

Relative Concentrations of Placental Lactogen II and PRL-Like Protein-A in Stressed Rats Placenta

Concentraciones del Lactógeno Placentario II y la Proteína A Ligada a Prolactina en Placentas de Ratas Estresadas

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SUMMARY: The chronic stress induces functional adaptations in the hypothalamo-pituitary- adrenocortical (HPA) and in the sympathetic-medullary-adrenal axis (SAM). Both axis are considered vital regulators of the homeostasis in vertebrates (Seyle, 1936; Ostrandrer *et al.*, 2006). On the other hand, the placenta provides highly specialized functions during gestation that are critical for the normal development of the embryo/fetus (Soares *et al.*, 1991). We hypothesized that the chronic immobilization (IMO) stress in pregnancy rats produces alterations in prolactin concentrations in placental tissue and also changes in the response of SAM axis. Chronic stress by IMO was applied on days 12, 17 and 21 of pregnancy rats. Relative concentrations and localization of placental lactogen-II (PL-II) and the PRL-like protein A (PLP-A) in chorioalantoic placenta were estimated by Immunoblotting and Immunocytochemical analysis. The levels of catecholamines metabolite, acid 3-metoxi 4-hidroxi mandélico (VMA), were analyzed in stressed rats urines on 6,12,17,21 days of pregnancy, by HPLC, in order to determine the response of SAM axis. During the days of the pregnancy studied, chronic stress did not induce any changes neither in the localization nor in placental concentrations of PL-II and PLP-A. The VMA values in stressed mothers urines increased on the day 6 respecting the control ones at the same time of pregnancy. VMA values in stressed rats at 21 days of pregnancy are smaller than the respective controls. We conclude that the chronic stressed mothers activated the SAM axis at the beginning of pregnancy and then they diminished the metabolites catecholamines that were interpreted as a stress adaptation coincident with normal concentrations of both placental prolactines at this stage of the pregnancy.

KEY WORDS: Chronic stress; Placental lactogen; Prolactin; Rat; SAM

INTRODUCTION

During pregnancy by maintaining both maternal and fetal homeostasis, mammals need to reorganize their metabolism. Successful maturation of the mammalian embryo is largely dependent upon the functioning of a specialized extraembryonic tissue of pregnancy, the placenta. (Soares, *et al.*, 1991, Georgiades *et al.*, 2002).

The rodent possesses two placental structures: *choriovitelline* placenta, which develops first (until the 11 day of pregnancy) and *chorioallantoic* placenta, which shows considerable developmental changes as gestation advances. Two prominent regions are formed within the chorioallantoic placenta: (1) *junctional zone* (JZ) and (2) *laberynth zone* (LZ). Four differentiated trophoblast cell phenotypes

comprising the rat chorioallantoic placenta can be readily identified: (1) trophoblast giant cells, (2) spongiotrophoblast cells (3) glycogen cells, and (4) syncytial trophoblast cells (Davies & Glaser, 1968; Soares, *et al.*, 1993); Ain *et al.*, 2003; Georgiades *et al.*; Pijnenborg & Vercruysse, 2005).

Trophoblast giant cells and spongiotrophoblast cells represent endocrine cells of the rat placenta which belong to prolactines (PRL) family (Soares, *et al.*, 1991,1996). The PRL family consists of 19 genes in rats (Soares & Linzer, 2001); Wiemers, *et al.*, 2003).

The predominant placental lactogen in the second half of pregnancy rat is known as placental lactogen-II (PL-II),

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(25kDa) glycosylated protein which has biological actions similar to those of pituitary PRL (Colosi, *et al.*, 1982; Faria, *et al.*, 1990; Robertson & Friesen, 1982).

PLP-A is a glycoprotein synthesized as two distinct mol wt species 29 and 33 kDa. which participates in the association of natural killer lymphocytes of uterine decidua (Cambell *et al.*, 1989; Deb & Soares, 1990; Ain *et al.*; Rupsari *et al.*, 2003).

The rats placenta synthesizes and secretes a number of polypeptide and steroid hormones (Soares, *et al.*, 1991). Many of which are identical or at least, they resemble those produced by the hypothalamic-pituitary-target (Jaffe, 1993).

Despite it is well known that stressing situations simultaneously activate both axes: the hypothalamo-pituitary-adrenocortical (HPA) and the sympathetic-medullary-adrenal axis (SAM), it has been postulated that both respond to differential characteristics of stressing stimuli (Vigas *et al.*, 1980; Kant *et al.*, 1987; Armario *et al.*, 1986; Martí *et al.*, 1993, 1994; Shulkin, 1999). The activation of the sympathetic nervous system by the corticotropin-releasing hormone (CRH) results in the release of noradrenaline (NA) from the peripheric sympathetic nervous ends. Sympathetic innervation reaches the adrenal medulla which releases adrenaline (A) in a greater proportion and also something of NA Nankova *et al.* (1996), De Boer *et al.*, 1989; Curtin *et al.*(1996); Kvetnansky *et al.* (1980). Increased plasma levels of A and NA are present in stress reaction, in order of importance, represent the stress second hormone. Our aims are: i. to determine the effects of the chronic stress applied to pregnant rats on relative concentrations of PL-II and PLP-A protein in placenta. ii. To evaluate the response of SAM axis in pregnant females subjected to IMO chronic stress.

