DR. NATASA RISTIC (Orcid ID : 0000-0003-0994-1280)

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Adverse effect of dexamethasone on development of the fetal rat ovary

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Nataša **Ristić**[a], Nataša **Nestorović**[a], Milica **Manojlović-Stojanoski**[a], Svetlana **Trifunović**[a], Vladimir **Ajdžanović**[a], Branko **Filipović**[a], Lazo **Pendovski**[b], Verica **Milošević**[a]

[a]University of Belgrade, Institute for Biological Research "Siniša Stanković", Department of Cytology, Belgrade, Serbia

[b]Faculty of Veterinary Medicine, Skopje, FYR of Macedonia

***Corresponding author**

Nataša Ristić, Ph.D.

Institute for Biological Research "Siniša Stanković"

142 Despot Stefan Blvd.

11060 Belgrade

Serbia

Tel: 381-11-2078323

Fax: 381-11-2761433

E-mail: negicn@ibiss.bg.ac.rs

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ABSTRACT

Dexamethasone (Dx) is often used in obstetric practice to promote fetal lung maturation and to prevent respiratory distress syndrome when the risk of preterm delivery persists. This therapy enables survival of the newborn, but also is associated with deleterious effects on the offspring, such as reproductive disorders. The aim of this study was to determine specifically whether prenatal exposure to Dx disturbs the physiological balance between proliferation and apoptosis of germinative cells (GC) in the ovary of 19 and 21 day old fetuses and thus induces developmental programing of the female reproductive system. Pregnant Wistar rats (n = 10/group), separated into control (vehicle) and Dx-treated (0.5 mg/kg body mass) groups, received injections on gestational days 16, 17 and 18. Exposure to Dx lowered the volume of the fetal ovary by 30% ($p<0.05$) in 21 day old fetuses, as well as the total number of GC in the ovary by 21% ($p<0.05$). When compared to the controls, in Dx-exposed fetuses the total number of PCNA positive GC was 27% lower at 19 days and 71% lower at 21 day old ($p<0.05$), while total numbers of caspase-3 positive GC were 2.3 fold and 34% higher respectively, ($p<0.05$). Our results demonstrate that prenatal exposure to Dx diminished proliferation but increased the rate of germinative cell apoptosis, with consequently reduced total germinative cell number and ovary volume. Impairment of fetal oogenesis and fewer GC in the fetal ovary compromise the oogonial stock and thus may constitute a risk for female fertility.

INTRODUCTION

Ovary development begins early during fetal life in mammals. This process involves segregation of the primordial germ cells (PGC) from the somatic lineages, migration from the yolk sac and colonization of the genital ridges, where they continue extensive mitotic proliferation reaching a peak value at 18.5 days post conception in rats. Subsequently, mitotic activity declines, most oogonia enter meiosis to produce a pool of oocytes arrested at the diplotene stage of the first meiotic division, while apoptotic activity resumes [1]. Normal ovarian development provides a balance between proliferation and apoptosis of germinative cells, which is the major phenomenon in establishing the maximal reproductive potential of adult females [2].

Exposure to drugs during intrauterine life may have an influence on normal progression of development of the fetal ovary and the future of adult fertility [2]. Pathological conditions during pregnancy involve assessment of the benefits and risks of drug usage, which is frequently indispensable, as in the case of glucocorticoids. Glucocorticoids have a strong influence on growth and final maturation of fetal organ systems [3]. One of the most important events during intrauterine life, which represents a critical developmental trigger, is the increase of endogenous fetal glucocorticoids [4]. Endogenous glucocorticoids are

essential for normal maturation of the fetal brain and lung as well as developmental switching in the pituitary, thyroid gland and kidney [5, 6]. When the risk of preterm delivery persists, a pregnant woman needs to be treated with exogenous glucocorticoids to promote differentiation and maturation of fetal organ systems, to mimic normal intrauterine conditions [7, 8]. Moreover, glucocorticoid application is routine in the prevention and management of conditions that threaten maternal and fetal health, such as maternal asthma [9] and autoimmune diseases [10]. Dexamethasone (Dx) is considered to be the glucocorticoid of choice for antenatal therapy [2]. In addition to its beneficial effects on growth and differentiation, the risks of such therapy are well known. Prenatal exposure to Dx is associated with several systemic long-term deleterious effects on the offspring, such as cardiovascular, metabolic and neuroendocrine disorders [11, 12, 13]. This concept is known as developmental programming, and implies linkage between adverse environmental signals during prenatal development with a greater incidence of pathophysiological conditions in postnatal life [14]. Dx is used in numerous experimental protocols to induce developmental programming [11, 15, 16]. Lately, interest in the field of developmental programming has been focused on reproductive dysfunction in adult females and males.

