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The effects of prenatal dexamethasone exposure and fructose challenge on pituitary-adrenocortical activity and anxiety-like behavior in female offspring

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Abstract

Prenatal glucocorticoid overexposure could largely influence pituitary-adrenal activity and anxiety-like behavior in offspring. Our aim was to study the possible potentiating effect of moderate dose of fructose – common ingredient of today's diet – on prenatal glucocorticoid treatment-induced hypothalamo-pituitary-adrenal (HPA) axis changes. Pregnant female rats were treated with multiple dexamethasone (Dx) doses (3x0.5mg/kg/b.m. Dx; 16th-18th gestational day). Half of female offspring from control and Dx treated dams were supplemented with 10% fructose solution, from weaning till adulthood. Immunohistochemistry, unbiased stereological evaluation and hormonal analysis are used to provide the morpho-functional state of pituitary and adrenal gland. Anxiety-like behavior was assessed using the light/dark box test and the elevated plus maze test. Prenatally Dx exposed females, with or without fructose consumption, had markedly reduced adrenocortical volume ($p<0.05$) comparing to controls. Increased basal plasma ACTH level in these females ($p<0.05$) maintained corticosterone concentration at control level produced by smaller adrenal glands. In parallel, anxiety-like behavior was shown by both tests used. In conclusion, prenatal Dx exposure cause negative psychophysiological outcome reflected in increased HPA axis activity and anxiety behavior in female offspring, while moderately increased fructose consumption failed to evoke any alteration or to potentiate effects of prenatal Dx exposure.

1. Introduction

In obstetric practice synthetic glucocorticoid usage essentially reflected in decreasing mortality and morbidity rates of newborns by influencing organ growth and maturation patterns, particularly the fetal lungs and heart (Liggins and Howie, 1972; Roberts and Dalziel, 2006; Agnew et al., 2018). On the other hand, human and animal studies have shown that *in utero* glucocorticoid overexposure signalized adverse environmental conditions for the developing fetus that result in growth restriction, low birth weight and numerous alterations of the neuroendocrine system, all of which influences susceptibility to later diseases (Barker, 2002; Rabadán-Diehl and Nathanielsz, 2013). Fetal programming, as a sum of irreversible metabolic, endocrine and physiological responses, represents adaptation to environmental adversity in order to enhance ability to survive after birth (Cottrell and Seckl, 2009). Long-term adverse side effect, could be additionally potentiated depending on the postnatal conditions (Cottrell and Seckl, 2009; Harris and Seckl, 2011).

Fructose is readily absorbed and rapidly metabolized by liver where stimulates lipogenesis, and dietary fructose does not stimulate insulin release (Basciano et al., 2005). A marked increase in fructose consumption caused by added sweeteners, such as sucrose and high-fructose corn syrup, is a characteristic of modern diets, which lead to increased energy intake (Tappy and Lê, 2010). As a result, the prevalence of metabolic disorders continues to rise dramatically in adults, and especially in adolescents (Nousen et al., 2013; Harrell et al., 2015). Obesity, impaired glucose homeostasis, insulin resistance and other forms of metabolic irregularity are risk factors highly associated with mental health and mood disorders, including anxiety (Garcia-Rizo et al., 2015; Gancheva et al., 2017). Disregulation of the hypothalamo-pituitary-adrenal (HPA) axis, especially when it comes to the circulating or tissue glucocorticoid impacts, is certainly part of the underlying mechanism that links metabolic and mental disorders (Culpepper, 2009; Nousen et al., 2013).

Anxiety-like behavior, as a complex physiological disorder, is characterized by numerous neuroendocrine, neurotransmitter-mediated and neuroanatomical disturbances, especially in the limbic system (Martin et al., 2009). Hippocampus, amygdala and hypothalamus, structures which, among others, comprise the limbic system, are involved in the control of fear, aggression, emotions, memory or learning (Raglan et al., 2017). The presence of mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) determines these regions to be especially sensitive to glucocorticoid action, realizable through both of the receptors, providing that behavioral outcomes are under significant glucocorticoid control (Fuller and

Young, 2009; Huang, 2011). Beside glucocorticoids, an increased energy intake influences the limbic system, including dopaminergic, serotonergic and hypothalamic neuropeptide Y signaling, that also reflects on behavioral responses (Nousen et al., 2013).

Sex specific differences of the HPA axis activity are shown both under basal and stress conditions, as well as after prenatal exposure to glucocorticoids, where females exhibit significantly increased stress reactivity (Alexander et al., 2012, Hiroi et al., 2016). Females also have a more pronounced response to deleterious effects of fructose overconsumption, in terms of the increase of adiposity and triglyceride plasma concentrations (Kovačević et al., 2014). However, males represent the main subject of experiments in the field of hormonal and metabolic disturbances (Goel et al., 2014). In general, women are more susceptible than men to developing morbid obesity and metabolic syndrome (Tonstad et al., 2007) that, together with high comorbidity between metabolic disturbances and mental health disorders, including behavioral disorders (Nousen et al., 2013; Gancheva et al., 2017) underline the need for gender-specific studies.

Antenatal glucocorticoid treatment, with or without postnatal metabolic overload, increases susceptibility to later stress-related diseases, including metabolic and psychiatric ones. Therefore, the aim was to investigate possible potentiating effect of moderate dose of fructose challenge on the latter HPA axis related, hormonal and behavioral responses induced by prenatal glucocorticoid Dx exposure. The present study has in its focus adrenal gland structure and functionality, in parallel with ACTH circulating level together with assessment of anxiety-like behavior, in 90 days old female offspring.

2. Material and methods

2. 1. Animals and experimental design

Female Wistar rats, 2-2.5-month-old, were mated in the laboratory of the Institute for Biological Research, Belgrade, Serbia, during the night, under standard conditions (23±2°C, 60-70% relative humidity, 12 h light/dark intervals (at 7am the lights switched on), food and water provided *ad libitum*). The morning when sperm positive vaginal smear was detected was indicative of pregnancy and considered as day 0 of gestation. Gravid females were randomized into two groups, each consisting of 12 dams. Experimental group received 0.5 mg/kg/b.m./day dexamethasone (Dx) on gestational days 16, 17 and 18, while control females were treated with the same quantity of saline. This dosing paradigm has been previously used and correspond to the clinical human exposure (Roberts and Dalziel, 2006; Carbone et al.,

2012). No differences in litter size were observed, and each consisted of 10-14 pups. Three weeks after parturition, immediately after weaning, females were randomly chosen from each of 12 control litters and from 12 litters of Dx-treated mothers, and further divided into two more groups. One group was fed with commercial standard chow and drinking water was available *ad libitum*, and other group had *ad libitum* access to the same chow and 10% (w/v) fructose solution instead of drinking water, till the third month of life. Thus, four groups were formed: control female offspring (C), female offspring supplemented with fructose in drinking water (F), female offspring from Dx treated dams (Dx) and female offspring from Dx treated dams supplemented with fructose in drinking water (Dx-F). Each group consisted of six females. All females were sacrificed by rapid decapitation in adult period of life *i.e.* female offspring were three months old.

The all animal procedures were in compliance with Directive 2010/63/EU on the protection of animals for experimental and other scientific purposes, complying ethical standards of the Low animal welfare No 41/2009 as national guides on the care and use of laboratory animals, and were approved by the Ethical Committee for Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković", University of Belgrade.