MATERIAL AND METHOD

Animals. We experimentally used young female primipar Wistar-albino rats (90-120 days) of 200 to 300 g. They were housed four by cage and maintained under lab standard conditions at $22 \pm 2^\circ\text{C}$, controlled photoperiod of 12 h (lights on 7:30 a.m. to 7:30 p.m.), room humidity. Food and water were administered *ad libitum*. The rats were cycled by colpocytograms in fresh to determine the estro day between 10:00 a.m. and 11:00 a.m. Females in estro were kept with a male of the same strain and copula was verified by the presence of spermatozoid in fresh vaginal exudates. To carry out the programmed sacrifice, the day after spermatozoid were detected in vaginal fluid was considered as day zero of

pregnancy. Pregnant females were separated in two groups: the control (C) and the experimental stress (E). Control groups females remained in their cages until sacrifice.

Experimental treatment. Chronic stress by immobilization (IMO) was applied to the experimental group females. Rats were immobilized on a wooden board (20 x 20 cm) by taping their four limbs to metal mounts, according to the method described Michajloskij *et al.* (1988). Each female was subjected to a 45 min stress session, from day 4 of gestation, 3 times a week, in the morning and at variable times. This process lasted until their sacrifice.

VMA concentrations were determined considering the volumes of 24 h urine and the animal body weight. The animal was checked on the days 6, 12, 17 and 21 of pregnancy.

Pregnant rats of the control and stress groups were sacrificed by decapitation on days 12, 17 and 21 of gestation. Placenta were removed and kept on ice until processing.

Obtention of placentary homogenates to apply immunoblot technique. Placenta homogenates were obtained by the method -with some modifications- described by Campbell *et al.* Placenta of the animals of the different experimental groups were rapidly removed and kept at -70° until use. These samples were then homogenized in ammonium bicarbonate buffer [100 mM NH_4HCO_3 (pH 9.3), 100 mM NaCl and 0.5mM methyl sulphonic fluoride (PMSF)]. Homogenates were centrifuged in the cold at 12000 X g for 15 min. Supernatant was recovered and centrifuged at 4°C , for 15 min at 100000. It was fractioned and the aliquots of each sample were separated for proteins measurement and kept at 20°C until use.

Immunoblotting. Relative concentrations of PL-II and PLP-A protein in the placenta of control and stressed rats, during the second half of gestation were estimated by immunoblotting.

Samples were suspended in sweep buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue and 50 mM DTT) and 5 μg of proteins from each sample were seeded on a analytical gel of 12% polyacrilamide (zone were 27-33 kDa prolactines are resolved). Protein concentrations of the cytosol preparations were estimated by method of Bradford. Cytosol preparations were separated by SDS-polyacrilamide gel electrophoresis in 12.5% (zone were 27-33 kDa prolactines are resolved). A marker of an appropriate molecular weight was seeded on a lane of the gel and an extra run lane of homogenate that was used as negative control. Electrophoresis was done

on sweep buffer (0.3% Tris base, 1.44% glycine, 0.1% SDS, pH 8.3), for 90 min (27 mA/gel, 100 V) and then transferred to nitrocellulose membranes (60 min in the cold, 100 V). Efficiency of transfer was confirmed with 0.5% Ponceau Red. The membrane was blocked with a TBS buffer solution (100 mM Tris base, 8 g/l de NaCl, pH 7.6) with 0.05% Tween-20 (TBS-T) and 5% low fat milk (Molico) and agitated for 1 h at room temperature. Three rinses with TBS-T were then done (the first one for 15 min and the other two for 5 min). The membrane was incubated either overnight (in cold chamber with agitation) or for 1 hour at room temperature with a solution of the first antibody (anti PL-II and anti PLP-A respectively) prepared on TBS-T with 1% BSA. PL-II and PLP-A protein were detected with anti-peptide antisera directed to aminoacids 56-70 of PL-II, Deb (1989), and aminoacids 152-164 of PLP-A, Deb (1990), respectively. The next day this membrane was thrice rinsed with TBS-T three rinses washes with TBS-T were performed. It was incubated for 1 hour at room temperature, with the second corresponding antibody (rabbit anti-IgG, prepared in goat, 1:2000 bounded to alkaline phosphatase enzyme). The membrane was then rinsed and revealed using a commercial kit (Dako, Co, Carpinteria, Ca). Bands images were swept with an AGFA scanner integrated to a PC and the program Sigma-Gel™ was used. Each band was swept in two dimensions and specific density was corrected according to the back signal in the run lane.

