the first sampling fraction from 1/10th to 1/5th (sampling fraction 1(f1) =1/5 = 0.2). For the estimation of primordial and primary follicle numbers in ovaries, two independent unbiased counting frames were used (Mayers et al., 2004; Ristić et al., 2008) to ensure 100-200 follicles of both types *per* animal. For primary follicles, an unbiased counting frame measuring 150 × 150 μm (22,500μm^2) was used, and for primordial follicles an unbiased counting frame measuring 94.8 × 94.8 μm (8,987 μm^2). After defining tissue boundaries, meander sampling was set to analyze 70% of the tissue (sampling fraction 2 for primary and primordial follicles was f2 = 0.7). For primordial follicles, sampling fraction 3 was f3 =8,987/22,500.

The number of healthy and atretic primordial follicles = Q^{-}(primordial) · (1/f1) · (1/f2) · (1/f3) = Q^{-}· 1/0.2 ·1/0.7· 1/(8,987/22,500).

The number of healthy and atretic primary follicles = Q^{-} (primary) · (1/f1) · (1/f2) = Q^{-} (primary)· 1/0.2 ·1/0.7.

Using the super-image acquisition option within the newCAST software pack and a 5× objective lens, a micrograph of the whole ovary section in 38-day-old offspring was obtained (Fig. 2).

Statistical analysis

All results are expressed as mean values for 10 animals *per* group (± SD). Data was tested for normality of distribution using the Kolmogorov-Smirnov test and analyzed by 2-way analysis of variance, with the age of female offspring (16 and 38 days) and applied treatment (C and Dx) as factors. The *post hoc* Bonferroni test was used to determine significant differences between groups. The minimum level of statistical significance was set at $P < 0.05$.

Results

Body mass and the timing of vaginal opening

Exposure to Dx during the last third of pregnancy did not affect the length of gestation, litter size, ratio of male to female pups or pup viability, while it did reduce birth mass by 34% ($P < 0.05$) compared to controls. When compared to control females, the reduced birth mass lasted up to postnatal day 16 (by 27%, $P < 0.05$) and postnatal day 38 (by 21%, $P < 0.05$) (Table 1). Despite a more rapid mass gain in the offspring prenatally exposed to Dx, which may be considered as catch-up growth, control values were not reached. From postnatal days 16 to 38, the body mass of female Dx-treated offspring rose 3.9-fold ($P < 0.05$), whereas in females from the control group the body mass increased 3.6-fold ($P < 0.05$; Table 1).

The day of vaginal opening in control females was 33.6±1.8 days of age, while in the offspring prenatally exposed to Dx it was delayed and did not occur until day 38.

Ovarian morphology

In the ovaries of 16-day-old control females, primordial, primary and secondary follicles (multilaminar follicles class A - Ma, multilaminar follicles class B - Mb and multilaminar follicles class C – Mc) were present. Atretic follicles corresponding to each stage of folliculogenesis were also observed (Fig. 1a). Multilaminar follicles were mostly located in the central core of the ovary, while primordial and primary follicles were limited to a thin peripheral rim. This pattern of follicle alignment is typical of immature ovaries and is not apparent in the adult ovary (Fig. 1c). During this period, the *zona pellucida* appears in Mc follicles (Fig. 1b). In the infantile female offspring of Dx-treated dams, the ovaries contained healthy and atretic follicles at all stages of development as seen in the control females, but in lower numbers, followed by reduced ovarian size (Fig. 1d, e).

In the peripubertal stage, the ovaries were mature, with a well-developed interstitium and a balanced distribution of healthy follicles at all stages of folliculogenesis. Atretic follicles in different stages of degeneration were also present (Fig. 2a). The hallmark morphologic feature of the ovary in the peripubertal period was the appearance of preovulatory follicles in the outer cortex (Fig. 2b). Puberty occurs with the first ovulation of these follicles and the *corpora lutea* of a recent ovulation were the only type of *corpora lutea* initially present in the ovaries of control peripubertal females (Fig. 2c). In the ovaries of 38-day-old female offspring prenatally exposed to Dx, *corpora lutea* were not observed, indicating that there had been no ovulations (Fig. 2d). Since the vaginal opening did not occur up to day 38, the absence of ovulations and *corpora lutea* was a confirmation that the onset of puberty was delayed. Due

to the absence of *corpora lutea*, the size of the ovaries was reduced, while the number of multilaminar and large follicles was increased in comparison to controls (Fig. 2d, e).