2. 2. Tissue preparation, histochemistry and immunohistochemistry

The adrenal glands were excised, weighed and fixed in 4% paraformaldehyde solution for 24h. After dehydration in a series of increasing ethanol gradient, the adrenals were enlightened in xylene, embedded in paraplast (Histolab Product AB, Göteborg, Sweden) and serially sectioned (5μm thick sections). After hematoxylin-eosin and Novelli staining, which enable easy identification of the tissue vascular profile, stereological measurements were performed. Novelli histochemical staining involves deparaffinization and rehydration of adrenal sections, followed by incubation in hot 1N HCl (60 °C, 3 min), 1% acid fuchsine (30 s) and 1% light green (30 s). After each individual incubation, the sections were rinsed with distilled water. At the end of the staining process sections were dehydrated and mounted. As a result, purple erythrocytes were clearly visible against the bright green background of the adrenal cortex (Ajdžanović et al., 2015).

Immunohistochemically labeled sections with vascular endothelial growth factor (VEGF) (as an angiogenic peptide) and proliferating cell nuclear antigen (PCNA) provided insight into the capacity for capillary network forming/branching and the proliferative activity of the cortical cells, respectively. After deparaffinization and rehydration, antigen retrieval procedure was performed by incubating sections in 0.01 M citrate buffer (pH 6.0) in microwave (750W) for 21 min. Endogenous peroxidase activity was blocked in 0.3% H_2O_2 in methanol for 15 min,

followed by blocking of non-specific staining by 1 h incubation with 10% normal swine or donkey serum (Dako, Glostrup, Denmark) for immunostaining of VEGF or PCNA, respectively. Then, sections were incubated with rabbit polyclonal anti-VEGF antibody (Abcam®, ab46154; Cambridge, MA, USA; 1:100) or mouse monoclonal antibody ([PC10] ab29, Abcam®, Cambridge, MA, USA; 1:10000), overnight at 4°C. After washing in PBS, sections were incubated with secondary antibodies: either polyclonal swine-anti-rabbit IgG/HRP (Dako A/S, Glostrup, Denmark; 1:300) or polyclonal donkey-anti-mouse IgG/HRP (Dako, Glostrup, Denmark; 1:250) for 1 h, at room temperature; antibody localization was visualized with 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin.

2.3. Stereological measurements

Adrenal cortex volume, volumes of individual zones of the adrenal cortex (zona glomerulosa - ZG, zona fasciculata - ZF, zona reticularis - ZR), and volume of vascular tissue was determined on every 30 section of serially sectioned adrenal gland (microscope Olympus BX-51, Olympus, Japan), using the new CAST software (Visiopharm Integrator System-version 5.3.1.1640, Visiopharm, Denmark). The volumes were estimated according to formula:

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

where a(p) is the area associated with each sampling point (59366,25 μm^2); BA the block advance is the mean distance between two consecutively studied sections (141μm) as true section thickness was determined to be 4.7 μm; n is the number of sections studied for each pituitary; and ΣPi is the sum of points hitting a given target.

Volumes of the individual cells of ZG, ZF and ZR were estimated using a planar rotator as an unbiased local estimator, at objective magnification 100x, measuring 100 cells in each adrenocortical zone *per* animal (Dorph-Petersen et al., 2001). The computer generates cell volume estimate from the formula:

$$V = \sum li^2 \cdot t \cdot \frac{\pi}{2}$$

where *t* represents distance between the half lines (test lines) and *li* is individual distance between the intersections of half lines with the cell boundary and the first line.

Volume density estimation was used to determine the percentage of immunohistochemically labeled cells with VEGF in the ZG, ZF and ZR. Four central sections were analyzed *per* animal, with a spacing of 10 sections apart. Morphometric assessment was performed using an interactive test grid for histomorphometric assessment that was provided by the newCAST

software. Volume densities (V_V) were calculated as the ratio of the number of points hitting VEGF positive cells divided by the number of points hitting analyzed area *i.e.* analyzed section:

$$V_V\ (\%) = Pp / Pt \times 100,$$

where Pp represents counted points hitting the VEGF-positive cells; Pt total points hitting each adrenocortical zone. Volume density was calculated for each adrenal zone *per* analyzed section. Then, the average value for four sections was calculated (for each component separately) representing the volume density of VEGF cells *pe*r animal.

2.4. Quantification of the cell proliferation

Mitotic index represents the proliferative activity of adrenocortical cells that was expressed as number of PCNA positive cells *per* 1000 adrenocortical cells, *per* each zone. In the first instance, the PCNA-positive cells were counted for the each adrenocortical zone separately, over the entire surface of adrenal sections. Also, the total number of adrenocortical cells in each zone is counted. Than the number of PCNA positive cells, expressed *per* 1000 cells in the ZG, ZF and ZR of adrenal gland cortex, was calculated, to prevent bias resulting from the heterogeneous PCNA distribution over the gland, and different cells size in each zones. Counting was performed separately on the three central sections *per* animal, for the each group.

2.5. Biochemical parameters

Blood was collected from the trunk, in the morning hours, between 09:00 and 09:30 h, and separated sera/plasma samples of all the animals were stored at the same time at -70 °C until ACTH, aldosterone and corticosterone level determination.

Plasma ACTH concentration was determined without dilution, by a chemiluminescence method using an IMMULITE automatic analyzer (DPC, Los Angeles, CA, USA), in duplicate samples within a single assay, with an intra-assay coefficient of variation (CV) of 9.6%. Serum aldosterone concentrations were determined by enzyme immunoassay for direct quantitative determination ELISA (IBL Internetional GmbH, Hamburg, Germany), in duplicate samples within a single assay, with an intra-assay CV of 7.4%. Serum corticosterone concentrations were measured without dilution by immunoassay (R&D Systems Inc., Minneapolis, USA), in duplicate within single assays, with an intra-assay CV of 8.0%.

2.6. Behavioral tests

The light/dark and elevated plus maze test box test were used for assessment of anxiety-like behavior in three months old females from control and experimental groups. Each animal tested for behavior have 4 days resting period between each test. Females were sacrificed for

a week after their behavior was tested in order to avoid any influence on histological pictures and hormonal status of the animals. Animal behavior was monitored and recorded by a video camera connected to a PC, and thereafter analyzed by ANY-maze software (version 5.1, Stoelting Co., Wood Dale, USA). All tests were performed between 11:00 AM – 13:00 PM in an isolated room away from any external interferences and noises with a low-intensity white light source. After each trial, the apparatus was cleaned with 70% ethanol and allowed to dry before the next animal was tested.

In the light/dark box test, two choices are confronted: the preference to stay in the dark and the challenge to explore a new environment. These two when conflicted may lead to observable anxiety-like symptoms by measuring certain parameters. The apparatus was consisted of a Plexiglas box divided into two equal chambers (20.5 x 41 x 41 cm) by a partition with a small opening (10 x 10 cm) through which the animal could move freely between the chambers. The light chamber consisted of white Plexiglas walls without a ceiling and was illuminated at 40 lx, while the dark chamber was bounded by black walls and a ceiling and not illuminated. Each rat was placed in the light chamber at the beginning of the test and allowed to move freely for 5 min. The following parameters were automatically scored and analyzed: time spent in the light chamber, distance travelled in the light chamber and number of entries to the light chamber. Higher activity in the light chamber was an indicator of lower anxiety.

Elevated plus maze test is based on two conflicting innate tendencies: exploring a novel environment and avoiding elevated and open spaces. The apparatus, made of blue acrylic, was consisted of two open (50 x 10 cm) and two closed arms (50 x 10 cm) with 40 cm walls, connected by a central platform (10 x 10 cm). The open arms were opposite to each other and the cross platform was elevated to a height of 50 cm. Each animal was placed individually in the central square of the apparatus facing the open arm and allowed to explore the maze freely for 5 min. The following parameters were automatically scored and analyzed: time spent in the open arms, distance travelled in the open arms and number of entries into the open arms. A locomotion independent anxiety parameter was calculated and expressed as number of open arm entries / number of open + closed arm entries x100. Higher activity in the open arms was an indicator of lower anxiety.

