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Chronic stress and its effects on adrenal cortex apoptosis in pregnant rats

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Abstract

The model of chronic intermittent stress by immobilization during pregnancy may produce alterations in the mechanisms that maintain adrenal gland homeostasis. In earlier investigations using this model, significant variations in plasma prolactin and corticosterone levels, and adrenal gland weights were observed. We hypothesized that chronic stress causes changes in apoptosis in the adrenal glands of pregnant rats. We identified and quantified apoptotic cells in the adrenal cortex and examined their ultrastructural characteristics using transmission electron microscopy. Adrenal glands of pregnant rats at gestation days 12, 17 and 21 were studied for control and experimental (stressed) rats. Immunolabelling techniques, stereological analysis and image quantification of adrenal gland sections were combined to determine differences in apoptosis in the different cell populations of the adrenal cortex. The apoptotic index of the experimental rats showed a significant reduction at gestation day 17, while at days 12 and 21 there were no differences from controls. Moreover, the apoptotic index of the reticular zones in control and experimental animals showed a significant increase compared to the glomerular and fascicular zones at the three gestation times studied. Chronic stress by immobilization reduced the caspase-dependent apoptotic index at gestation day 17, which may be related to variations in plasma concentrations of estrogens and prolactin.

Key words: adrenal cortex, apoptosis, caspase 3, chronic stress, electron microscopy, gestation, rats, TUNEL

On the cellular level, life and death are inseparable phenomena. The numbers of cells that compose the tissues of an adult organism are confined within constant bounds. Cells that die are replaced by others. This process is regulated to ensure the maintenance of appropriately balanced cell loss, renewal and differentiation (Lizarbe Iracheta 2007).

Apoptosis, or programmed cell death, is a physiological process that plays a fundamental role in tissue development and homeostasis. Apoptosis is regulated by a cascade of signals that function in induction, transduction and amplification of intra-cellular signals. Caspases are specific cysteine-proteases that are closely associated with apoptosis. They are found as inactive precursors in the cytoplasm. When they receive the apoptotic signal, they undergo a proteolytic breakdown and generate subunits that constitute the active enzyme. Active caspases break down several proteins, which lead to cell death (Rastogi et al. 2009). This process is characterized by a series of typical morphological events including cell shrinkage, fragmentation into apoptotic bodies surrounded by membrane, and rapid phagocytosis by neighboring cells (Saraste and Pulkki 2000).

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The adrenal gland responds to stress and is subject to dynamic structural changes that include both cell proliferation and death. Balance between these two processes is vital for gland integrity and function (Goldstein 2010).

Several theories have been proposed to explain cellular replacement in the different zones of the adrenal cortex. The migration theory describes cell proliferation in the external part of the cortex, migration of these cells from the glomerular to the fascicular zone, and from there to the reticular zone where eventually they degenerate (Idelman 1978, Wolkersdorfer and Bornstein 1998). Alternatively, the transformation theory suggests that the transformation may occur either between the glomerular and fascicular zones or between fascicular and reticular zones (Wolkersdorfer and Bornstein 1998) whereby zonal tissue is replaced by proliferative cells from an intermediate zone. The intermediate zone is between the glomerular and fascicular zones and cell migration occurs in two opposite directions: toward the medulla and toward the capsule. Finally, zonal theory (Swann 1940) postulates that both cellular proliferation and apoptosis occur independently in each cortical zone; therefore, each zone might be regulated locally and have no effect on the functions of other zones (Vinson 2003).

These three hypotheses are based on histological observations of mammalian adrenals under experimental conditions such as pharmacologic manipulation, castration or unilateral adrenalectomy. These experimental conditions generally produce increased cell proliferation in the adrenal cortex. Because renewal of cells in the adrenal glands of mammals is slow under normal conditions, these experimental procedures are useful for studying cell turnover. Despite numerous studies, however, knowledge of adrenocortical cellular kinetics remains limited (Kataoka et al. 1996).

When chronic intermittent stress of sufficient intensity, such as immobilization, is applied, increased adrenal gland size is observed. The size increase seems to be related to a large increase in corticosterone levels as a result of the trophic effect of adrenocorticotrophin hormone (ACTH) secretion. It is likely, however, that other factors may contribute to the response observed (Nadal and Armario 2010). The increased adrenal gland size is accompanied by increased adrenal cortex response to ACTH in vivo and in vitro. In clinical studies in humans and animals it has been shown that circulating levels of ACTH are correlated positively with adrenal gland size (Kobayashi et al. 2006). It has not been determined whether the increased weight of the adrenal gland under chronic stress conditions is produced by hypertrophy, hyperplasia or reduced levels of apoptosis. If the latter, it is not clear whether apoptosis occurs everywhere in the cortex or whether it is localized in some zones.