Ovarian volume

The volume of the ovaries from 16- and 38-day-old females prenatally exposed to Dx was significantly decreased compared to the control values, by 30% ($P<0.05$) and 27% ($P<0.05$), respectively (Table 2). Follicular parenchyma volume was also significantly decreased, by 34% ($P<0.05$) and 39% ($P<0.05$), respectively, whereas the volume of ovarian stroma remained unchanged (Table 2).

Number of follicles

In the ovaries of 16-day-old offspring, prenatal exposure to Dx induced a significant decrease in the number of primordial (nongrowing) follicles by 41% ($P<0.05$) when compared to the corresponding control values (Fig. 3a). This decrease was followed by a significant reduction in the number of growing follicles, namely, primary (by 49%; $P<0.05$) and secondary (multilaminar) follicles class A - Ma (by 27%, $P<0.05$), class B - Mb (by 23%, $P<0.05$) and class C - Mc (by 33%; $P<0.05$) (Fig. 3a). The total number of small growing follicles decreased by 41% ($P<0.05$) as compared to the control values (Fig. 3a).

In the ovaries of infantile females prenatally exposed to Dx, a decrease in the number of atretic follicles was also detected. The number of atretic primordial and small growing follicles decreased by 30% ($P<0.05$) and 56% ($P<0.05$), respectively, in comparison with the

controls (Fig. 3b). The percentage of atretic follicles in the total number (healthy+atretic) of primordial and small growing follicles is shown in Fig. 5a.

In the ovaries of 38-day-old females prenatally exposed to Dx, all classes of follicles were present like in the control animals, while *corpora lutea* were not observed. The numbers of primordial and primary follicles were reduced by 26% ($P<0.05$) and 34% ($P<0.05$), respectively, in comparison with control values (Fig. 4a). This decrease was followed by an increase in the number of multilaminar and large follicles of all classes. The numbers of secondary (multilaminar) follicles class A - Ma, class B - Mb, class C - Mc and class D - Md significantly increased (3-fold; 35%; 39%; 56%, $P<0.05$, respectively). The total number of small growing follicles was significantly decreased by 24% ($P<0.05$) in comparison with control values (Fig. 4a).

The number of antral follicles of classes I and IV increased 3- and 5-fold ($P<0.05$), respectively, while the number of large follicles of classes II, III and V wasn't significantly changed in comparison with control values. The total number of large follicles increased 2.3-fold ($P<0.05$), as compared to the control values (Fig. 4b).

Compared to controls, the number of atretic primordial and small growing follicles in the ovaries of peripubertal offspring prenatally exposed to Dx remained unchanged. Moreover, the total number of large atretic follicles increased 2.5-fold ($P< 0.05$) (Fig. 4c).

The percentage of atretic follicles in the total number (healthy+atretic) of primordial, small growing and large follicles is shown in Fig. 5b.

Numerical density

The numerical density (NV) of each class of follicles and the significances of the value differences are summarized in Table 3. In 16-day-old female offspring prenatally exposed to Dx, significant ($P<0.05$) changes in numerical density were observed in the groups of primordial and primary follicles, as well as in groups of classes Mb and Mc and in the sum of small atretic follicles. In 38-day-old females from Dx-treated mothers, significant changes were observed in multilaminar follicles class D, large follicles classes I, IV and V, as well as in the sum of large healthy and large atretic follicles (Table 3).

Discussion

This study has deepened our knowledge about the programming effects of prenatal exposure to Dx on ovarian development in infantile and peripubertal offspring. By using a structural (histological and stereological) approach, we demonstrated that Dx exposure during fetal development decreased the volume of the ovary in 16- and 38-day-old offspring. The number of follicles was decreased in 16-day-old offspring, while a disrupted process of folliculogenesis and delayed onset of puberty were noticed in 38-day-old females. Changes in ovarian structure, established during fetal development after exposure to Dx, are long lasting and persist postnatally throughout the neonatal, infant and peripubertal periods. The established structural changes occurring early in life may be related to the reduced reproductive potential of females in adulthood.