2.7. Statistical analysis

Morphometric, hormonal and behavior data obtained for each group of female rats were averaged and SD was calculated with STATISTICA® version 10.0 (StatSoft Inc., USA). Data were tested for normality of distribution by the Kolmogorov–Smirnov test, whereas

homogeneity of variances was evaluated by Levene's test. Data were analyzed by two-way analysis of variance, with the Dx exposure and fructose supplementation as factors. The *post hoc* Bonferroni test was used to determine significant differences between groups. A probability value of 5% or less was considered statistically significant.

3. Results

3.1. Body mass and adrenal gland weights

Two-way ANOVA did not show significant main effect for Dx exposure, fructose consumption and their interaction. Significant main effect of Dx exposure on absolute ($F_{(1,20)}$=77.364, p<0.001) and relative adrenal weight ($F_{(1,20)}$=52.305, p<0.001) was only observed, while main effect of fructose consumption as well as Dx x F interaction was not significant on these parameters. *Post hoc* test showed significant decrease of absolute and relative adrenal weights of Dx and Dx-F female offspring, comparing to females that were not exposed to Dx (Table 1).

3.2. Histochemistry, immunohistochemistry and stereological measurements

Qualitative histological analysis of the central sections of the adrenal gland clearly demonstrated a decrease of the adrenocortical area in the Dx and Dx-F groups relative to the control, whereas no differences were present in the F group (Figure 1, *a-d*).

Statistical data cocncerning the results of two-way analysis of stereological parameters is given in Table 2. Significant main effect of Dx exposure was expressed in volume of adrenal gland and cortex, volume of ZF, ZR and vascular tissue. Fructose overconsumption had weak main effect on volume of ZF cell nuclei. No interaction between factors was observed.

Post hoc analysis showed that adrenal gland and adrenal cortex volume were decreased in the Dx exposed, comparing to unexposed females. Determination of the individual volume of cortical zones showed that after either prenatal Dx exposure or prenatal Dx exposure followed by postnatal fructose overconsumption, there were a significant decrease (p<0.05) of ZF volumes, in relation to values found in unexposed females. Volume of ZR was found to be significantly decreased only in Dx-F group in comparison to control and fructose-supplemented females. There were no changes of adrenal cell and nuclear volume between control and experimental groups (Figure 2).

After Novelli histochemical staining vascular network is identified to be particularly pronounced in ZF and ZR regions i.e. in inner most portion of the cortex where cord-like cell lines are surrounded by fenestrated capillaries. In relation to controls, blood vessels network is

especially prominent after prenatal Dx exposure, while in Dx-F group circulation network is expressed to a lesser degree, shown as blue violet erythrocyte arrays on the light green background of the steroidogenic cells. (Figure 3, a-d).

Volume of vascular tissue in adrenal cortex was markedly increased ($p<0.05$) in females prenatally exposed to Dx, with and without fructose consumption postnatally, comparing to control group (Figure 3 *e*).

VEGF immunostaining was very intensive in the ZG cells of all groups. Prenatal Dx exposure, regardless of fructose consummation, led to VEGF expression in greater number of ZF and ZR cells, localized individually or in scattered clusters, comparing with control group. Immunopositivity, positioned in cytoplasm differs among the cells of inner adrenal zones: in small number of cells intense immunostaining was noted, while diffuse cytoplasmic immunopositivity and presence of lipid droplets were evident in other cells. After fructose overexposure, VEGF immunopositivity is present in few cells, while majority of steroidogenic ZF and ZR cells are immnonegative (Figure 4, a-d).

Volume density of VEGF immunopositive cells in inner adrenocortical zones *i.e.* ZF and ZR, were markedly increased ($p<0.05$) in females prenatally exposed to Dx, with and without fructose consumption postnatally, comparing to control group (Figure 4 *e*).

3.3. Mitotic index

Immunohistochemical analysis revealed that PCNA-expressing cells were found in all adrenal zones of control females, with majority expression in the ZG cells and outer part of ZF. PCNA nuclear labeling, with varying intensity of immunoreactive staining among steroidogenic cells, is characteristics for all examined groups (Figure 5). In Dx and Dx-F groups less number of labeled cells was observed in the entire cortex; this decrease is particularly noticeable in the outer region of the cortex, as the most proliferative active part. Similarly to controls, F group is characterized by a relatively abundant PCNA expression in the ZG and outer ZF (Figure 5, *a-d*).

Mitotic index (MI) determination confirmed histological observation. Two-way ANOVA showed significant main effect of Dx exposure on mitotic index in ZG ($F_{(1,20)}= 118.852$, $p<0.001$) and ZF ($F_{(1,20)}=46.471$, $p<0.001$), while fructose supplementation had significant main effect only on mitotic index in ZF ($F_{(1,20)}=6.987$, $p<0.05$). Significant interaction between factors was observed in mitotic index of ZG ($F_{(1,20)}=8.113$, $p<0.001$). Neither Dx, nor fructose showed significant main effect on mitotic index in ZR.

Post hoc analysis showed that in relation to the control and fructose supplemented group, in both groups of Dx overexposed females the number of PCNA positive cells in the ZG and ZF

was significantly decreased ($p<0.05$), while ZR values remained at the control level. Comparing adrenocortical proliferation rate of females on fructose-enriched diet and controls it has been established that the number of PCNA positive cells in all cortical zones is as in control (Figure 5, *e*).

3.4. Biochemical parameters

Two-way ANOVA revealed significant effect of Dx exposure ($F_{(1,20)}=14.673$, $p<0.01$) and fructose-enriched diet ($F_{(1,20)}=11.584$, $p<0.01$) on ACTH blood concentration, and *post hoc* test showed significant increase of this parameter Dx and Dx-F groups by 44.6% and 75.5%, respectively in comparison with control values. On the other hand, corticosterone and aldosterone levels were unchanged by Dx prenatal exposure or fructose overconsumption. Fasting blood glucose concentration was unchanged following both Dx exposure and fructose supplementation (Table 3).

3.5. Behavioral tests

In the light/dark box test, Dx exposed females without and with fructose consumption stayed in the light chamber for a significantly shorter time then controls and significant main of this factor was revealed by two-way ANOVA ($F_{(1,20)}=75.725$, $p<0.001$). In both Dx exposed groups, the time spent in the light chamber was by two-fold decreased in relation to control values. In these animals, although reduced, the number of entries to the light chamber and the distance travelled in the light chamber were not significantly changed (Figure 6).

In the elevated plus maze test, analyzed parameters were markedly decreased in all Dx-exposed females. This was confirmed by two-way ANOVA that showed significant main effect of Dx exposure on number of entries to the open arms ($F_{(1,20)}=27.957$, $p<0.001$), time spent in the open arms ($F_{(1,20)}=34.037$, $p<0.001$) and distance travelled in the open arms ($F_{(1,20)}=33.680$, $p<0.001$). Comparing with controls, number of entries to the open arms was reduced by five-fold and four-fold, as well as the time spent in the open arms that was three-fold decreased in Dx and Dx-F group, respectively. Distance that rats travelled in the open arms was also decreased for five-fold in Dx and four-fold in Dx-F group, in relation to control values ($p<0.05$) (Figure 6). A locomotion independent anxiety parameter was markedly decreased in both Dx-exposed groups. This was confirmed by two-way ANOVA that showed significant main effect of Dx exposure ($F_{(1,20)}=18.381$, $p<0.001$) and fructose (($F_{(1,20)}=5.577$, $p<0.05$) on the calculated parameter. In relation to the control (39±4) the values in Dx (11±7) and Dx-F group (8±4) were significantly decreased ($p<0.05$).