Our recent results, obtained by exploiting a rat model, indicate that prenatal exposure to Dx leads to novel developmental programming of the reproductive system at the level of the pituitary gland and ovary. Namely, 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 [17]. Changes established during fetal development are long lasting and persist postnatally throughout neonatal, infant and peripubertal periods [18]. Prenatal exposure to Dx also decreased the number of ovarian primordial, primary and secondary follicles in neonatal offspring [19], which indicates reduced reproductive potential for each female. The aim of the present study was to elucidate if this situation is the consequence of a disturbed balance between proliferation and loss by apoptosis of germinative cells in the fetal ovary after exposure to Dx.

Herein, we have investigated the expression of markers of cell proliferation (proliferating cell nuclear antigen - PCNA) and apoptosis (caspase-3) in the developing rat ovary following exposure to Dx. Estimation of the total number of proliferating germinative

cells and germinative cells with signs of apoptosis, using design-based stereology, may provide evidence about whether prenatal exposure to Dx impairs development of the rat ovary, and thus influences developmental programing of the female reproductive system.

MATERIALS AND METHODS

Animals and Experimental Groups

Adult female Wistar rats (average body mass of 200–250 g; bred at the Institute for Biological Research "Siniša Stanković", Belgrade, Serbia) were maintained under controlled conditions (12 h:12 h light-dark cycle at 22°C) with food and water available *ad libitum*. Two nulliparous females were mated *per* fertile male. Detection of sperm in a vaginal smear was considered as gestational day (GD) 1. The gravid females were randomized into a control and an experimental group, each consisting of 20 animals. During the key period of rat ovarian development on days 16, 17 and 18 of pregnancy, each experimental dam received 0.5 mg Dx (Dx phosphate dissolved in 0.9% saline (Krka FARMA d.o.o., Belgrade, Serbia)/kg body mass (BM) in 0.4 ml *s.c.*. This dosing regimen was designed to fall within the range of clinical exposure in women (0.1–0.5 mg/kg) [20]. The control gravid females received the same volume of saline. On days 19 or 21 of gestation pregnant dams were anaesthetized with diethylether (Merck, Kenilworth, USA) and the fetuses were removed from the uterus. Their sex was determined by external examination (anogenital distance) and the males were discarded. One female fetus was randomly chosen from each of the 10 control and 10 litters of Dx-treated dams, at 19 and 21 days old. Randomization obviated any potential litter bias. The ovaries were excised and fixed with 4% paraformaldehyde for 24 h. After embedding in Histowax (Histolab Product AB, Göteborg, Sweden), each tissue block was serially sectioned, at 2-μm thickness, on a rotary microtome (RM 2125RT Leica Microsystems, Wetzlar, Germany). All animal procedures complied with the European Communities Council Directive (86/609/EEC) and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković", University of Belgrade (No. 2-12/13).

Immunohistochemical Analyses

Expression of proliferating cell nuclear antigen (PCNA) and caspase-3 in the fetal ovaries was investigated using immunohistochemical methods [18]. After deparaffinization and rehydratation, two-micrometer-thick serial sections were pretreated with citrate buffer for antigen retrieval according to standard protocols. For inactivation of endogenous peroxidase, 0.3% hydrogen peroxidase in methanol was used, followed by incubation with normal donkey serum (Abcam, Cambridge, UK, 1:10) for 45 min to reduce nonspecific background staining. The samples were then incubated overnight with the primary antibodies: anti-PCNA (mouse monoclonal, Abcam, Cambridge, UK, 1:10000) or anti-caspase-3 goat anti-human,

Santa Cruz Biotechnology, USA, 1:200). The HRP conjugated secondary antibodies were donkey anti-mouse and donkey anti-goat, (Abcam, Cambridge, UK; 1:100). Staining was visualized with DAB (3,3'-diaminobenzidine tetrahydrochloride; Dako A/S Glostrup, Denmark). As a control for nonspecific binding of the secondary antibody, parallel sections were incubated without the primary antibody.