Normal development of reproductive organs is crucial for successful reproduction. In rats, early ovarian development occurs during the fetal period. It is a highly regulated process whose disruption can lead to adverse reproductive outcomes (Wear et al., 2016), such as a

smaller primordial follicle pool, incomplete development of follicles and disturbed sexual differentiation (Edson et al., 2009; Sarraj and Drummnon, 2012). Exposure to Dx during this vulnerable period was shown to decrease the proliferation rate of germinative cells, while increasing their apoptotic rate (Ristić et al., 2019). Disturbed balance between these physiological processes in the fetal ovary may lead to an imbalance in germinative cell proliferation and death, resulting in impaired oogenesis. As a consequence, the total number of germinative cells in the fetal ovary becomes reduced and the oogonial stock is compromised (Ristić et al., 2019). As the final number of oocytes potentially available for fertilization throughout the fertile lifespan is primarily determined during fetal development (Fyndlay et al., 2015), a lowered number of germinative cells in the fetal ovary after exposure to Dx could indicate reduced reproductive potential of the offspring. Decreased numbers of ovarian primordial, primary and secondary follicles in neonatal offspring, as we have reported previously (Ristić et al., 2008), and in infantile offspring, as demonstrated in this study, validate this assumption.

In 38-day-old offspring prenatally exposed to Dx, puberty onset was delayed. The vaginal opening did not occur up to day 38. In the ovaries, *corpora lutea* were not observed, indicating that there were no ovulations. Since puberty is defined as the period when the vaginal opening and first ovulation occur (Picut et al., 2015), absence of these signs of pubertal onset signaled that puberty had not yet started. The developmental process that leads to puberty in the female rat is based on an extraordinarily complex series of interrelated events. Body weight is a very important determinant of sexual maturation. It has been reported that the onset of puberty was delayed in both undernutrition-induced and dexamethasone-induced IUGR rodent models (Iwasa et al., 2010; Politch and Herrenkohl,

1984; Smith and Waddell, 2000). In prenatally undernourished female rats, the action of the hypothalamic kisspeptin system was suppressed. Kisspeptin, a Kiss1 gene product (Kotani et al., 2001; Ohtaki et al., 2001), stimulates gonadotropin releasing hormone (GnRH) release in rats and mice (Irwig et al., 2004; Matsui et al., 2004) and is a key factor in pubertal onset in humans, mice, rats and monkeys (de Roux et al., 2003; Navarro et al., 2004; Seminara et al., 2003; Shahab et al., 2005). Thus, decreased hypothalamic kisspeptin action contributes to retarded reproductive function development and delayed onset of puberty in prenatally undernourished female rats (Iwasa et al., 2010). Increased leptin resistance after rapid growth in female offspring might be involved in these alterations (Iwasa et al., 2010). Female offspring from a dexamethasone-administered mother showed a significant reduction in body mass from birth to the prepubertal period (Iwasa et al., 2011), which was confirmed in our experimental conditions. It is possible that a prenatal environment which contains excessive levels of glucocorticoids disturbs the development of body mass regulation systems and that these alterations persist throughout the neonatal and prepubertal periods (Iwasa et al., 2011). In this IUGR model, delayed onset of puberty might have occurred independently of the hypothalamic kisspeptin system, as the mRNA level of hypothalamic Kiss1 factor and serum concentration of leptin were not altered (Iwasa et al., 2011). Since puberty is the final stage of maturation of the hypothalamic-pituitary-gonadal (HPG) axis, culminating in an adult phenotype (Odell, 1992), a potential mechanism for delaying the onset of puberty in experimental conditions is *via* direct action of dexamethasone on its development (Smith and Waddell, 2000). Acting at all the three levels of the HPG axis, Dx leads to developmental programming of the reproductive system in female rats. Major development and migration of GnRH neurons in rats occur between days 18 and 19 of fetal development (Jennes, 1989).

Exposure to Dx during late gestational stages has been shown to decrease the number and disrupt the migration and distribution of GnRH neurons in fetal brain, which can be linked to delayed reproductive development and altered reproductive function and behavior in adulthood (Lim et al., 2014; Schwanzel-Fukuda et al., 1989; Wierman et al., 2011). Also, exposure to Dx during the most vulnerable period in pituitary development markedly reduced gonadotropic cell number, which partly contributed to diminished volume of the entire gland in rat fetuses (Ristić et al., 2014). Changes established during fetal development persist postnatally, throughout the neonatal, infant and peripubertal periods, accompanied by a decrease in the serum concentrations of FSH and LH (Ristić et al., 2016). Decreased total germ cell number in the fetal ovary and consequently decreased number of ovarian follicles in neonatal and infantile offspring after exposure to Dx (Ristić et al., 2008, 2019) confirm the programming effect of Dx at the level of the ovary.