4. Discussion

The results of this study showed that Dx programming effects were manifested through an increased plasma ACTH level that forced some aspects of adrenal steroid production. This resulted in maintaining the circulating corticosterone concentration at the control level despite a significantly reduced volume of the adrenal gland cortex in adult females prenatally exposed to glucocorticoids. In parallel, by applying behavioral tests, the light/dark box test and the elevated plus maze test, the anxiety-like behavior was noted. The effects were the same if offspring was exposed to synergistic action of prenatal glucocorticoid and postnatal fructose overexposure, indicating that fetal programming had a prevailing influence in the applied experimental model.

Applied metabolic challenge, in the form of excessive fructose intake in drinking water, from weaning till adulthood, represented moderately increased fructose consumption which mimics the unhealthy diet habits, causing increased adiposity and triglyceride plasma level (Kovačević et al., 2014). Evidently, the fructose dosage and time regime were not of high enough intensity to provoke changes of HPA axis activity, as well as behavioral alterations. On the other hand, in rats fed with a significantly higher proportion of fructose in comparison to that applied in our study, anxiety-like behavior, increased HPA axis activity and the reprogram of hypothalamic transcript expression that particularly affects genes related to stress and feeding were all provoked (Harrell et al., 2015; Gancheva et al., 2017). Even offspring anxiety was modulated during the juvenile and adult periods as a result of the maternal feeding, but again, dams were exposed to a high fructose diet, with even 60% fructose (Bukhari et al., 2018). Thus, the level of consumption that led to an energetic overload has a central role in causing and potentiating adverse side effects referring to HPA axis and behavior.

The natural fetal protection against high levels of maternal glucocorticoids was ensured by the action of the 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), the placental enzyme that inactivates them. Contrary to maternal, synthetic glucocorticoids pass the enzymatic placental barrier, and so the applied Dx reached the fetal circulation, decreased birth mass and changed the developmental trajectory (Holmes et al., 2006; Kapoor et al., 2008). Due to catch-up growth there were no significant differences in three month old female offspring from different experimental groups.

Unlike body mass, the adrenal gland weight remained significantly reduced during adulthood, in absolute and relative terms. As a consequences of glucocorticoid exposure *in utero*, a significant decrease of PCNA positive cells number *i.e.* number of replicating cells in the outer portion of the adrenal cortex, the region that is proliferatively most active (Engeland et

al., 2005; Vinson, 2016), was established. Considering that there were no changes in the size of cells and their nuclei in the entire cortex, the decreased proliferation rate might have been the cause of the marked decrease of ZF and ZR volumes and the size of the entire gland as well as adrenocortical cell number.

In parallel with the decreased volume of the adrenal cortex, the markedly increased volume of vascular tissue has been measured in offspring of Dx treated dams, after Novelli histohemical staining. Having in mind that the adrenal gland represents one of the most extensively vascularized organs, the VEGF immunostaining pattern was further analyzed as the growth factor which strongly supports angiogenesis and endothelial cell mitosis (Ferrara, 1999). The positive correlation of the VEGF expression in adrenocortical cells and the markedly increased vascular volume, in both Dx exposed groups, might confirmed the stimulating VEGF influence on the vascular network maintenance and extension. Literature showed that ACTH provoke VEGF synthesis in steroidogenic cells of ZF and ZR (Shifren et al., 1998), indicating that increased ACTH level, established after prenatal Dx exposure, could stimulate VEGF expression. This had profound physiologic consequences as the adrenal blood supply determines ACTH availability, substrate supply as well as steroid hormones diffusion across the cell membrane into fenestrated capillary network, thus influencing the circulating level of adrenal steroids.

In addition to indirect links, direct links between steroidogenesis in ZF and ZR cells and ACTH were shown. The elevated ACTH level *via* the activation of cAMP/protein kinase A signaling and chronic stimulation of steroidogenic enzyme genes transcription (Ruggiero and Lalli, 2016) resulted in the production of the corticosterone that was at the control level despite the significantly decreased ZF volume. However, the possibility of establishing homeostasis in extreme conditions by adrenal glands with decreased volume remains questionable. The increased ACTH level in Dx exposed offspring shown here was most likely caused via altered neurogenesis during a critical period of development in the regions that control pituitary corticotroph function such as the hypothalamus, amygdala and hippocampus (Carson, 2016).

The assessment of the light/dark box test and the elevated plus maze test revealed that increased anxiousness and lower exploratory behavior caused by fear of open space and height have been present in both groups that were Dx exposed prenatally. Literature data demonstrated anxiety-like behavior in offspring, which was under different types of prenatal adversity in parallel with unchanged activity of HPA axis (Takahashi et al., 1992; Holmes et al., 2006; Nagano et al., 2008). In our study we have found that anxiety-like behavior was

accompanied with increased HPA activity, documented by increased ACTH blood concentration in basal conditions, and unchanged glucocorticoid level in the circulation despite the significantly decreased adrenocortical volume. Permanent modification of brain structures and functions, in regions that control both HPA axis activity and anxiety, caused by prenatal glucocorticoids exposure, possibly represent the explanation in this respect (Huang, 2011). The hippocampal inhibitory control over HPA axis is reduced in offspring after exposure to glucocorticoid excess during the fetal period due to permanent deficit in a number of proliferating cells and decreased expression of the GR and /or MR (Shoener et al., 2006; Noorlander et al., 2014). On the other hand, prenatal glucocorticoid overexposure increased CRH mRNA, as well as GR and MR mRNA expression in selected regions of the amygdala, exerting stimulatory influence on HPA axis activity (Flandreau et al., 2012). As a result, the elevated hypothalamic CRH mRNA and protein level, together with the increased granule density and their intensive migration in pituitary ACTH cells of adult female rats following antenatal Dx treatment were found (Shoener et al., 2006; John et al., 2006). Thus, it is indicated that all these alterations reflected on increased ACTH circulating level and anxiety.

Conclusion

Comparing both applied treatments and their synergistic action, it can be concluded that antenatal glucocorticoid exposure has been a potent programming factor with prevailing impact on both HPA axis activity and anxiety behavior. Increased ACTH circulatory level, decreased volume of adrenal gland and unchanged corticosterone concentration were accompanied with anxiety-like behavior. Insignificant effects of moderately increased fructose consumption suggest that the detrimental effect of fructose depends on its dosage. The adverse effects of prenatal glucocorticoid action are not additionally potentiated if postnatal fructose slightly increased in diet.

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Figure legends

Figure 1. Histological appearance of adrenal gland of *a*. control females (C), *b.* prenatally Dx exposed female offspring (Dx), *c*. fructose-supplemented females (F) and *d*. prenatally dexamethasone exposed and postnatally fructose-supplemented females (Dx-F). H&E; ZG-zona glomerulosa; ZF-zona fasciculata; ZR-zona reticularis; M-medulla; bar-550μm.

Figure 2. Volumes of adrenal gland, cortex, zona glomerulosa (ZG), zona fasciculata (ZF), zona reticularis (ZR) as well as volumes of individual cells and nuclei in control females (C), prenatally Dx exposed females (Dx), fructose-supplemented females (F) and Dx exposed and fructose-supplemented females (Dx-F). All values are provided as the mean ± SD; n=6. $^{a}p<0.05$ *vs*. C; $^{b}p<0.05$ *vs*. F.

Figure 3. Representative micrographs of adrenal gland vascularity *a.* control females (C), *b*. prenatally Dx exposed female offspring (Dx), *c*. fructose-supplemented females (F) and *d*. prenatally dexamethasone exposed and postnatally fructose-supplemented females (Dx-F). Novelli staining. ZF-zona fasciculata; ZR-zona reticularis; bar-200μm. *e.* Volume of adrenal gland vascular network in control females (C), prenatally Dx exposed females (Dx), fructose-supplemented females (F) and Dx exposed and fructose-supplemented females (Dx-F). All values are provided as the mean ± SD; n=6. $^{a}p<0.05$ *vs*. C; $^{b}p<0.05$ *vs*. F.