We used pregnant rats to determine the effects exerted by hormones that are released during pregnancy and under chronic stress conditions on apoptosis in the adrenal cortex. We determined effects of chronic stress on apoptosis in the adrenal cortex of pregnant rats using immunocytochemical techniques (TUNEL and activated caspase) and we examined ultrastructural characteristics of apoptosis in the adrenal cortex of experimental rats using transmission electron microscopy (EM).

Materials and methods

Animals and laboratory conditions

Thirty Wistar 200–300 g 90–120-day-old female rats were used for the experiment. Animals were maintained under controlled laboratory conditions at $20 \pm 2^{\circ}$ C, with a 12/12 h light/dark cycle, and food and water available *ad libitum*. The Conclusions and Recommendation on the Reduction, Refinement and Replacement of Laboratory Animals Procedure from the Declaration of Bologna (Russell and Burch 1959) were followed for animal experimentation. All experiments were conducted according to the principles and procedures of the NIH Guide for the Care and Use of Laboratory Animals.

Rats were cycled using colpo-cytograms and were mated during pro-estrous with a male of the same strain. Pregnancy day zero was defined by presence of spermatozoa in vaginal fluid. Pregnant rats were separated into control and experimental groups.

Experimental treatment

Experimental rats were subjected to sessions of immobilization stress in a tubular clamp made of perforated plastic. The clamp was anchored to a wood base and padded for animal comfort. Each immobilization session lasted 45 min. Rats were subjected to these sessions every other day beginning with the fourth day of gestation to prevent the embryo resorption prior to the day before sacrifice. The method was adapted from the National University of Rio Cuarto biotherius under ethical rules for manipulation of animals for experimentation.

Five animals for each group and for each gestational stage were sacrificed by decapitation without anesthetic at gestation days 12, 17 and 21. For TUNEL and activated caspase 3 studies, five control and five experimental animals were used. After

decapitation, the adrenal glands of each pregnant rat were excised and fixed in 10% buffered formaldehyde for 12 h and processed according to conventional histological technique. Alternate 5 μm thick sections were cut using a Reichert-Young 2065 microtome and mounted with Vectabond adhesive (Vector Laboratories, Inc., Burlingame, CA). Three microscope slides per animal, each with three sections of the same gland were analyzed. Glomerular, fascicular and reticular zones of the adrenal cortex were identified by their histological characteristics.

TUNEL assay

Twelve alternate sections were processed from each animal of both control and experimental groups at all gestation stages studied. The hydrated sections were incubated with K-proteinase (Oncor, Gaithersburg, MD) for 8–10 min in a wet chamber. Endogenous peroxidase was blocked using 30% hydrogen peroxide according to the protocol included with the Apoptag Plus in Situ Apoptosis Peroxidase kit (Oncor). Negative controls omitted the terminal deoxynucleotidyl transferase (TdT); post-lactation mammary gland was used as the positive control. Normal nuclei were counterstained with 1% methyl green for 5 min.

Activated caspase 3 detection

Histological sections were de-waxed and hydrated in phosphate buffered saline, pH 7.4 (PBS), and antigen retrieval was carried out in a microwave oven at 850 w for 6 min. A 20% solution of hydrogen peroxide was used to block endogenous peroxidases. Nonspecific antibodies were blocked with horse serum (Vector) for 30 min. We used an anti-caspase primary antibody, rabbit polyclonal antibody (Chemicon International, Inc., Billerica, MA) diluted 1:10 in PBS, followed by a biotinylated secondary antibody and avidin-biotin-peroxidase complex (Vectastain ABC Elite Kit 6200; Vector). Diaminobenzidine (DAB) (Vector) was used for visualization and methyl green in sodium acetate (20 mg/ml) was used for nuclear counterstaining for 5 min. The negative control was the reaction applied to adrenal cortex without the primary antibody and the positive control was the reaction in post-lactation mammary gland.