Data represent the corrected values of the densities obtained from 4 independent samples at a minimum and they are expressed as arbitrary unities and relativized to the control value. Eight samples (4 for controls and 4 for stressed) were analyzed by immunoblots (n = 8).

Statistics. The values obtained in each immunoblots were analyzed by a two factors ANOVA with the statistical program SSPS9 Windows. These values were expressed as means (\pm SEM), the differences were considered as statistically significant if $p \leq 0.05$.

Immunocytochemistry. (Bullock & Petrusz, 1983) immunochemistry technique was used to identify the cells that synthesize placental prolactines PL-II and PLP-A.

The placenta obtained at days 12, 17, and 21 days of pregnancy were fixed in phosphate buffer (PBS, pH 7.6), for 24 hours. Then they were rinsed in PBS, dehydrated through an alcohol ascending grades cleared in xylene and paraffine embedded. Tissue cuts of μ m thick were deparaffinized, hydrated and H_2O_2 treated to block endogenous peroxidase. They were treated with horse normal serum (2% in PBS) and incubated overnight at 4°C with specific antibodies. After washing, they were treated

with the second biotinylated antibody (anti-rabbit developed in goat) (Vector Laboratories, USA) for an hour at room temperature and then rinsed again in PBS and treated with Avidine-Biotin-Immunoperoxidase kit (ABC, Vectastain, Vector Laboratories). Finally, the sections were incubated with 3-3'-diaminobenzidine, in the presence of H_2O_2 to reveal peroxidase expression sites. The reaction was stopped in distilled water. An antiserum to aminoacids 56-70 of rat PL-II, Deb *et al.* (1989), was used to determine the distribution of PL-II, and an antiserum to aminoacids 152-164 of PLP-A, Deb *et al.* (1989b), were used to determine the distribution of PLP-A. These antibodies were provided by Dr. Michael J. Soares. Department of Physiology. University of Kansas Medical Center. U.S.A.

RESULTS

Placental localization of PLII y PLP-A. Immunocytochemistry technique was applied on placental tissue of chronic IMO stressed rats and the localization and intensity of PL-II and PLP-A immunomarks were evaluated. The localization of both placental prolactines coincided with Campbell *et al.* findings.

The cytokine/hormone PLP-A was identified in small granules of the cytoplasm of the trophoblast giant cells (TGC) of the bounding zone of chorioallantoic placenta in both, control and stressed rats on day 12 of pregnancy (Fig. 1).

No differences were evidenced neither in immunomarked cellular types nor in the immunomark intensity between the stressed rats placenta respecting those of the control ones.

PL-II immunomarkation was localized in the cytoplasm of the giant trophoblastic cells in the decidua region associate to placental tissue in both groups the control and stressed rats at day 12 of pregnancy (Fig. 2).

The comparative analysis between placenta of control and stressed rats of 17 and 21 days of pregnancy did not show substantial changes neither in the distribution nor in the intensities of the immunomarks of PL-II and PLP-A proteins. These results are shown in (Figs. 1 and 2).

Determination of concentrations of PL II and PLP-A, by immunoblot, in placenta of stressed rats. The effects of chronic stress applied to gestating mothers, on the concentrations of PL II in the placental tissue at 12, 17 and 21 days of pregnancy, were analyzed using the immunoblot technique (Fig. 3).