Stereological analysis

All stereological analyses were performed using a workstation comprising a microscope (Olympus BX-51) equipped with a microcator (Heidenhain MT1201), a motorized stage (Prior) and a CCD video camera (PixeLink). The main objectives were a planachromatic 4×dry lens (numerical aperture 0.13), a 40×dry lens (numerical aperture 0.17) and a 100×oil lens (numerical aperture 1.30). Control of stage movements and interactive test grids and unbiased dissector frames were provided by the newCAST software package.

Volume of fetal ovary

Fetal ovary volume was estimated using Cavalieri's principle [21]. Sampling of sections (2 μm thick) was systematically uniform from the random start. Every 10th section from the whole fetal ovary was analyzed (the same sections were used in the subsequent estimation of PCNA and caspase-3 positive GC numbers by the physical dissector method). Fetal ovary volume was determined by multiplying the sum of the areas by the interval between the sections according to 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 within the grid (1671.024μm^2); BA (block advance) is the mean distance between two consecutively studied sections, *i.e.* real section thickness 2 μm × 10; n is the number of sections studied for each ovary, and ΣPi is the sum of points hitting a given target.

Quantification of GC, PCNA and caspase-3 positive germinative cell numbers

The total numbers of GC and immunostained GC in ovaries from control and Dx-treated dams were determined using a fractionator/physical disector design with two levels of sampling [19].

Sampling was systematically uniform from a random start [21, 22]. Sections defined as section pairs were first captured into a super image. After defining and linking, the pairs were aligned by translation and rotation using the montage option in the new-CAST software. A ×100 oil immersion objective was used for cell counting. One section in the pair was labeled the reference section and the other the 'look-up' section. Subsequently, they were analyzed in both directions with the reference section also becoming the look-up section. This doubles the first sampling fraction from 1/10th to 1/5th (sampling fraction 1(f1) =1/5 = 0.2). For estimation of the total number of GC, PCNA and caspase-3 positive GC in fetal ovaries, an unbiased counting frame measuring 40 × 40 μm (1,600μm^2) was used to ensure 150–200 cells *per* animal. After defining tissue boundaries, meander sampling was set to analyze 70% of the tissue. Therefore, the sampling fraction 2 was f2 = 0.7. The fields of vision were randomly selected and the percentage of tissue analyzed was controlled by the software. Additionally, new-CAST software facilitated matching of fields between the reference and the look-up section. When the field of vision was selected at the reference section, the matching position at the consecutive look-up section was scanned and a matching field of vision was generated from four images. GC, PCNA and caspase-3 positive GC were counted if their nuclei appeared within the unbiased counting frame applied to the reference section; they were not intersected by exclusion boundaries [23] and did not appear in the look-up section. Raw counts (Q^-) of GC, PCNA and caspase-3 positive GC numbers were multiplied by the reciprocals of the sampling fractions to estimate the total numbers of GC, PCNA and caspase-3 positive GC *per* fetal ovary.

GC/PCNA/caspase-3 positive GC (n) = Q^- (GC/PCNA/caspase-3 positive GC) × (1/f1) × (1/f2).

Cytological markers used for GC identification include typical diagnostic parameters such as cell size and shape (proportion of dimensions) and nucleus size, shape and position.

Statistical Analysis

All results are expressed as mean values for 10 animals *per* group (± SD). Data were evaluated for normality of distribution using the Kolmogorov-Smirnov test followed by 2-way analysis of variance (ANOVA), with the age of female fetuses (19 and 21 days) and the 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

Fetal body mass

As shown by 2-way ANOVA, there were significant effects of treatment (C/Dx, $F = 211.131$; $p<0.001$), age (19/21, $F = 1765.190$; $p<0.001$) and treatment × age (C/Dx*19/21, $F = 73.837$; $p<0.001$) on fetal body mass. Subsequent *post hoc* analysis revealed that exposure to Dx during the last week of fetal development reduced BM of 19 and 21 day old female fetuses by 20% ($p<0.05$) and 29% ($p<0.05$), respectively. From fetal day 19 to 21, BM of control female rat fetuses increased 2.7 fold ($p<0.05$), while in the same period that of Dx exposed fetuses increased 2.4 fold ($p<0.05$, Table 1). Exposure to Dx did not affect litter size or ratio of male to female pups (Table 1).