Changes induced by Dx exposure during fetal development at all three levels of the HPG axis may contribute to impaired reproductive development and function during adulthood and thus may constitute a risk for female fertility. These results indicate that the reproductive system is an important target for developmental programming induced by Dx.

The use of dexamethasone during pregnancy is considered one of the best advances in antenatal and neonatal medicine in recent times, but new epidemiological and animal studies are needed for a comprehensive assessment of risks. Since the early life environment is critical to maintaining reproductive capacity in offspring, extrapolation of animal data from our study to human and veterinary reproductive medicine may be of great importance.

Conflicts of interest

The authors declare no conflicts of interest.

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Table 1. Gestational length, litter size, female-to-male ratio, birth mass and postnatal mass in 16-day-old and 38-day-old control females (C16 and C38) and females prenatally exposed to dexamethasone (Dx16 and Dx38).

All results are given as mean ± SD, n=10,

	Gestational Length (day)	Litter size (No.)	Female:Male ratio	Birth Mass (g)	Postnatal Mass (g) Day 16	Day 38
C	22±0	11±2	1:1±0.1	6.5±0.4	30±3	109±6[b]
Dx	22±0	11±3	1:1±0.3	4.3±0.3[a]	22±2[a]	86±6[a, c]

[a]P<0.05, Dx *vs.* corresponding control; [b]P<0.05, C38 *vs.* C16, [c]P<0.05, Dx38 *vs.* Dx16.

Table 2. The volume of ovary (Vov), follicular parenchyma (Vfp), ovarian stroma (Vos) and the volume density of follicular parenchyma (V_{Vfp}) and ovarian stroma (V_{Vos}) in 16-day-old and 38-day-old control females (C16 and C38) and females prenatally exposed to dexamethasone (Dx16 and Dx38).

All results are given as mean ± SD, n=10

	Vov (mm^3)	Vfp (mm^3)	Vos (mm^3)	V_{Vfp} (%)	V_{Vos} (%)
C16	0.87±0.07	0.59±0.05	0.28±0.02	68	32
Dx16	0.61±0.03[a]	0.39±0.04 [a]	0.22±0.03	64	36
C38	4.20±0.08	3.30±0.96	0.90±0.05	78	22
Dx38	3.07±0.28 [a]	2.00±0.18 [a]	1.07±0.11	65	35

[a]P<0.05, Dx *vs.* corresponding control.

Table 3.Numerical density of follicles (N_V) in the ovaries of 16-day-old and 38-day-old control females (C16 and C38) and females prenatally exposed to dexamethasone (Dx16 and Dx38).