Electron microscopy

Blocks 1 mm^3 from adrenal glands of control and experimental rats were obtained for each gestation day studied. Tissues were fixed with 4% glutaraldehyde 0.1 M in sodium cacodylate buffer, pH 7.4. Blocks then were postfixed with 1% OsO_4 in the same buffer. Blocks were dehydrated with acetone and embedded in Epon 812. Coarse sections (1 μm) were cut and stained with toluidine blue for zone identification. Blocks were sectioned with a Porter Blumm Sorval MT1-A ultramicrotome and the grids were contrasted with 5% aqueus uranyl acetate and lead citrate. Sections were examined using an electron JEM 1200 EX II microscope and photographed.

Stereological analysis

For each adrenal cortex section, 30 fields of the glomerular zone with an average of 60 cells per field; 90 fields of the fascicular zone with an average of 170 cells per field; and 60 fields from the reticular zone with an average of 120 cells per field were scanned to build a raw image data base for the control and experimental groups for each gestational day studied. A Zeiss Axiophot microscope with a built-in AxioVision Zeiss digital camera and associated software was used (video-Printer Sony 3000 and Scion stereological image analysis software).

Qualitative analysis of TUNEL stained images was performed by comparing distribution of anti-digoxigenin labeled apoptosis and unlabeled cells, counterstained with methyl green, of the zones of the adrenal cortex of control and experimental animals at the gestation stages studied. Images were processed by quantifying both labeled and unlabeled anti-activated caspase 3 cells. The apoptotic index caspase-dependent (AIC) was estimated as the ratio of apoptotic to normal cells multiplied by 100. Finally, the nuclear cytoplasmatic area ratio was evaluated for cells of the zones of the adrenal cortex at gestation day 17. The morphometric analysis was carried out using the software Image J (NIH). Data were transferred to a data base in Excel software for further statistical analyses (Indelman 1978).

Statistical analysis

One- and three-way ANOVAs were applied. A logarithmic lineal model with Poisson distribution also was used to consider group, time and interaction effects. For comparison significant differences between zones or days, we realized a post hoc test (LSD fisher). Statistical analyses were performed using with InfoStat and SAS 9.1 software. Differences were considered significant at $p \leq 0.05$. When differences between zones were significant, we

performed a post-hoc test (LSD-Fisher) to determine between which zones the differences were significant. In the post hoc test, the results are expressed with letters: different letters indicate significant differences at $p < 0.05$; however, the p value was not specified specifically for each significant difference, i.e., for each zone.

Results

Apoptosis qualitative analysis

We used the TUNEL technique to identify apoptotic nuclei in the adrenal gland. A similar pattern of TUNEL marked nuclei was observed in both control and experimental groups. TUNEL positive nuclei were observed in all three zones of the adrenal cortex, but mainly in the reticular zone. In both control and experimental groups, cells labeled with activated caspase 3 were frequent in fascicular and reticular zones and sparse in the glomerular zone at all three gestation stages studied. TUNEL and activated caspase 3 labeling were weaker in the experimental group at gestation day 17, while at days 12 and 21, the pattern of the TUNEL labeled cells and activated caspase 3 labeled cells were similar in both groups (Fig. 1A–D). Differences between TUNEL labeled nuclei and caspase 3 labeled cells