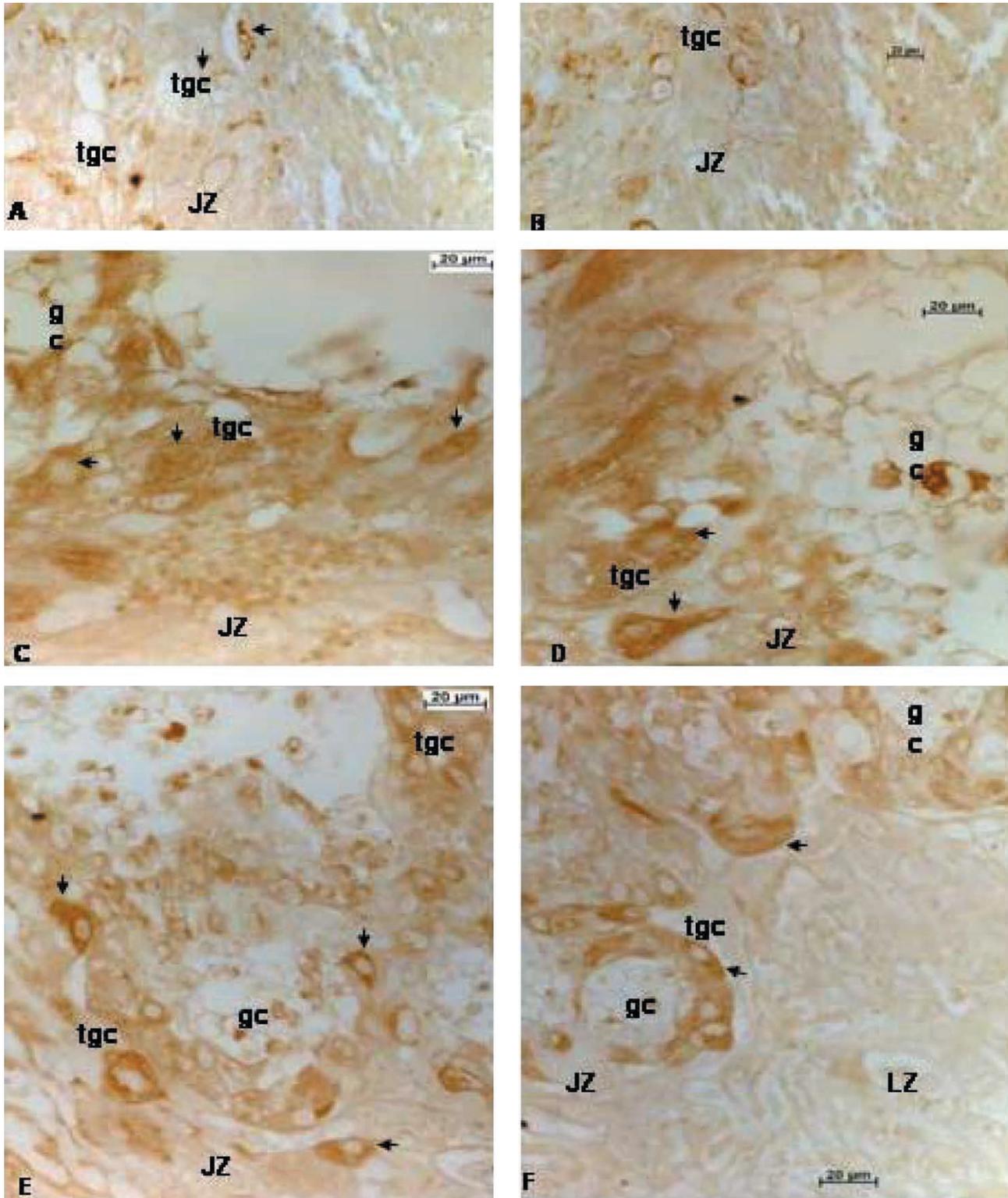


Fig. 1. Immunocytochemical analysis of PRL-like protein A (PLP-A) with sections of placental tissue from days 12, 17 and 21 (control and stressed) (A-F). PLP-A was staining in the trophoblast giant cells (tgc) cytoplasm located at the junctional zone (JZ), from day 12 of pregnancy rats (A-B). Note the intense staining in the tgc cytoplasm and nucleus, around glycogen cell (gc) in JZ and labyrinth zone (LZ), from days 17 and 21 control and stressed pregnancy rats (C-D-E-F).