Fetal ovary and germinative cells

Histological and stereological analysis

In 19 and 21 day old fetuses, definitive histological organization of the ovaries was not established. Germinative cells and somatic cells were arranged in clusters, separated from each other by connective tissue cells and blood vessels. The medulla of the ovary was filled with dense clusters of oocytes, while somatic cells were rare. In contrast, oocytes were scarce at the cortex, whereas epithelial cells were abundant. The germ cells were identified from their large size, round and lightly staining nucleus. The widespread distribution of PCNA indicated active GC proliferation in the fetal ovary. Apoptotic germ cells were identified as large cells that stained for caspase-3 (Figure 1 and 2). Exposure to Dx during this crucial period in ovary development did not affect the histological features of the fetal ovary.

Two way ANOVA showed that treatment, age, and treatment × age significantly influenced ovary volume, total number of GC and total number of PCNA and caspase-3 positive GC in the ovaries of 19 and 21 day old fetuses (Table 2). Applying Cavalieri's principle, we calculated that the fetal ovary volume in 19 day old control fetuses was $4 \times 10^{-2} \pm 3 \times 10^{-4}$ mm^3. *Post hoc* analysis revealed that Dx exposure did not affect ovary volume in 19 day old fetuses, while in 21 day old fetuses it was reduced by 30% ($p<0.05$) when compared to controls. As a result of near term physiological processes control fetal ovaries increased in volume by 49% between fetal days 19 and 21 ($p<0.05$, Fig. 3).

Using the fractionator/physical disector design we estimated that the total number of GC *per* ovary of 19 day old control fetuses was 3892 ± 63 cells, among which there were 2530 ± 146 PCNA positive GC and 428 ± 12 casp-3 positive GC. The *post hoc* test showed that Dx exposure did not affect the total number of GC in 19 day old fetal ovaries, while the total number of PCNA positive GC *per* ovary was 27% lower ($p<0.05$), and the total number

of casp-3 positive GC had increased 2.3 fold ($p<0.05$) when compared to controls (Table 3). In 21 day old fetuses exposure to Dx had reduced the total number of GC by 21% ($p<0.05$), as well as the total number of PCNA positive GC by 71% ($p<0.05$). Administration of Dx raised the total number of casp-3 positive GC by 34% ($p<0.05$) in comparison to control values (Table 3). Thus, from fetal day 19 to 21 the total numbers of GC, PCNA and casp-3 positive GC were significantly diminished by 57% ($p<0.05$), 84% ($p<0.05$) and 50% ($p<0.05$), respectively (Table 3).

In the ovaries of control 19 day old fetuses the relative numbers of PCNA and caspasa-3 positive/total GC (labeling index) were 65% and 11%, respectively. After Dx exposure the percentage of PCNA positive/total GC declined to 48%, while that of casp-3 positive /total GC increased to 25% (Figure 4). Between fetal day 19 and 21 relative numbers of PCNA and casp-3 positive/total GC cells reach to 25% and 13%, respectively, in control fetuses. Dx exposure enhanced the drop in proportion of PCNA positive/total GC cells in 21 day old fetuses to 9%, but increased that of casp-3 positive/total GC cells to 22% (Figure 4).

DISCUSSION

This work represents an experimental demonstration *in vivo* of the potential harmful effect of Dx exposure on the rat fetal ovary. Using a modern stereological approach, we demonstrated that Dx exposure decreased the volume of the fetal ovary and the proliferation rate of germinative cells, while it increased their apoptotic rate. Accordingly, Dx lowered the number of germinative cells in the rat fetal ovary, which can be linked with reduced reproductive potential in adulthood.

The fetal period is critical for ovary development and future adult fertility, since the final number of oocytes accessible for reproduction of the next generation is mostly defined at that time [2]. An important target of developmental programming is the reproductive system. If adverse impacts are experienced at a critical timeframe during fetal life, normal dynamics of the developmental process in the ovary may be changed, with long-term effects on ovary structure and function.

Under our experimental conditions overexposure to glucocorticoids caused intrauterine growth retardation, followed by reduced body mass of 21 day old female fetuses. Body mass is a crucial determinant of fetal growth. Low birth mass, as a marker of adverse environmental signals during prenatal development, is associated with increased frequency of pathophysiological conditions in adulthood [14]. An excessive level of glucocorticoids during

fetal life disturbs development of body weight regulatory systems and such alterations continue until prepuberty [16]. Low birth mass and durable reduced body mass in female offspring prenatally exposed to glucocorticoids may be related to delayed onset of puberty [24, 25], indicating that body mass is an essential component of reproductive maturation.