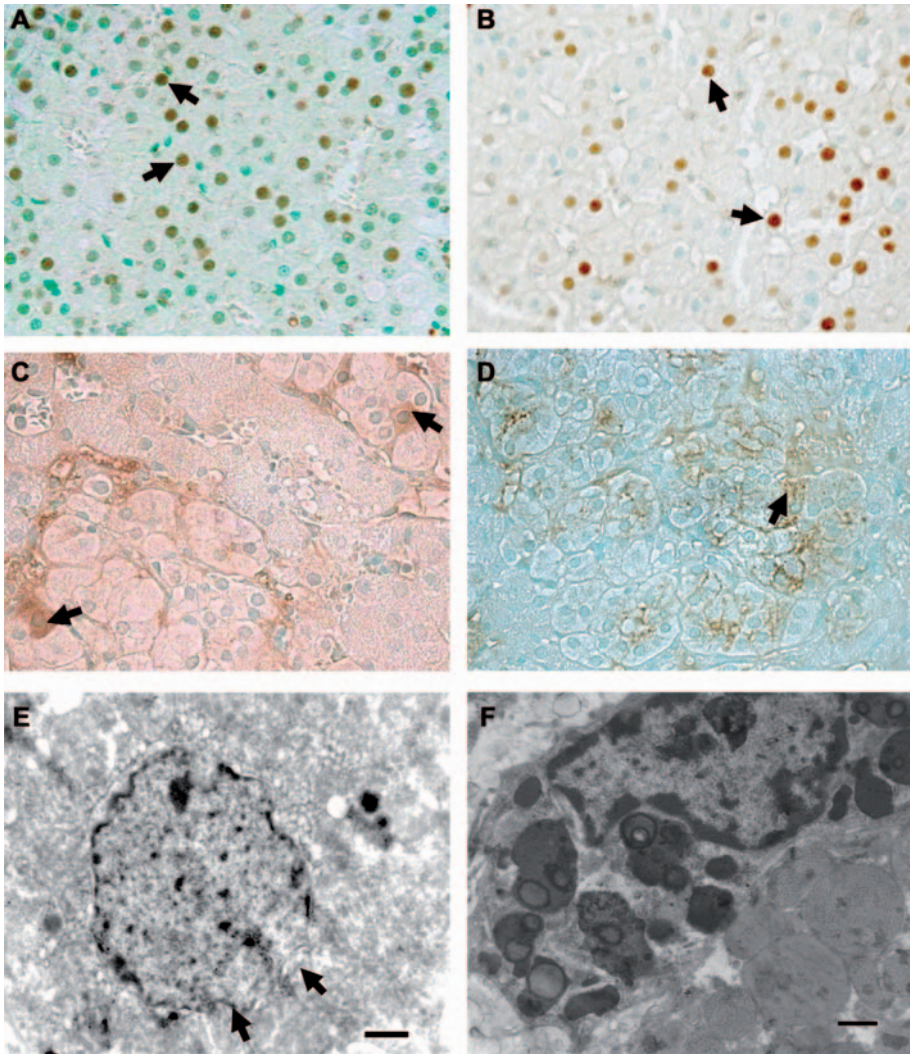

Fig. 1. A) Adrenal reticular zone of a control rat at gestation day 17. TUNEL and methyl green. ×400. B) Adrenal reticular zone of a stressed rat at gestation day 17. TUNEL and methyl green. ×400. C) Adrenal reticular zone of control rat at gestation day 17. Activated caspase 3 and methyl green. ×400. D) Adrenal reticular zone of stressed rat at gestation day 17. Activated caspase 3 and methyl green. ×400. E) Apoptotic nucleus from fascicular zone of the adrenal cortex of a stressed rat at gestation day 17. ×5000. F) Macrophage phagocytosing apoptotic bodies in the reticular zone of the adrenal cortex of control rat at gestation day 12. ×8000. Arrows in (A) and (B) indicate TUNEL positive nuclei. Arrows in (C) and (D) indicate positive caspase 3 nuclei. Arrows in (E) indicate loss of integrity of the nuclear membrane.

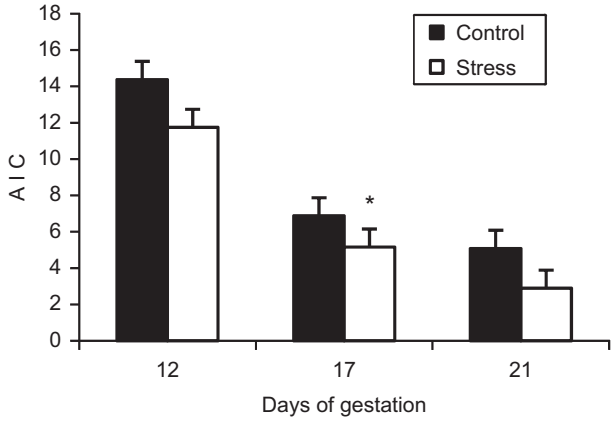

Fig. 2. AIC in adrenal cortex of pregnant rats.

are due to the fact that the TUNEL technique identifies all cells with inter-nucleosomal DNA fragmentation, while the caspase 3 technique shows only cells that undergo apoptosis through the caspase pathway.

We also investigated apoptosis using EM. Figure 1E shows the nucleus of an apoptotic cell from the fascicular zone that contains fragmented chromatin and loss of nuclear membrane integrity. (Fig. 1F) shows the phagocytosis of cytoplasmatic apoptotic bodies by a macrophage in the reticular zone. Different organelles and nuclear rests with plasmatic membrane integrity and mitochondria with vacuoles are also evident in the figure.