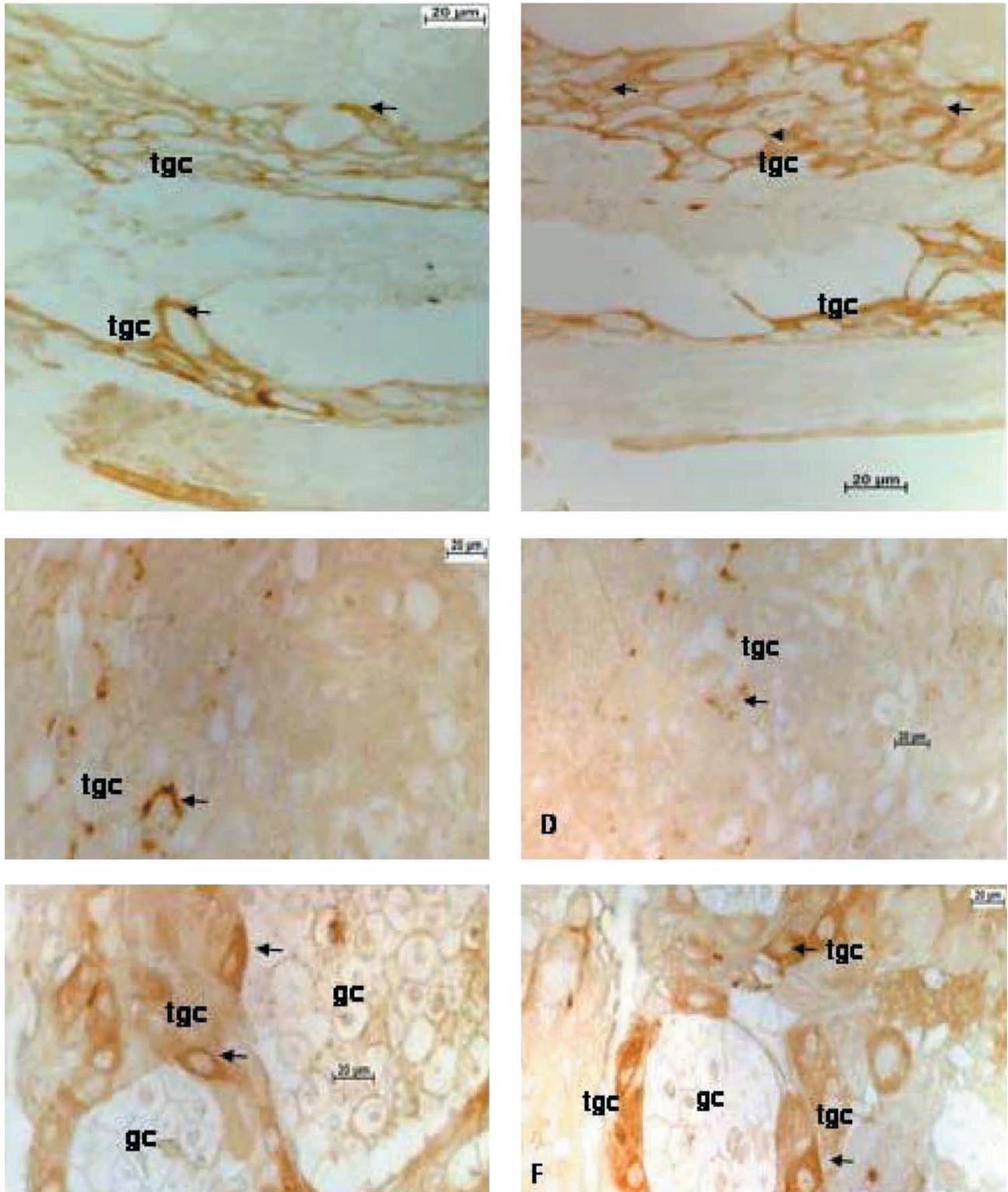


Fig. 2. Immunocytochemical analysis of PL-II with sections of placental tissue from days 12, 17 and 21 (control and stressed) (A-F). PL-II was staining in the trophoblast giant cells (tgc) cytoplasm, located at the interface between the chorioallantoic placenta and uterine deciduas, from day 12 of pregnancy rats (A-B). Note the intense staining in the tgc cytoplasm, around glycogen cell (gc) in JZ and LZ, from days 17 and 21 control and stressed pregnancy rats (C-D-E-F).

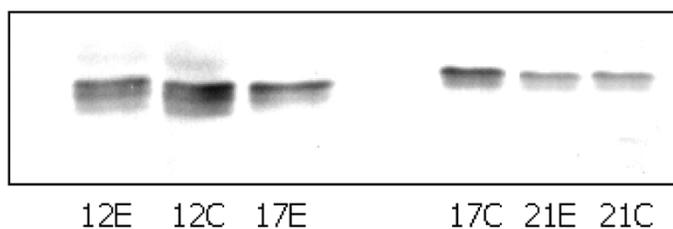


Fig. 3. Representative immunoblots, probed with antipeptide antisera specific to PL-II, in placental homogenates on days 12, 17, 21 of pregnancy, in stressed and control rats.

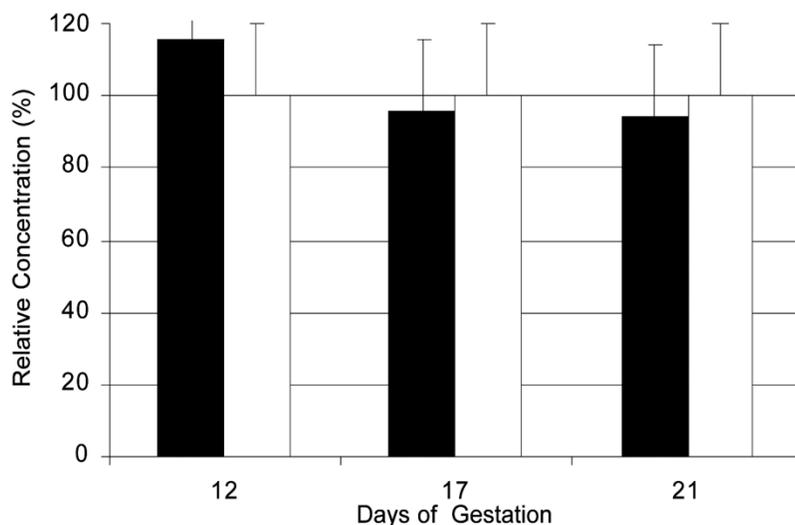


Fig. 4. Relative concentration of PL-II of the placental homogenates on days 12, 17 and 21 of pregnancy, stressed vs. control rats. Values represent desintometric reading from several replicate immunoblots and are expressed as a percentage of the highest value (100%).