Ovarian development during fetal life is a complex phenomenon determined by successive changes in cell kinetics. Progressive growth of germinative cells, as differentiation proceeds, and enlarged proliferation of follicular cells [26] contribute to the increased ovarian volume in the same period. Periods of rapid GC proliferation are followed by stages of stability and greater rates of apoptosis. High mitotic activity of oogonia was detected in our experimental conditions using the PCNA immunomarker. The widespread distribution of PCNA indicated active cell proliferation in the ovary of 19 day old fetuses. However, the decline in percentage of PCNA positive/total GC between fetal days 19 and 21 correlated with transition from mitosis to meiosis, *i.e.* from oogonia to primary oocytes.

Besides GC proliferation, an essential component of ovarian development is apoptosis. Prenatal loss of oocytes reflects selection at the cellular level and provides regulation of the size and quality of the germinative cell pool that will define reproductive potential of the individual [27]. Apoptosis occurs at all stages of oogenesis, with three main waves of loss described until primordial follicle formation [28]. The first wave appears at the oogonial stage and affects up to 3.6% of GC. We documented the second wave of loss in 19 day old fetuses, when oogonia cease mitosis and enter meiosis and the apoptotic rate of GC reaches 11%. Degeneration of germinative cells continues up to 48 hours after birth, when a third wave of degeneration occurs just before the formation of primordial follicles. In that period, the oocytes are in late pachiten [28]. The balance between apoptosis and proliferation during fetal ovarian development is the crucial phenomenon for establishing maximal reproductive potential in adulthood, since the overall number of oocytes is defined during the fetal period.

Dexamethasone is often used in obstetric practice to promote fetal lung maturation and to prevent respiratory distress syndrome, when the risk of preterm delivery persists [29]. Several epidemiological studies using Dx as prenatal treatment have shown that it does not have a teratogenic effect when applied according to the usual protocol [30, 31]. However, the effect of Dx on the fetal ovary and long-term fertility of women exposed *in utero* is unknown, as Dx treatment was initiated in the early 1980s. Therefore, animal studies can greatly illuminate this very important aspect of female reproduction.

Exposure to Dx during the most vulnerable period in ovary development decreased the relative number of PCNA positive germinative cells, while increasing that of caspase-3 positive germinative cells. Dx lowered the expression levels of v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (*KIT)* [2]. *KIT* is crucial for germinative cell survival [32] and reduction in *KIT* expression contributes to Dx-induced germinative cell apoptosis in the human female fetal germline [2]. Accelerated apoptosis of germinative cells may lead to an imbalance of cell proliferation and death, resulting in impaired oogenesis. Impairment of fetal oogenesis through an apoptotic mechanism after Dx exposure reduces the total number of GC in the fetal ovary, compromises the oogonial stock and thus may constitute a risk for female fertility.

Our data indicate that prenatal exposure to Dx reduces rat ovary volume and number of germinative cells in the fetal ovary. In accordance with the central dogma of mammalian reproductive biology, the number of oocytes at birth represents almost the complete reproductive potential of an individual. Fewer oocytes in the fetal rat ovary due to Dx exposure leads to a significant decrease of the overall follicle number in neonatal rat ovaries, as we reported earlier [19]. These data document the programming effect of Dx at the level of the ovary. Clear programming effects of Dx were also detected in the hypothalamus and pituitary gland [33, 17, 18]. Dx induced changes at all three levels of the hypothalamo-pituitary-gonadal axis may contribute seriously to impaired reproductive function during adulthood. It is clear that treating pregnant women with drugs, especially with those of hormonal nature which cross the placental barrier and may affect fetal development, requires a cautious approach. The short medical history of Dx use and lack of wide-ranging epidemiological studies elaborating the potential harmful effects of Dx treatment during pregnancy on the reproductive system of female offspring indicate that our findings have important implications for humans.

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Table 1. Litter size, female:male ratio, body mass in 19 and 21 day old control fetuses (C) and fetuses exposed to dexamethasone (Dx).