Total AIC in the adrenal cortex of pregnant rats

There was a significant decrease in the AIC in the experimental group (5.5 ± 1.73) compared to the control group (6.87 ± 2.07; $p < 0.05$) at gestation day 17, but no significant differences were detected at days 12 and 21 (Fig. 2).

AIC in the zones of adrenal cortex of pregnant rats

The AIC in the three cortical zones in both control and experimental groups are shown in Table 1. For the control group, the AIC in the reticular zone was significantly higher than in the fascicular and glomerular zones ($p < 0.05$) at day 12. At day 17, significant differences among the three cortical zones were observed ($p < 0.05$). At day 21, the AIC in the glomerular zone was significantly lower than in the fascicular and reticular zones ($p < 0.05$) (Fig. 3).

For the experimental group, no significant differences among the three cortical zones were observed at day 12. At day 17, the AIC in the glomerular zone was significantly lower than in the fascicular and reticular zones ($p < 0.05$). At day 21, the AIC in the glomerular zone was significantly lower than in the fascicular and reticular zones ($p < 0.05$) (Fig. 4).

Relation of zone and treatment on AIC

There was no relation among zones, treatment and AIC at gestation days 12 and 21 (Figs. 5, 7). At gestation day 17, however, the AIC of experimental rats was significantly less than the AIC of control rats ($p < 0.05$) (Fig. 6).

Discussion

The homeostasis of the adrenal gland is regulated by both proliferative and apoptotic processes (Hoeflich and Bielohuby 2009). The gland can adapt to various acute and chronic stresses (Nussdorfer and Gotardo 1998). After activation of the HPA axis, ACTH triggers physiologic and morphologic responses in the adrenal cortex, which cause release of glucocorticoids with attendant structural changes in the gland that involve vascularization, cellular hypertrophy and hyperplasia (Bornstein 1996). There is evidence that suggests that in rats

Table 1. Apoptotic index in the zones of the adrenal cortex in pregnant rats

Group	Day of gestation	Glomerular zone	Fascicular zone	Reticular zone
Control	12	3.44 ± 1.14	12.64 ± 1.17	34.68 ± 3.56*
	17	0.10 ± 0.01*	7.37 ± 0.82*	15.67 ± 1.94*
	21	0.15 ± 0.01*	6.12 ± 0.64	7.13 ± 0.98
Stressed	12	7.09 ± 1.21	12.78 ± 1.89	15.76 ± 1.24
	17	0.13 ± 0.02*	3.05 ± 1.26	6.02 ± 2.73
	21	0*	6.31 ± 2.07	7.53 ± 2.41

Data are means $\pm$ SD.
*Significant differences between zones at each day of pregnancy.

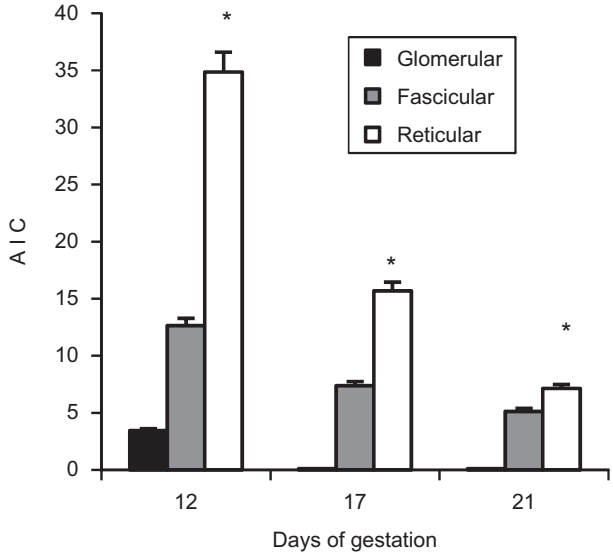

Fig. 3. AIC in zones of adrenal cortex of control pregnant rats.

subjected to chronic stress, the association between central activation of HPA axis and adrenal cortex is disrupted (Ehrhart-Bornstein et al. 1998, Pignatelli et al. 1998). Exposure to chronic stress is accompanied by a strong response of the HPA axis and a progressive return to normal plasma levels of ACTH despite persistent exposure to the stressor. Under the same conditions, CORT levels remain elevated (Mizoguchi et al. 2001). Therefore, ACTH levels often are neither related to elevated glucocorticoid concentrations nor to hyperplasia or hypertrophy of the adrenal gland (Bornstein and Chrousos 1999). The cause of the dissociation of CORT and ACTH levels is unknown (Mizoguchi et al. 2001).