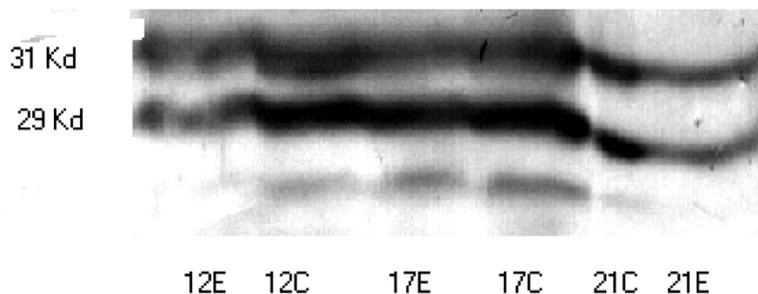


Fig. 5. Representative immunoblots, probed with antipeptide antisera specific to PLP-A of 33 kDa and PLP-A de 29 kDa, in placental homogenates on days 12,17,21 of pregnancy, in stressed and control rats.

No significant statistical differences were observed between the mean values of the concentrations of PL-II between the placental homogenates of stressed rats and control ones without treatment at days 12, 17 and 21 of pregnancy (Fig. 4).

PLP-A concentrations in placental tissue of rats chronically stressed were quantified. Fig. 5 shows a representative immunoblot of PLP-A. In this immunoblot it can be observed the two bands corresponding to the two molecular species PLP-A of 33 kDa and PLP-A of 29 kDa, respectively. The values of PLP-A of 33 kDa and PLP-A

of 29 kDa obtained when quantifying the bands of each immunoblot until n=8 was completed were statistically analyzed and graphicated. No significant statistical differences were found between the mean values (\pm SEM con un $p < 0.05$) in neither the concentrations of PLP-A of 29 kDa nor the PLP-A of 33 kDa of the placental homogenates of stressed mothers when they were compared with the control group in each one of the three days of pregnancy studied (Figs. 6 and 7).

Response of the sympathetic-adrenomedullary axis of rats subjected to stress during gestation. Considering

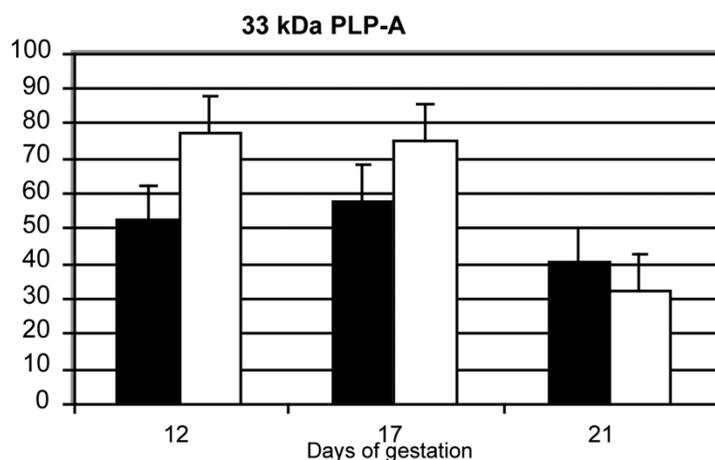


Fig. 6. Relative concentration of PLP-A (29kDa) of the placental homogenates on days 12, 17 and 21 of pregnancy (stressed vs control rats). Values represent densitometry reading from several replicate immunoblots and are expressed as a percentage of the highest value (100%).

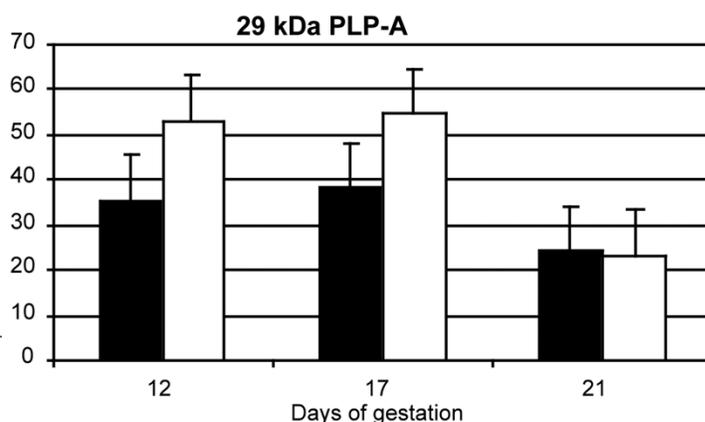


Fig. 7. Relative concentration of PLP-A (33kDa) of the placental homogenates on days 12, 17 and 21 of pregnancy (stressed vs control rats). Values represent densitometry reading from several replicate immunoblots and are expressed as a percentage of the highest value (100%).