	Litter size	Female:male ratio	Fetal body mass (g) 19 day	21 day
C	12±3	1:1±0.5	1.5±0.1	4.1±0.5[b]
Dx	12±2	1:1±0.3	1.2±0.2[a]	2.9±0.3[a,c]

All results are given as mean ± SD, n=10, [a]p<0.05, Dx *vs.* corresponding control; [b]p<0.05, C21 *vs.* C19, [c]p<0.05, Dx21 *vs.* Dx19 (Bonferroni test)

Table 2. Results of the two way ANOVA

	Df	Ovary volume	GC	PCNA+GC	Casp-3+GC
treatment	1	F=125.6 p<0.001	F=134.6 p<0.001	F=405.3 p<0.001	F=2640.8 p<0.001
age	1	F=173.2 p<0.001	F=22409.8 p<0.001	F=6356.8 P<0.001	F=5547.4 p<0.001
treatment×age	1	F=26.6 p<0.001	F=95.9 p<0.001	F=61.6 P<0.001	F=1510.8 p<0.001

Table 3. Total number of germinative cells, PCNA positive and caspase-3 positive germinative cells in ovary of 19 and 21 day old control fetuses (C) and fetuses exposed to dexamethasone (Dx).

	Σ GC	Σ PCNA +	Σ casp-3 +
C19	3892±63	2530±146	428±12
Dx19	3863±48	1854±35[a]	966±24[a]
C21	1661±39[b]	415±24[b]	216±23[b]
Dx21	1320±49[a]	119±13[a]	290±12[a]

All results are given as mean ± SD, n=10, [a]$p<0.05$, Dx *vs.* corresponding control; [b]$p<0.05$, C21 *vs.* C19 (Bonferroni test)

Figure 1.

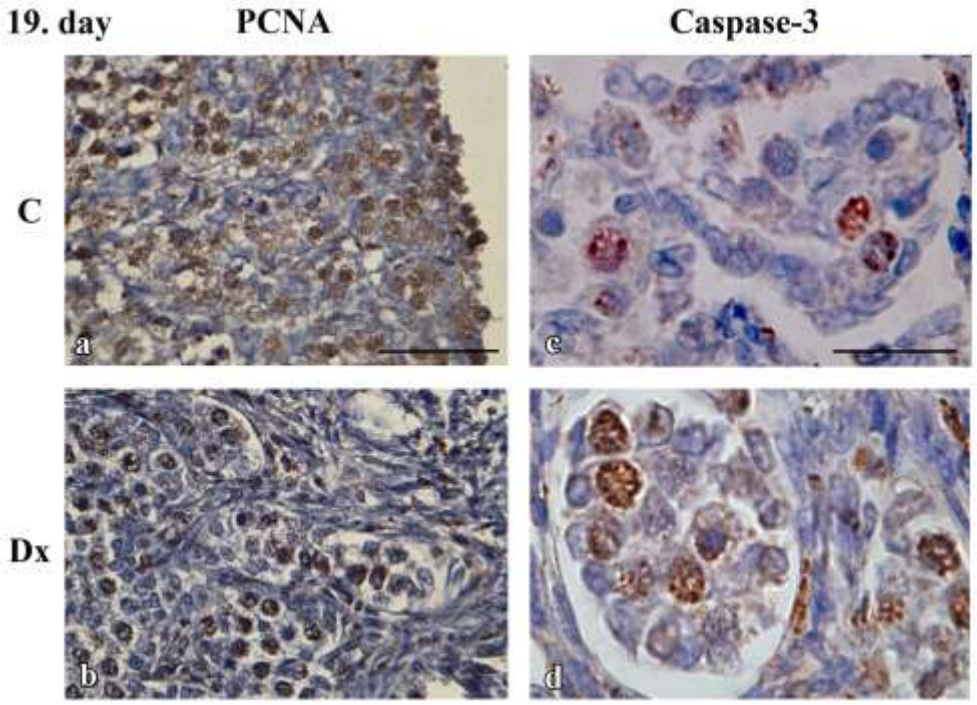

Figure 1. Immunohistochemical localization of PCNA and caspase-3 positive germinative cells in ovary of 19 day old control fetuses (C) and fetuses exposed to dexamethasone (Dx); scale bar- 55μm (a, b) and 20 μm (c, d)

Figure 2.