All results are given as mean ± SD, n=10

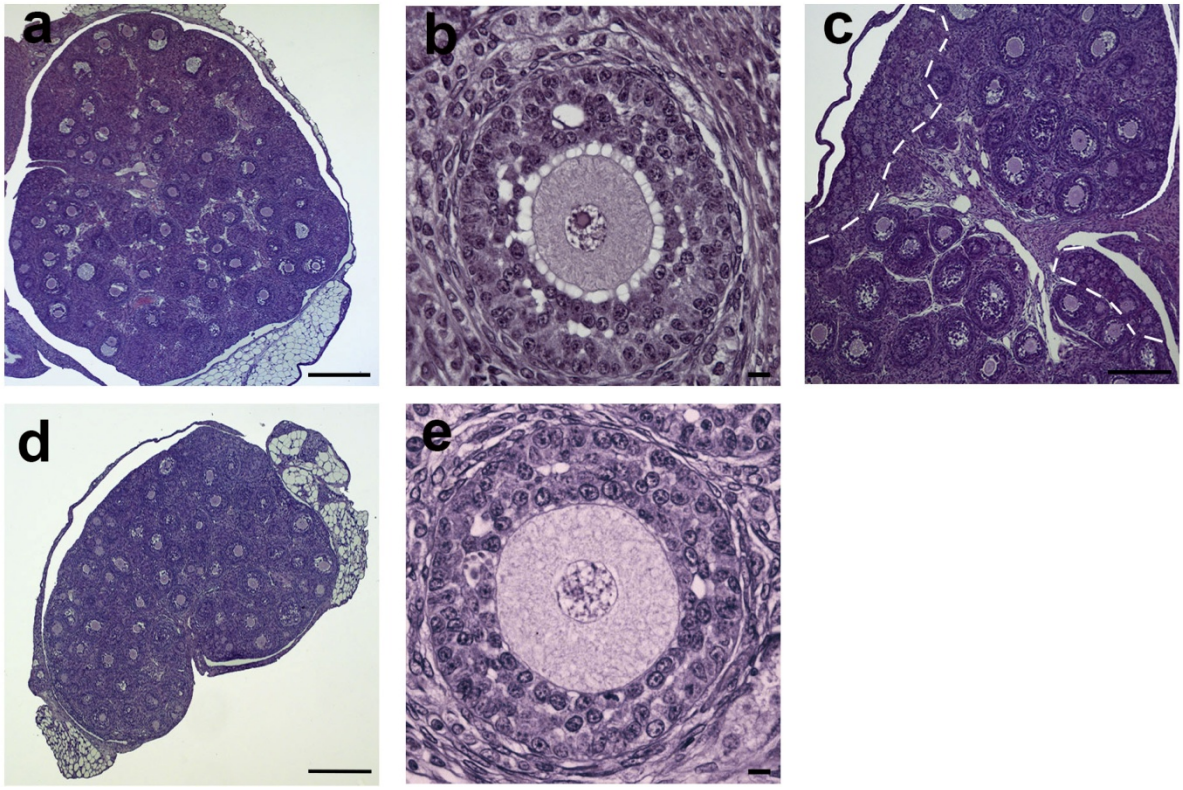
Follicle class	N_V (No/mm^3)			
	16. day		38. day	
	C	Dx	C	Dx
Primordial follicles	3714.8±253.8	3128.6±127.8[a]	731.4±28.4	708.2±56.9
Primary follicles	859.6±42.7	622.2±57.5 [a]	186.1±4.6	160.7±19.2
Ma	120.4±6.5	125.8±7.0	1.0±0.4	3.8±1.0
Mb	256.3±20.2	282.5±8.3 [a]	13.5±0.8	23.9±2.2
Mc	36.6±2.2	35.5±7.7 [a]	7.0±0.9	12.6±2.0
Md			4.7±1.0	9.5±1.7 [a]
Small healthy follicles (Σ)	1202.6±142.7	1065.0±59.5	212.4±4.3	210.6±16.2
Atretic primordial follicles	71.2±11.3	60.8±3.0	16.5±2.0	20.9±2.7
Small atretic follicles (Σ)	51.3±5.2	31.0±6.7 [a]	15.8±1.7	22.0±1.6
class I			1.9±0.6	7.7±2.7 [a]
class II			2.3±0.6	3.7±2.3
class III			0.7±0.4	1.23±0.50
class IV			0.4±0.2	2.6±0.8 [a]
class V			0.1±0.1	1.8±1.6 [a]
Large healthy follicles (Σ)			5.4±1.2	16.8±4.0 [a]
Large atretic follicles (Σ)			2.8±0.8	9.0±1.3 [a]

[a]P<0.05, Dx *vs.* corresponding control.

Ma - multilaminar follicles class A (<75 μm); Mb - multilaminar follicles class B (76 to 150 μm); Mc - multilaminar follicles class C (151 to 200 μm); Md - multilaminar follicles class D (201 to 274 μm).