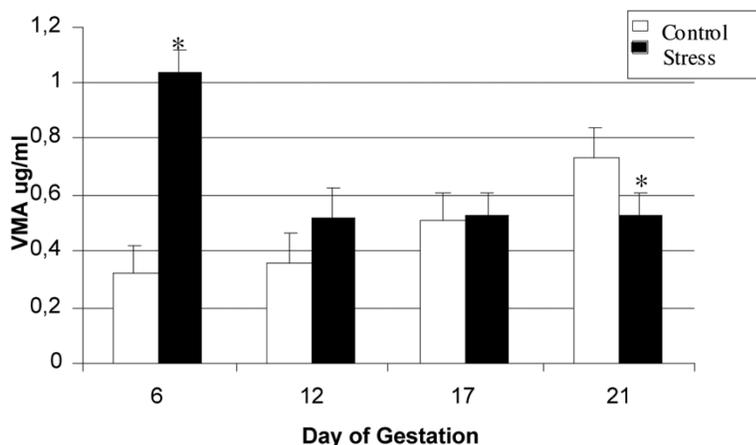


Fig. 8. Levels of VMA in urine of 24 h. Expressed for 100 g of body weigh, in control and stressed pregnancy rats, and the values are expressed: medium \pm SEM * $p \leq 0.05$.

that the second of the peripheral systems of the response to stress, is the activation of SAM and that one of the indicator parameters of this axis functioning are the concentrations of catecholamines A and NA, the levels of 3-methoxy 4-hydroxymandelic (VMA) acid were evaluated. Since this acid is one of the metabolites of catecholamines, it was evaluated in the urine of pregnant rats to relate these peripheric values to the results obtained from the different variables analyzed in placental homogenates from mothers under the effects of chronic stress.

The catecholamine metabolites, 3-methoxy 4-hydroxymandelic (VMA) acid as well as homovanilic acid were found in animals urine. VMA is produced as metabolite of both A as well as NA from the sympathetic nervous system, central and peripheric and from the adrenal gland (Amam, *et al.*, 1981; Fukuda *et al.*, 1996).

On day 6 of gestation the pregnant females began being stressed (n=8). IMO stress sessions were performed and on days 6, 12, 17 and 21 of pregnancy the urines corresponding to both groups studied were extracted.

The values of VMS metabolite obtained by High Performance Liquid Chromatography (HPLC) in samples of rat urine were analyzed using repeated measures ANOVA which showed a significant interaction between treatment and time ($p \leq 0.05$) (Fig. 8).

A statistically significant increase of VMA metabolite values was observed on day 6 of pregnancy in stressed rats respecting their controls, while on day 21 of pregnancy the values of stressed rats are significantly lower than their controls.

As pregnancy develops VMA values in stressed rats urine decreased on times 12, 17 and 21 compared to day 6 of gestation.

A significant increase of VMA levels was observed in the control animals on day 21 compared to days 12 and 17 of pregnancy.

DISCUSSION

Placenta synthesizes hormone/cytokines of PRL family, which characterize by their structural similitude and biological actions similar to the prolactines synthesized in the anterior pituitary (Soares *et al.*, 1996).

Studies on the response of hypophisiary PRL stress are not concluding. Williams *et al.*, (1999) did not find any variations in the levels of maternal PRL in stressed rats when applying IMO chronic stress and accute stress by means of several stressors. However, Armario *et al.* demonstrated a significant decrease of maternal PRL in stressed rats ending gestation.

We have demonstrated an increase in the plasmatic concentration of maternal PRL on stressed rats in their 17 day of pregnancy followed by a significant decrease towards the end of gestation (Soñez *et al.*, 1996).

PL-II is first expressed in placenary JZ and the LZ which would favor its access to maternal blood and to fetal vessels. Cambell *et al.*; Soares *et al.* (1991). On the other hand, there are receptors of high affinity to estrogen and progesterone in placenary JU which leads to presume some direct effect of these steroids on the PL II expression in this area. Besides, the fetectomy reduces PL II levels in maternal serum in the second stage of gestation (Cambell *et al.*).

PL-II participates in the signals translation to activate the receptor of pituitary PRL; these ligands are critical to maintain pregnancy during the half of pregnancy and just before delivery when a decrease of hypophisiary PRL levels takes place (Soares *et al.*, 1996). Some factors liberated by decidua that have not been identified yet, control the gradual declination of expression of PL II and an increase of PLP-A in the bounding zone of rat placenta (Cambell *et al.*).

Neither concentrations of PL-II nor the PLP-A concentrations showed alterations in placenary tissue of rats subjected to chronic IMO stress at the three stages of pregnancy studied when they were compared with their respective controls. Even when changes are produced in the plasmatic levels of steroid hormones in IMO stressed mothers, the concentrations of PL-II and PLP-A are not affected.