Figure 4. VEGF immunohistochemical staining of adrenal gland in *a*. control females (C), *b*. prenatally Dx exposed female offspring (Dx), *c*. fructose-supplemented females (F) and *d*. prenatally dexamethasone exposed and postnatally fructose-supplemented females (Dx-F). *e.* Volume density of VEGF immunopositive cells in control females (C), prenatally Dx exposed females (Dx), fructose-supplemented females (F) and Dx exposed and fructose-supplemented females (Dx-F). All values are provided as the mean ± SD; n=6. $^{a}p<0.05$ *vs*. C; $^{b}p<0.05$ *vs*. F. ZG-zona glomerulosa; ZF-zona fasciculata; bar-100μm.

Figure 5. PCNA immunohistochemical staining of adrenal gland in *a*. control females (C), *b*. prenatally Dx exposed female offspring (Dx), *c*. fructose-supplemented females (F) and *d*. Dx exposed and fructose-supplemented females who drank fructose solution (Dx-F). ZG-zona glomerulosa; ZF-zona fasciculata; bar-50μm. *e.* Mitotic index expressed *per* 1000 cells *per* zone in control females (C), prenatally Dx exposed females (Dx), fructose-supplemented females (F) and Dx exposed and fructose-supplemented females (Dx-F). ZG-zona glomerulosa; ZF-zona fasciculata; ZR-zona reticularis. All values are provided as the mean ± SD; n=6. [a]$p<0.05$ *vs*. C; [b]$p<0.05$ *vs*. F.

Figure 6. Results of the elevated plus maze and the light/dark box tests in control females (C), prenatally Dx exposed females (Dx), fructose-supplemented females (F) and Dx exposed and fructose-supplemented females (Dx-F). All values are provided as the mean ± SEM; n=6. [a]$p<0.05$ *vs*. C; [b]$p<0.05$ *vs*. F.

Figure 1.

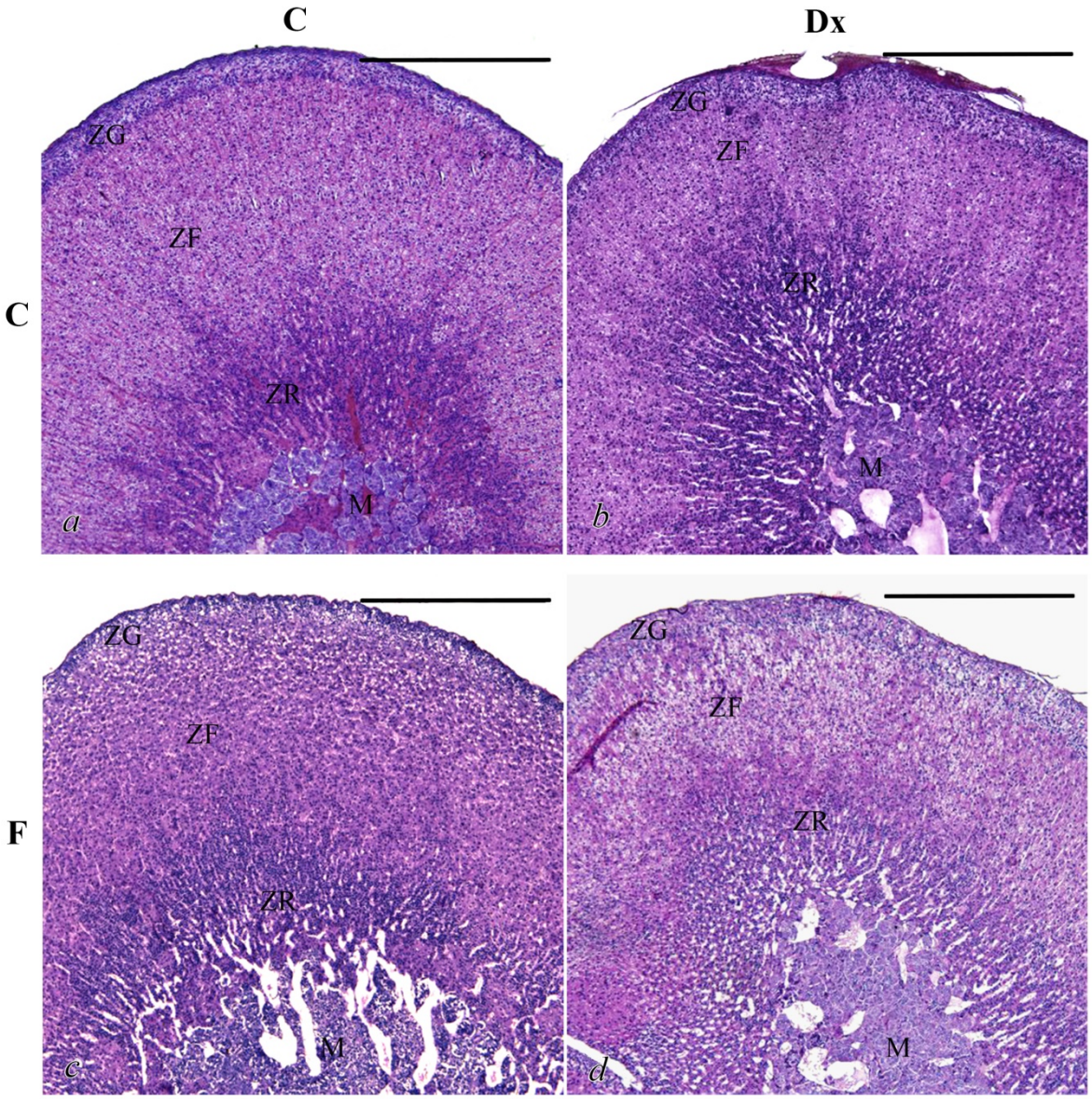


Figure 2.