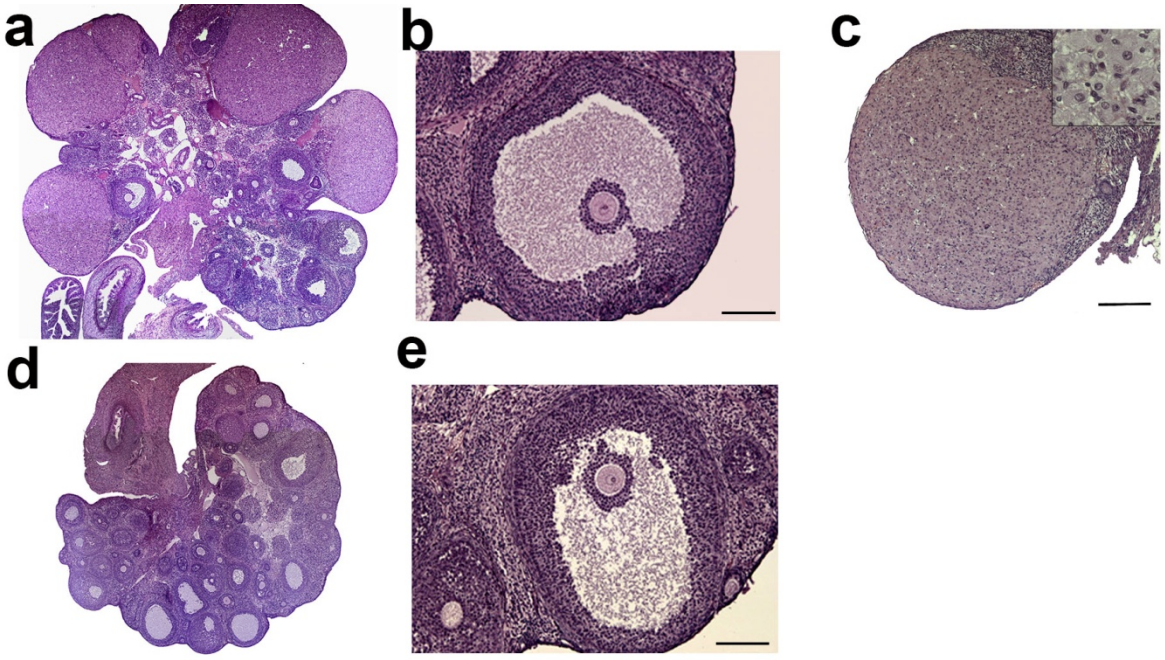
The large follicles class 1 (276 to 350 μm); class 2 (351 to 400 μm); class 3 (401 to 450 μm); class 4 (451 to 575 μm); class 5 (>575 μm).

Figure 1.



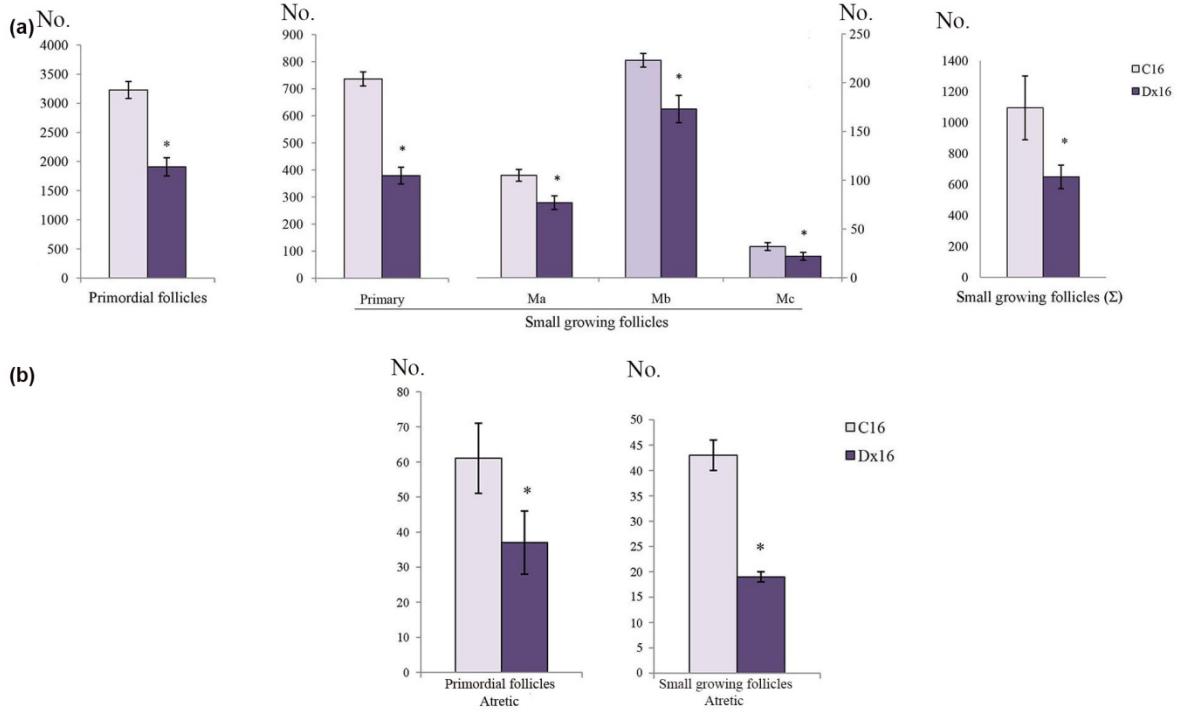
Representative micrographs of hematoxylin and eosin-stained ovarian tissue sections (3µm) of 16-day-old offspring; a) in control offspring ovaries had a definite histological organization, bar 200 µm; (b) multilaminar follicles Mc with *zona pellucida* in control offspring, bar 20 µm; (c) in control offspring primordial and primary follicles are limited to a thin peripheral rim (dashed line) and multilaminar follicles are located in the central core of the ovary - typical of immature ovaries, bar 100 µm; (d) in the group prenatally exposed to dexamethasone ovaries of smaller size were observed, bar 200 µm; (e) multilaminar follicles Mc in offspring prenatally exposed to dexamethasone, bar 20 µm;