PL-II placed in the different cellular types of JZ and LZ, modifying its location according to the stage of pregnancy. At first it placed in TGC of JZ and in the TGC of LZ in advanced pregnancy. This result coincides with those obtained by some other authors (Cambell *et al.*).

PLP-A placed in TGC cytoplasm in the JZ of the chorionviteline placenta while in the corionallantoic placenta in advanced pregnancy it has nuclear and cytoplasmatic localization in TGC of JZ and in the spongiotrophoblast cells. The nuclear detection of PLP-A suggests that it participates in regulating mechanisms that occur in the TGC nucleous.

The localization of this hormone/cytokine coincides with the findings of Cambell *et al.*; Deb & Soares. It is known that the PLP-A biological activity is to bound to "natural killer" lymphocytes, in a specific way, in the mesometrial compartment of the uterus of pregnant rats. "natural killer" lymphocytes participle in the immune response, they increase in the half of pregnancy and then decrease at the end of this (Muller Heiner Liu *et al.*,1991; Soñez *et al.*; Ain *et al.*).

One of the peripheral stress markers are the levels of catecholamines metabolites, indicators of SAM axis response. High concentrations of VMA were found in urine of stressed pregnant rats when analyzing this at the beginning of (day 6) of pregnancy. The increase observed in the first stage of pregnancy would be probably due to the liberation of A and NA, which are precursors of the metabolite VMA produced by IMO (Curtin *et al.*).

According to Fukuda *et al.*, NA is liberated under psychological stimulus and since, IMO stress is a very strong physical stimulus, the VMA increase at the beginning of pregnancy would be a consequence of the liberation of A by the sympathetic nervous system. When the stimulus is repeated in a chronic way there is a decrease in catecholamines production that might be explained as a consequence of the habituation to the same stimulus (Kvetnasnsky *et al.*; Kant *et al.*).

A VMA decrease may be also observed on day 21 of pregnancy in stressed rats respecting to the control. This demonstrates a rapid loss of VMA profile towards the end of pregnancy. This might be due either to an exhaustion of the SAM axis or to the fact that repeated expositions to chronic stress produce a decrease in A and NA liberation what is interpreted as an adaptation of the axis to this system (Rodríguez, 2000).

Considering our results of the SAM axis response to the chronic stress effects in pregnant rats, we may conclude that a SAM axis profile decay is produced. This is probably due to a habituation of the animals to the repeated stimulus.

Although alterations are produced in the plasmatic levels of hypophisiary PRL in stressed pregnant rats, we did not find differences neither in the localization nor in the concentrations of PL-II and PLP-A in the different cellular types of placenary tissues of stressed mother. This result might imply a series of

complex mechanisms of autocrine and/or paracrine molecular signals that would act via specific receptors in the placental cells in order to maintain tissue homeostasis and protect the embryo of the stress harmful effects.

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RESUMEN: El estrés crónico induce adaptaciones funcionales en los ejes hipotálamo-pituitario-adrenal (HPA) y en el simpático médulo adrenal (SAM). Ambos ejes son considerados reguladores vitales de la homeostasis en los vertebrados (Seyle, 1936; Ostrandrer *et al.*, 2006). Por otro lado, el desarrollo y crecimiento fetal de los mamíferos dependen en gran medida del buen funcionamiento de la placenta (Soares, 1991). Nosotros hipotetizamos que el estrés crónico por inmovilización (IMO) aplicado a las ratas gestantes produce alteraciones en las concentraciones de las prolactinas en el tejido placentario y cambios en la respuesta del eje SAM. Se le aplicó estrés crónico por IMO a las hembras en los días 12, 17 y 21 de la preñez y se analizó por inmunocitoquímica e inmunoblotting la localización y concentraciones del lactógeno placentario dos (PL-II) y la proteína A ligada a la prolactina (PLP-A) en la placenta. Se analizaron por HPLC, en las orinas de ratas preñadas (6,12,17,21 días), los niveles del metabolito de las catecolaminas, (ácido 3-metoxi 4-hidroximandélico) (VMA), a fin de determinar la respuesta del eje SAM al tratamiento. El estrés crónico no indujo cambios tanto en la localización como en las concentraciones de PL-II y PLP-A en las placentas en los días de la preñez estudiados. Los valores de VMA en las orinas de las madres estresadas se incrementaron en el día 6 con respecto al control del mismo tiempo de preñez. Mientras que a los 21 días los valores de VMA de las ratas estresadas son menores que los controles respectivos. Concluimos que en las madres estresadas crónicamente, no se alteraron las concentraciones de ambas prolactinas placentarias. En cambio se activó el eje SAM al comienzo de la preñez ante el primer estímulo estresante y luego una reducción de la respuesta del eje ante el estrés crónico, a medida que avanza la preñez.

PALABRAS CLAVE: Estrés crónico; Lactógeno placentario; Placenta; Prolactina; Rata; SAM.

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