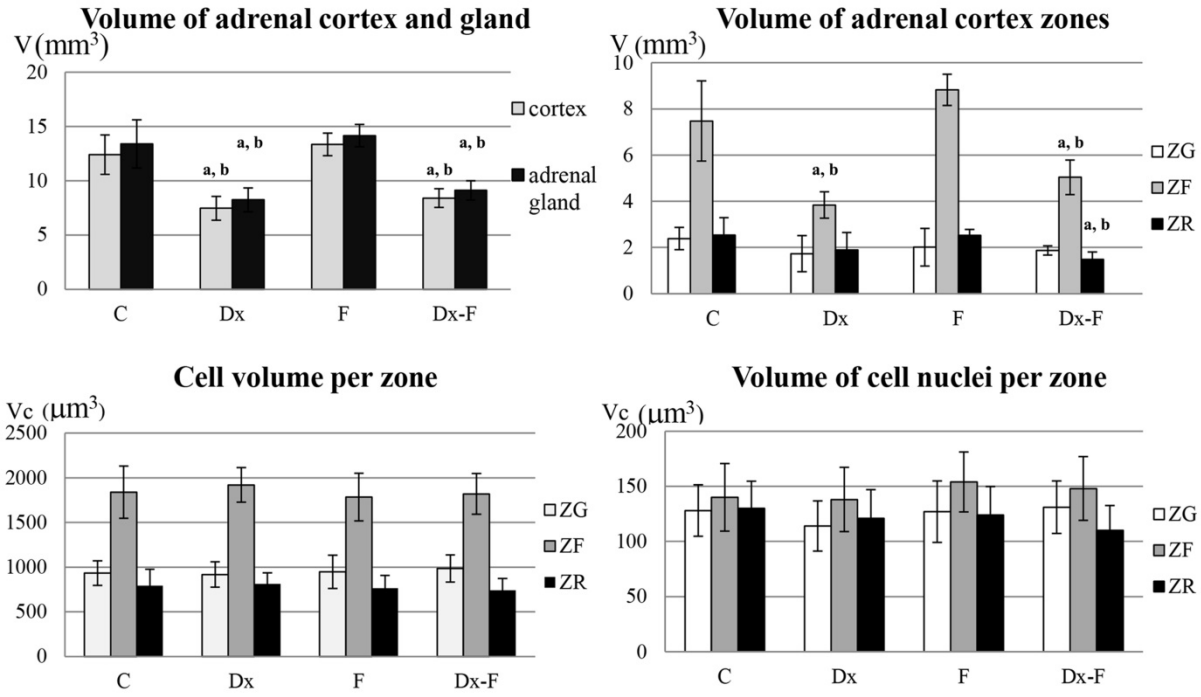
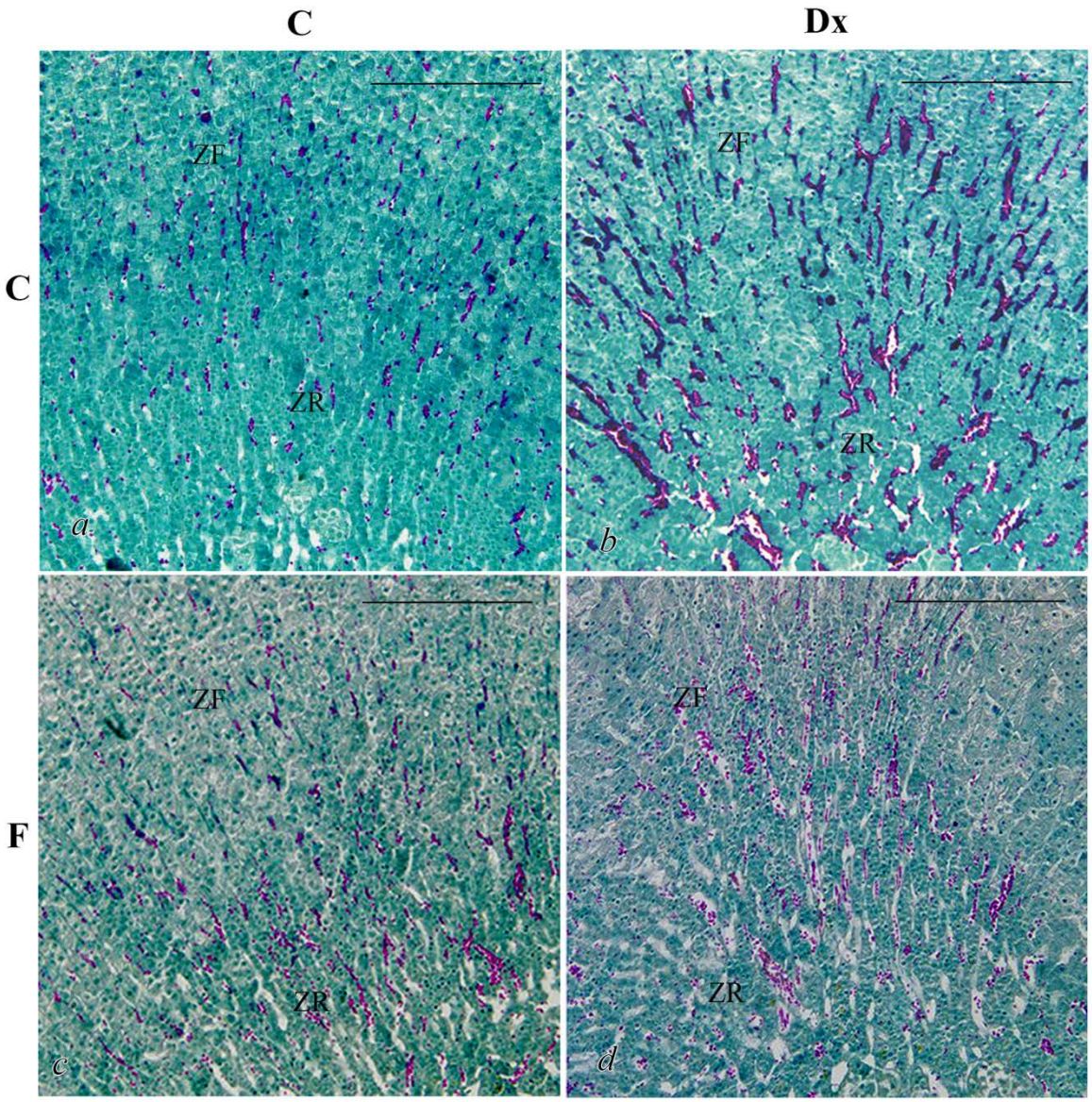

Volume of adrenal cortex and gland
V (mm3)
20
15
10
5
0
a, b
a, b
a, b
a, b
cortex
adrenal gland
C
Dx
F
Dx-F
Volume of adrenal cortex zones
V (mm3)
10
8
6
4
2
0
a, b
a, b
a, b
ZG
ZF
ZR
C
Dx
F
Dx-F
Cell volume per zone
Vc (µm3)
2500
2000
1500
1000
500
0
ZG
ZF
ZR
C
Dx
F
Dx-F
Volume of cell nuclei per zone
Vc (µm3)
200
150
100
50
0
ZG
ZF
ZR
C
Dx
F
Dx-F

Figure 3.


C
Dx
C
F
ZF
ZR
a
b
c
d

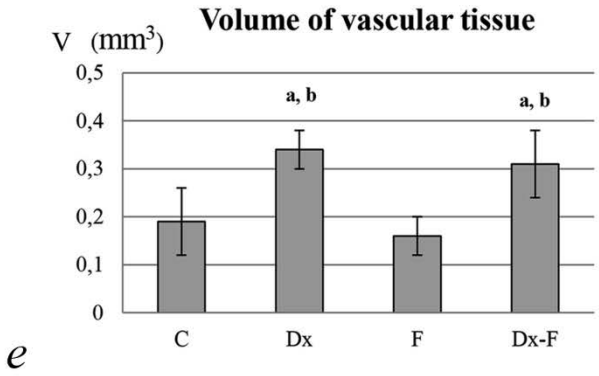

Volume of vascular tissue
V (mm3)
0,5
0,4
0,3
0,2
0,1
0
a, b
a, b
C
Dx
F
Dx-F
e

Figure 4.