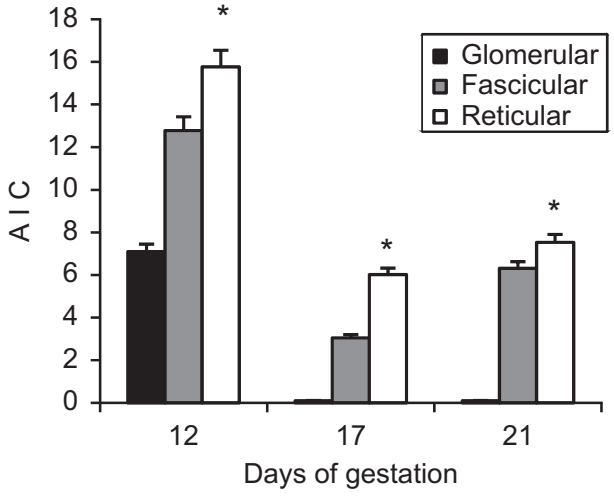

Fig. 4. AIC in zones of adrenal cortex of stressed pregnant rats.

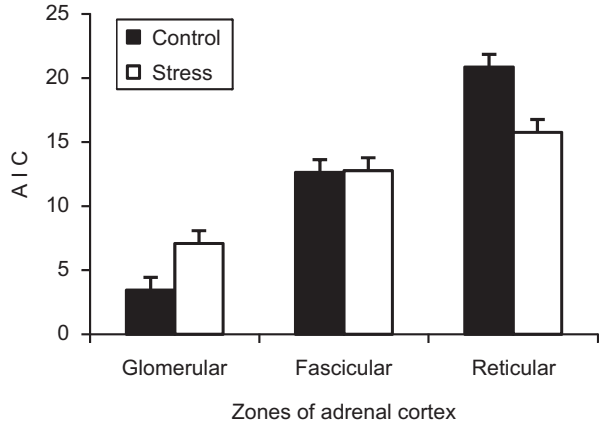

Fig. 5. AIC in adrenal cortex of rats at gestation day 12.

The increased CORT response to ACTH has been associated with a proportional increase in adrenal mass. In fact, enlargement of the adrenal cortex has been described after several types of chronic stress (Aguilera 1996, Gosney 1985, Scaria and Premalatha 1967, Schmidt 1992, Tharp 1975), e.g., cell hypertrophy in the fascicular zone occurs after chronic exposure to toluene (Gotohda et al. 2005), and both hypertrophy and hyperplasia have been observed after streptozotocin induced diabetes (Rebuffat et al. 1998). These data suggest that chronic stress induces adrenal growth in a particular way depending on the cortical zone (Ulrich-Lai et al. 2006).

It has been demonstrated earlier that after chronic stress caused by immobilization of pregnant rats, the total adrenal gland weights showed a pattern similar to that of control rats after immobilization. There was a significant increase of adrenal weight, however, at gestation day 17 in pregnant rats (Soñez 2001). The mechanism for this increase is unknown.

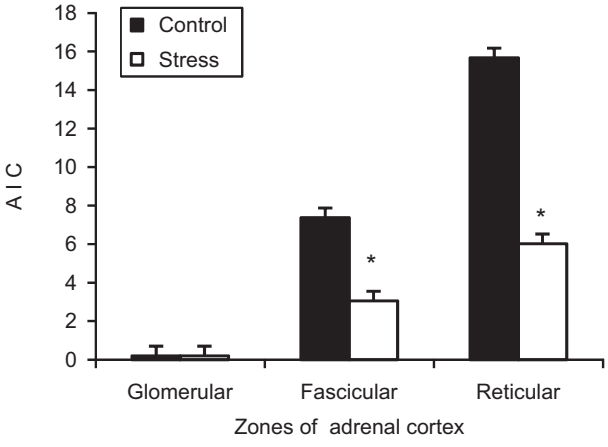

Fig. 6. AIC in adrenal cortex of rats at gestation day 17.