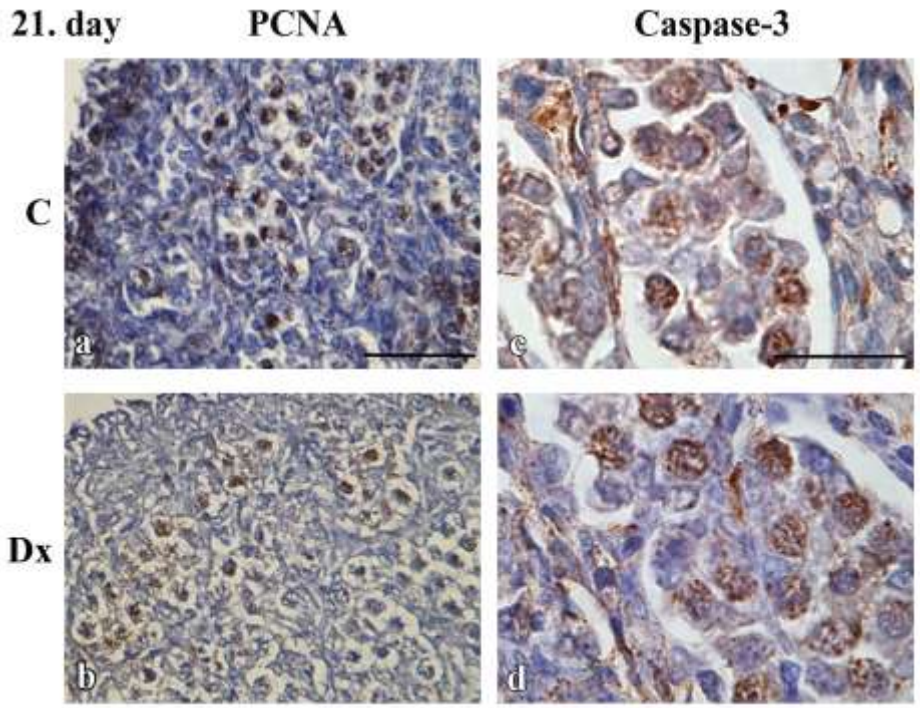

Figure 2. Immunohistochemical localization of PCNA and caspase-3 positive germinative cells in ovary of 21 day old control fetuses (C) and fetuses exposed to dexamethasone (Dx); scale bar-55μm (a, b) and 20 μm (c, d)

Figure 3.

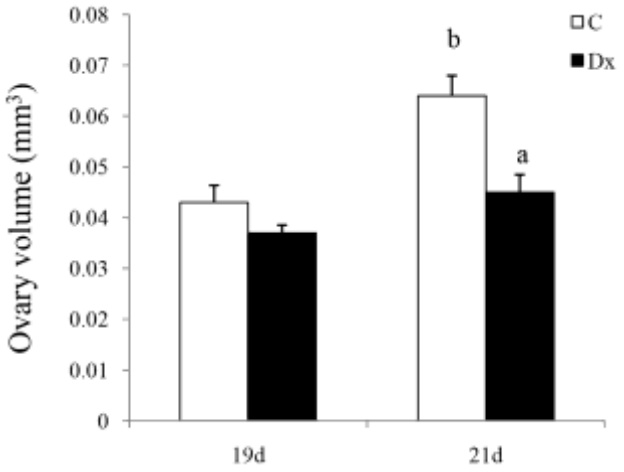

Figure 3. Volume of fetal ovary in 19 and 21day old control fetuses (C) and fetuses exposed to dexamethasone (Dx). All results are given as mean ± SD, n=10, [a]p<0.05, Dx *vs.* corresponding control, [b]p<0.05, C21 *vs.* C19 (Bonferroni test)

Figure 4.

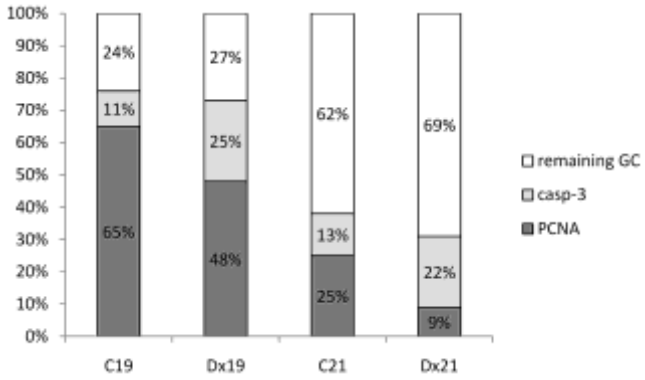

Figure 4: Graphical presentation of PCNA and caspase-3 labeling index in germ cells, and remaining germinative cells, in the developing ovary of 19 and 21 day old control fetuses (C) and fetuses exposed to dexamethasone (Dx)