Figure 2.



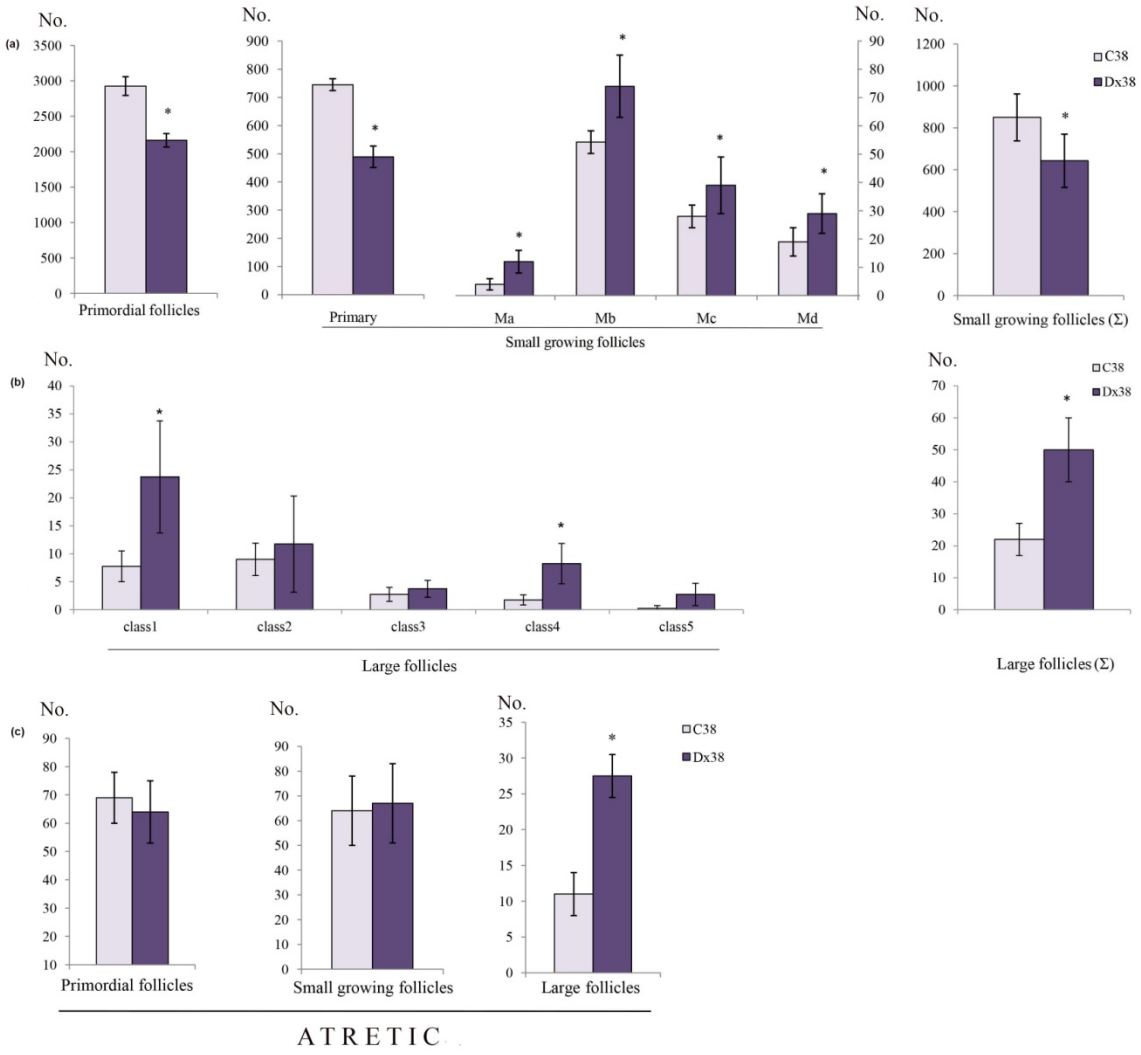
Representative micrographs of hematoxylin and eosin-stained ovarian tissue sections (3µm) of 38-day-old offspring; a) in control ovary the hallmark morphological feature was the appearance of preovulatory follicles and *corpora lutea* in the outer cortex of the ovary, bar 200 µm; (b) preovulatory follicles in control ovary, bar 100 µm; (c) *corpora lutea* in control ovary, bar 20 µm; (d) after prenatal exposure to dexamethasone, *corpora lutea* were not observed, bar 200 µm; (e) preovulatory follicles in offspring prenatally exposed to dexamethasone, bar 100 µm.