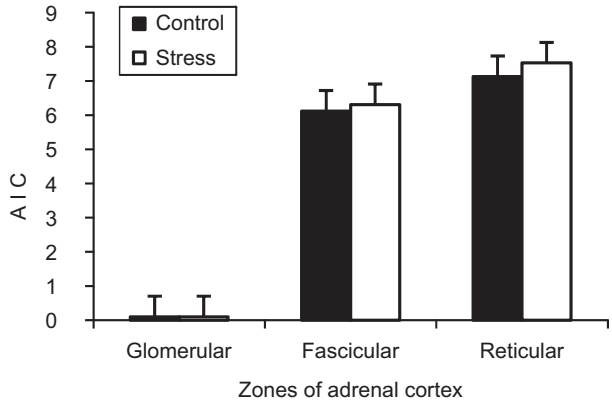

Fig. 7. AIC in adrenal cortex of rats at gestation day 21.

We demonstrated that the AIC was significantly reduced in the adrenal cortex at gestation day 17, which suggests that the AIC is related directly to changes in the plasma concentration of hormones associated with pregnancy and stress. We infer that the AIC is related to adrenal gland weight, because at gestation day 17, experimental rats showed low AIC in the fascicular and reticular zones, which may contribute to the increased weight of the gland.

Analysis of the normal apoptotic process in the different zones of the adrenal cortex is complex. Blanco et al. (2001) found apoptotic nuclei in all three adrenocortical zones, but they were more frequent in the reticular zone. On the other hand, Carsia et al. (1998) found apoptotic nuclei in the fascicular and reticular zones, but not in glomerular zone. Wolkersdorfer et al. (1996) also detected apoptotic nuclei in all three zones of the adrenal cortex, but the AIC was highest in glomerular zone and lowest in reticular zone.

We found that the AIC was higher in the reticular zone and lower in the glomerular zone in both control and experimental rats. Earlier we demonstrated that the cell proliferation index (CPI) of the reticular zone in both control and experimental rats was significantly decreased compared to the glomerular and fascicular zones for all three stages of gestation studied (Bozzo et al. 2011).

Our findings are consistent with the cell migration theory that describes cortical cell proliferation in the external part of the adrenal cortex, and migration and differentiation of glomerular zone cells to fascicular zone cells, then from fascicular cells to reticular cells, where they degenerate and die (Vinson 2003). In this way, cell death controlled by the adrenal gland may be essential for the functional zonal architecture, because cells undergoing apoptosis are found mainly in the reticular zone. For these reasons, the reticular zone has been described as a cell aging and death zone (Willenberg et al. 1998).

ACTH stimulates the expression and secretion of thrombospondin 2 that is found in the fascicular and glomerular zones, but is absent from the reticular zone. This protein promotes cell adhesion and inhibits apoptosis in the zones where it is present. Therefore, thrombospondin 2 may act as an anti-apoptotic protein that is controlled by ACTH (Feige et al. 1998, Otis et al. 2007).

In experimental rats subjected to immobilization stress, plasma levels of prolactin (PRL) are increased significantly at gestation day 17 (Soñez et al. 1996). Increased prolactin may reduce apoptosis as reflected by the lower AIC observed in fascicular and reticular zones of experimental rats compared to controls. These interpretations are supported by the fact that prolactin promoted cell proliferation and blocked apoptosis (Krishnan et al. 2003). Prolactin also stimulated Bcl-2 expression and caused adrenal hypertrophy; hyperprolactinemia increases adrenal weight as a consequence of cell hypertrophy (Silva et al. 2004). Moreover, it has been demonstrated that activation of caspase 3 is a characteristic of glucocorticoid-induced apoptosis and that apoptosis is inhibited by high plasma levels of prolactin (Krishnan et al. 2003).

High concentrations of plasma estrogens have been demonstrated at gestation day 17 in experimental rats (Soñez et al. 1996). It has been reported also that estrogens reduce Bax pro-apoptotic protein in the adrenal cortex and that DNA fragmentation is reduced by 50% compared to controls (Tronko et al. 2009).

Bcl-2 is related to inhibition of apoptosis, because it prevents loss of cytochrome c from mitochondria. Estradiol stimulates Bcl-2 production in several tissues including the adrenal cortex. Estradiol treatment increases the expression of estrogen receptors and reduces the activity of pro-apoptotic proteases such as calpain and caspases 3 and 9 (Tronko et al. 2009). This suggests that estrogens may exert an anti-apoptotic effect on the adrenal cortex at gestation day 17.

We also found that chronic stress in pregnant rats caused a reduction in proliferation (Bozzo et al. 2011) and apoptosis in the adrenal cortex. Our results indicate a lower degree turnover of adrenal cortical cells.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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