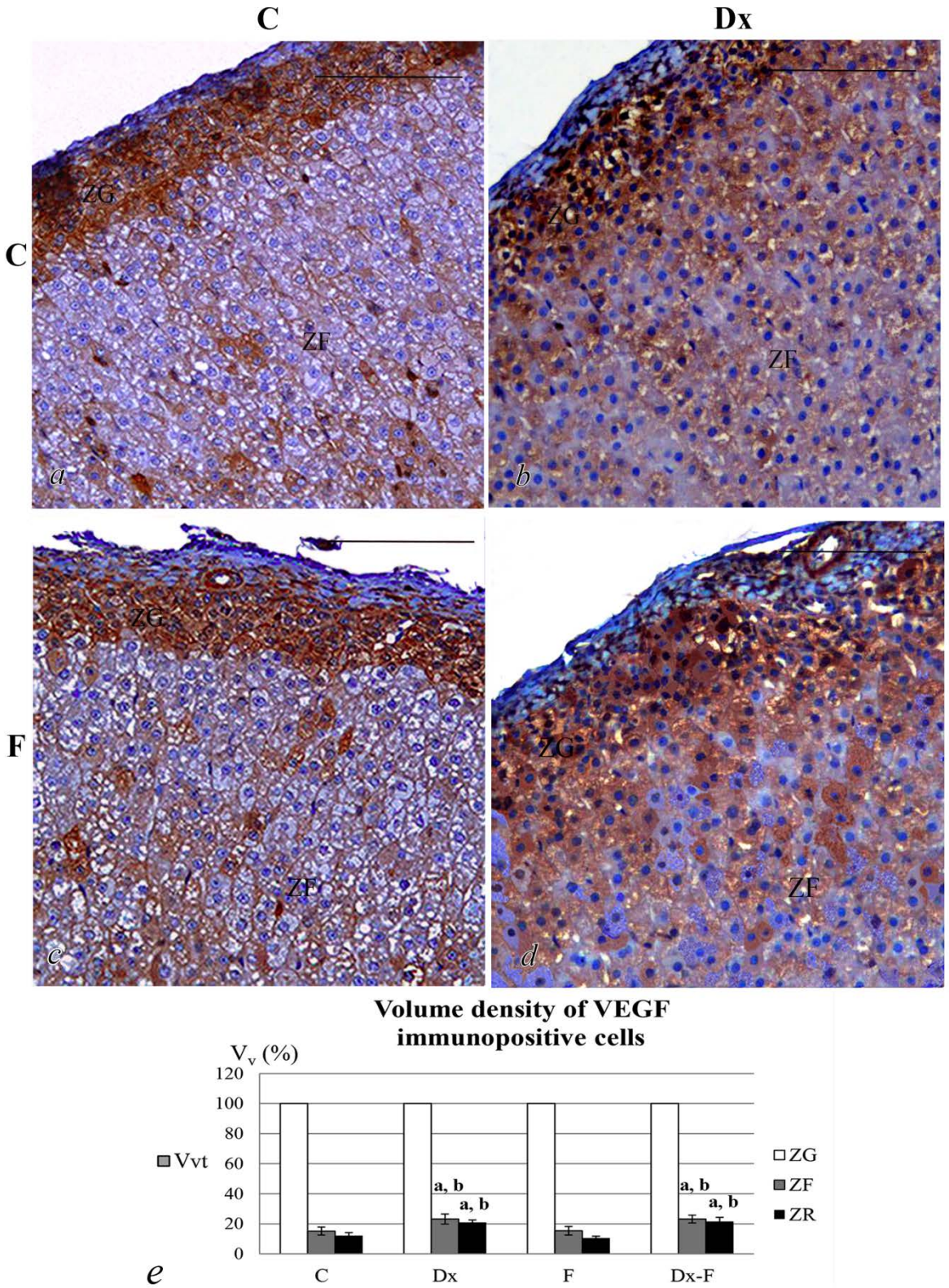
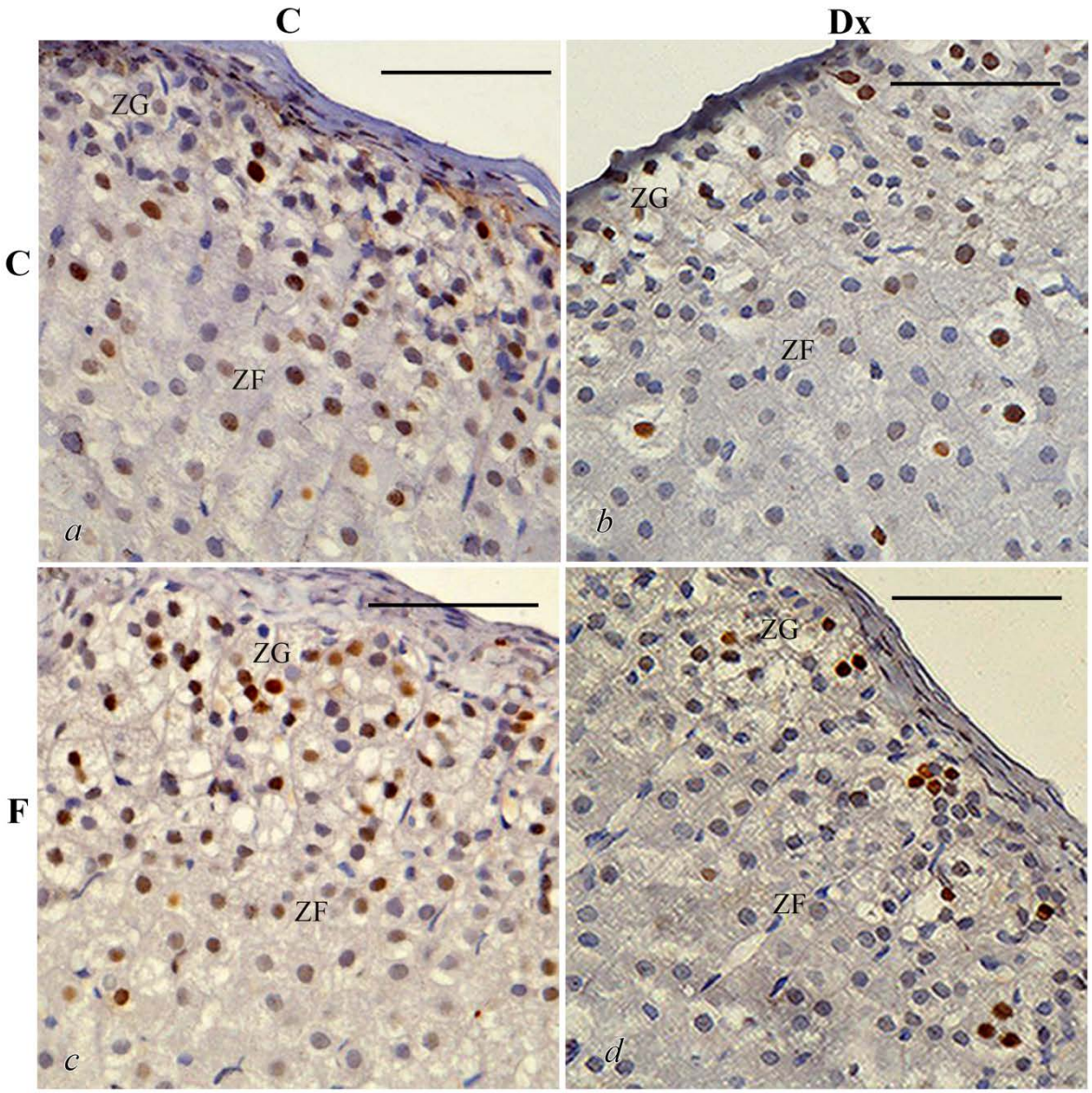

C
Dx
C
F
ZG
ZF
a
b
c
d
Volume density of VEGF immunopositive cells
Vv (%)
120
100
80
60
40
20
0
Vvt
a, b
a, b
a, b
a, b
C
Dx
F
Dx-F
ZG
ZF
ZR
e

Figure 5.


C
Dx
C
F
ZG
ZF
a
b
c
d

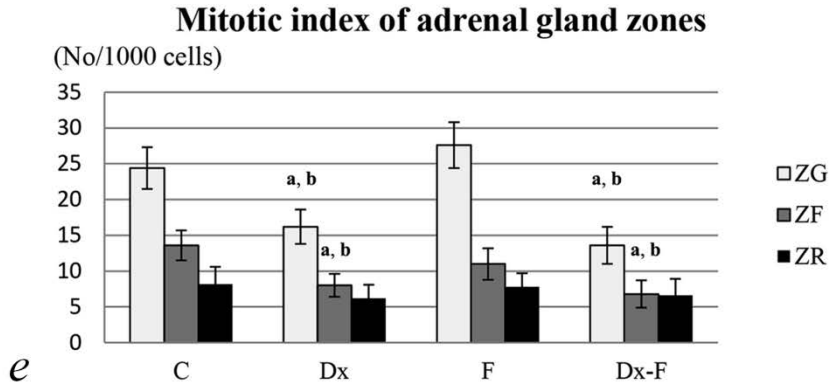

Mitotic index of adrenal gland zones
(No/1000 cells)
35
30
25
20
15
10
5
0
a, b
a, b
a, b
a, b
C
Dx
F
Dx-F
ZG
ZF
ZR
e

Figure 6.