Figure 3.



The number (No.) of healthy (a) and atretic (b) follicles per ovary in 16-day-old control (C16) and female offspring prenatally exposed to dexamethasone (Dx16). All results are given as mean ± SD, n = 10, *P <0.05, Dx vs. corresponding control. Ma - multilaminar follicles class A (<75 μm); Mb - multilaminar follicles class B (76 to 150 μm); Mc - multilaminar follicles class C (151 to 200 μm).

Figure 4.

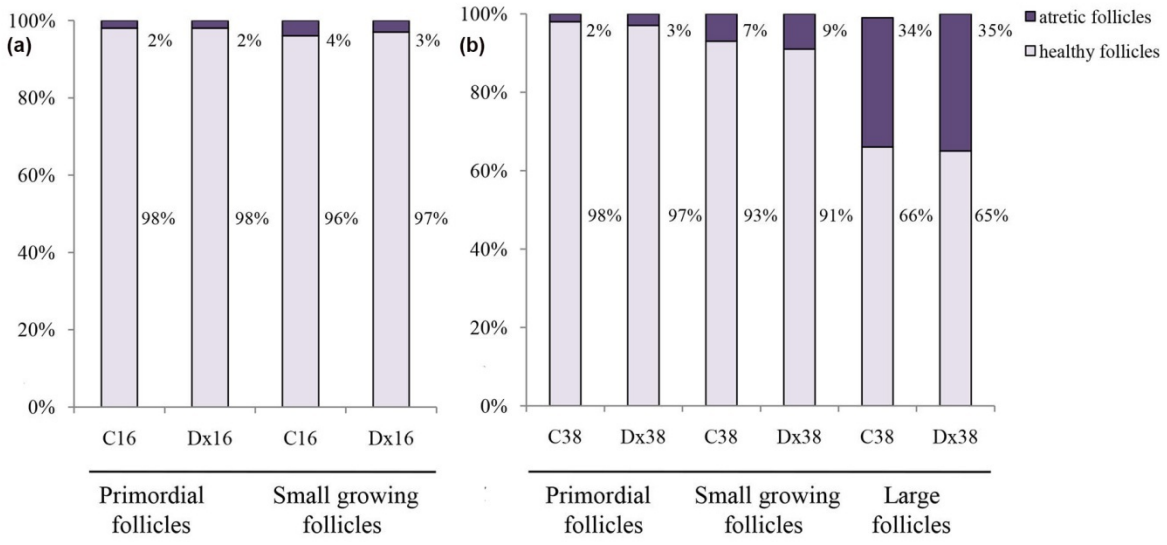


The number (No.) of healthy (a, b) and atretic (c) follicles per ovary in 38-day-old control (C38) and female offspring prenatally exposed to dexamethasone (Dx 38). All results are given as mean ± SD, n = 10, *P <0.05, Dx vs. corresponding control.

Ma - multilaminar follicles class A (<75 μm); Mb - multilaminar follicles class B (76 to 150 μm); Mc - multilaminar follicles class C (151 to 200 μm); Md - multilaminar follicles class D (201 to 274 μm).

The large follicles class 1 (276 to 350 µm); class 2 (351 to 400 µm); class 3 (401 to 450 µm); class 4 (451 to 575 µm); class 5 (>575 µm).

Figure 5.



The percentages of healthy and atretic follicles in the ovaries of 16-day-old and 38-day-old control (C16 and C38) and female offspring prenatally exposed to dexamethasone (Dx16 and Dx38).