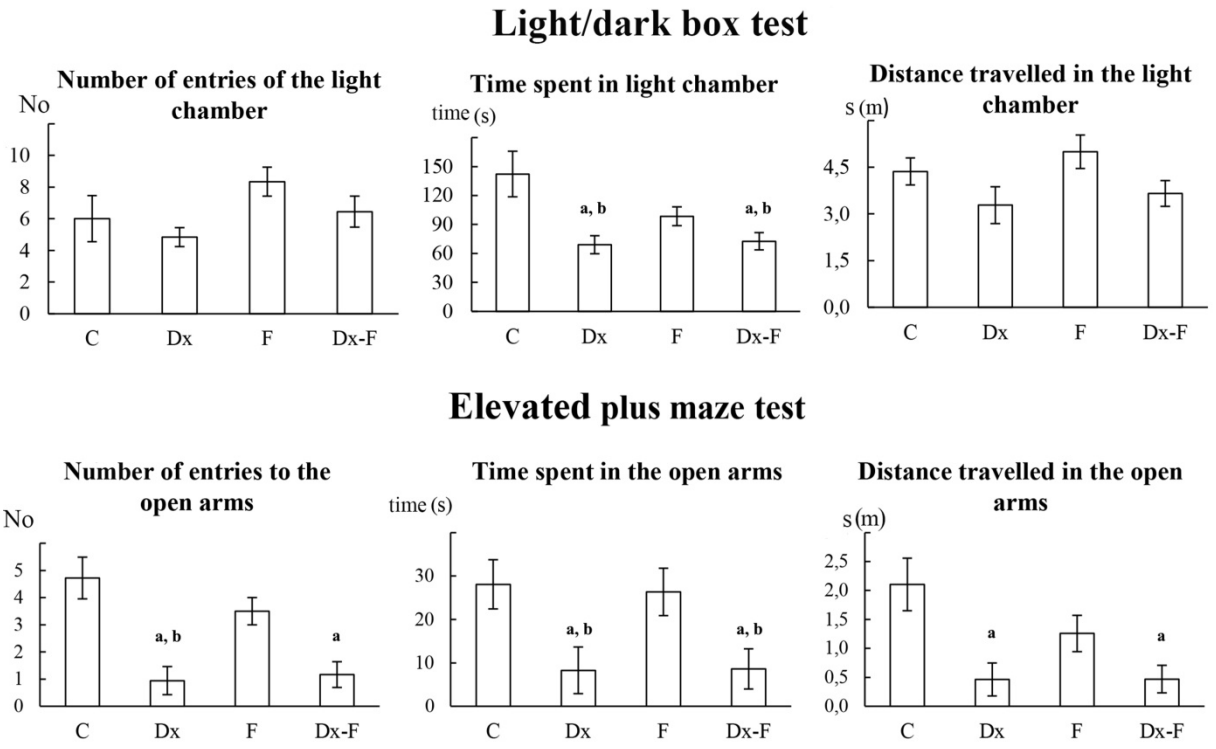

Light/dark box test
Number of entries of the light chamber
No
10
8
6
4
2
0
C
Dx
F
Dx-F
Time spent in light chamber
time (s)
150
120
90
60
30
0
a, b
a, b
C
Dx
F
Dx-F
Distance travelled in the light chamber
s (m)
4,5
3,0
1,5
0,0
C
Dx
F
Dx-F
Elevated plus maze test
Number of entries to the open arms
No
5
4
3
2
1
0
a, b
a
C
Dx
F
Dx-F
Time spent in the open arms
time (s)
30
20
10
0
a, b
a, b
C
Dx
F
Dx-F
Distance travelled in the open arms
s (m)
2,5
2,0
1,5
1,0
0,5
0,0
a
a
C
Dx
F
Dx-F

Table 1. Body mass, absolute and relative adrenal gland weight in control females (C), prenatally dexamethasone exposed females (Dx), fructose-supplemented females (F) and prenatally dexamethasone exposed and postnatally fructose-supplemented females (Dx-F).

Groups	Body mass (g)	Absolute adrenal gland weight (g)	Relative adrenal gland weight (mg/g)
C	198 ± 11	32.2 ± 2.1	16.2± 0.9
Dx	193 ± 8	26.3 ± 2.2[a,b]	13.6± 1.0[a,b]
F	201± 5	34.5 ± 1.2	17.4± 0.7
Dx-F	199 ± 10	26.5 ± 2.3[a,b]	13.4± 1.7[a,b]

All values are provided as the mean ± SD; n=6. [a]$p<0.05$ *vs*. C; [b]$p<0.05$ *vs*. F.

Table 2. Significance of the main effects resulting from Dx exposure (Dx) and fructose supplementation (F), as well as from their interaction (Dx x F), on adrenal gland stereological parameters.

Factors / Parameters	Dx	F	Dx x F	df
Volume of adrenal cortex	F=114.987 p<0.001	F=4.213 p>0.05	F=0.000 p>0.05	1. 20
Volume of adrenal gland	F=113.811 p<0.001	F=3.145 p>0.05	F=0.003 p>0.05	1. 20
Volume of ZG	F=2.996 p>0.05	F=0.284 p>0.05	F=1.331 p>0.05	1. 20
Volume of ZF	F=94.966 p<0.001	F=11.215 p<0.001	F=0.035 p>0.05	1. 20
Volume of ZR	F=16.263 p<0.001	F=1.024 p>0.05	F=0.843 p>0.05	1. 20
Volume of vascular tissue	F=46.0.24 p<0.001	F=1.987 p>0.05	F=0.009 p>0.05	1. 20
Volume of ZG cells	F=0.65 p>0.05	F=2.478 p>0.05	F=2.148 p>0.05	1. 20
Volume of ZF cells	F=1.76 p>0.05	F=2.4 p>0.05	F=0.24 p>0.05	1. 20
Volume of ZR cells	F=0.53 p>0.05	F=7.55 p>0.05	F=1.45 p>0.05	1. 20
Volume of ZG cell nuclei	F=0.09 p>0.05	F=1.48 p>0.05	F=3.024 p>0.05	1. 20

Volume of ZF cell nuclei	F=0.01 p>0.05	F=0.41 p>0.05	F=0.47 p>0.05	1. 20
Volume of ZR cell nuclei	F=1.4 p>0.05	F=1.232 p>0.05	F=0.035 p>0.05	1. 20
Volume density of VEGF immunopositive cells in the ZF	F=57.00 p<0.001	F=0.006 p>0.05	F=0.006 p>0.05	1, 20
Volume density of VEGF immunopositive cells in the ZR	F=46.59 p<0.001	F=0.682 p>0.05	F=2.354 p>0.05	1, 20

Table 3. Results of hormonal data in control females (C), prenatally Dx exposed females (Dx), fructose-supplemented females (F) and Dx exposed and fructose-supplemented females (Dx-F).

Groups	Plasma level of ACTH (ng/L)	Serum level of aldosterone (ng/mL)	Serum level of corticosterone (ng/mL)
C	21.3±4.0	0.109±0.014	28.9 ± 9.1
Dx	30.8±6.7[a] ↑	0.096±0.007	22.2 ± 8.0
F	30.0±9.8	0.087±0.012	27.5 ± 8.6
Dx-F	37.4±5.3[a] ↑	0.091±0.009	21.7 ± 12.6

All values are provided as the mean ± SD; n=6. [a]$p<0.05$ *vs.* C.