

IL-2 is involved in immune response of prenatally stressed rats exposed to postnatally stimulation

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ABSTRACT. Environmental cues influence growth and development of mammals during prenatal and particularly early postnatal life and can exert long-lasting effects in adult life. High circulating concentration of glucocorticoids during pregnancy (prenatal stress) affects the activity of the hypothalamic-pituitary-adrenal axis (HPA) of offspring and has been linked to alter immune system responses. Early postnatal stimulation (handling) of prenatally stressed animals generates long-term beneficial effects on the reactivity of the HPA axis and immune system function. The aim of this study was to further investigate the effect of handling on immune response in prenatally stressed male rats and to elucidate a possible relationship with the HPA axis activity. Control and prenatally stressed (PS) offspring by immobilization (IMO) were handled during the first week of life. Animals from both treatments were subjected to acute stress by IMO. Corticosterone (COR) plasma concentration was measured by RIA assay, T lymphocyte proliferation by [³H] thymidine assay and IL-2 levels by direct ELISA technique. Chronic IMO prenatal stress caused an increase in mother plasma COR basal levels. Furthermore, prenatally stressed rats subjected to an acute stress session had lower T cell proliferation and decreased IL-2 release. In addition, early postnatal stimulation reversed the negative effects of prenatal stress on proliferation of T lymphocytes and IL-2 release.

Key words: early stimulation, prenatal stress, hypothalamic-pituitary-adrenal axis, immune system.

RESUMEN. Las condiciones ambientales influyen en el crecimiento y desarrollo de los mamíferos durante la vida prenatal, posnatal temprana y pueden ejercer efectos a largo plazo en la vida adulta. Altas concentraciones circulantes de glucocorticoides durante el embarazo (estrés prenatal) afecta a la actividad del eje hipotálamo-pituitario-adrenal (HPA) de la descendencia y se encuentra en relación con alteraciones de las respuestas del sistema inmunológico. La estimulación postnatal temprana de los animales estresados prenatalmente genera efectos beneficiosos a largo plazo en la reactividad del eje HPA y en la función del sistema inmunológico. El objetivo del estudio fue investigar el efecto de las manipulaciones posnatales tempranas sobre la respuesta inmune de ratas machos y dilucidar la posible relación con la actividad del eje HPA. Crías controles y estresadas prenatalmente (PS) por inmovilización (IMO) se manipularon durante la primera semana de vida. Los animales controles y PS fueron sometidos a estrés agudo por la IMO. Los niveles de corticosterona (COR) plasmática se midieron por el ensayo de RIA, la proliferación de linfocitos T por [³H] timidina y los niveles de IL-2 por la técnica de ELISA directa. El estrés prenatal crónico por IMO aumentó los niveles plasmáticos basales de COR en la madre, mientras que las crías PS sometidas a una sesión de estrés agudo posnatal mostraron disminuida la proliferación de células T y niveles menores de IL-2. En conclusión, las estimulaciones posnatales tempranas revertieron los efectos negativos provocados por el estrés prenatal sobre la proliferación de los linfocitos T y la liberación de IL-2.

Palabras claves: manipulaciones posnatales tempranas, estrés prenatal, eje hipotálamo-pituitario-adrenal, sistema inmune.

INTRODUCTION

Prenatal environment exerts a profound influence on the development of an organism and may predispose to adaptive disturbances in postnatal life. Stress during pregnancy can alter postnatal physiological responses in the offspring. The degree and extension of these alterations depend upon nature, intensity and duration of the stressor as well as on the stage of gestation at which the stressor is applied (Mayer *et al* 2011). Stress in the pregnant female produces numerous endocrine changes, chiefly increased in circulating levels of catecholamines (CA), namely epinephrine and norepinephrine and glucocorticoids (GC), all of which are considered the major endocrine mediators

of stress-induced immune changes (Maccari *et al* 2003). Releases of these hormones into the mother's blood stream directly act on fetal development through fetal gene regulation or indirectly through changes in the metabolism of the placenta (Maccari *et al* 2014). The placenta expresses 11 β -hydroxysteroid dehydrogenase type-2 enzyme, which limits fetal exposure to maternal GC inactivating corticosterone into the metabolite 11-dehydrocorticosterone. Nevertheless, chronic stress during pregnancy significantly reduces activity of this barrier enzyme and increases exposure of the foetuses to maternal glucocorticoids (Benediktsson *et al* 1997). As a consequence, GC are able to enter into fetal circulation and affect the ontogeny of hippocampal receptors. This change in GC levels might exert neurotoxic effects (Landfield *et al* 1996).

Besides, maternal stress induces hyperactivity of the offspring's HPA axis and increases amygdala corticotropin-releasing hormone (CRH) release (Keller-Wood 2015). These changes alter growth rhythm, produce behavioral changes, impair glucose tolerance and modify immune function (Merlot *et al* 2008). In addition, high levels of maternal CA induce vascular constriction that raises fetal

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placental hypoxia, which in turn, activates the fetal HPA axis and sympathetic nervous system. Consequently, the fetal stress response disrupts and interferes with intrauterine growth (Li *et al* 2012).

Specifically on immune system function, prenatal stress induces phagocytic dysfunction, decreases NK cytotoxicity and reduces the proliferative response of B cells (Veru *et al* 2014). Additionally, Merlot *et al* (2008) demonstrated that stress during pregnancy induced a size reduction of the thymus and a reduction in the number of circulation total CD4⁺ and CD8⁺ lymphocytes. Although stress has been reported to affect various functions of the immune system, the mechanisms that mediate these effects remain unclear. Interleukin 2 is considered the primary growth factor for antigen-activated T lymphocytes being the major inducer for the developmental production of suppressive T regulatory cells (Malek 2003).

Early stimulation or handling (H) of prenatally stressed offspring during the first week of life leads to long-term reduction of the stress response (Fenoglio *et al* 2005). The effect of H on HPA axis function in adult rats could be mediated by increased sensitivity of hippocampal neurons to surrounding GC and upregulation of GC receptors. A reduction in the number of GC receptors is associated with high GC plasma levels, which leads to less adaptive response to stressful situations and to a greater probability of neuronal death in the hippocampus and cognitive deficits (learning and memory) (Ferré *et al* 1994).

The aim of this work was to investigate the effects of early postnatal stimulation in prenatally stressed male rats on immune response and their relationship to the activity hypothalamic-pituitary-adrenal axis.

MATERIAL AND METHODS

ANIMALS

Albino Wistar rats were grown in the National University of Río Cuarto. Five male and 10 female rats were used, the animals were housed in plastic cages in groups of 3 under the following conditions: 12 h light/12 h dark, 22 °C, constant humidity and water and food *ad libitum*. Male-female (1:2) were mated overnight and the following morning a vaginal smear was performed and examined microscopically. The first day of pregnancy was determined by the presence of sperm in the smear and the pregnant females were located in separate home cages.

All animals (control, pregnant females and their offspring) were maintained according to the Guide for Care and Use of Laboratory Animals and the experiments were approved by the local Institutional Animal Care Committee.

PRENATAL STRESS

Chronic stress by IMO was applied to the experimental group of pregnant females. Experimental rats were stressed

during two weeks starting on the 6th day of pregnancy (Bertuzzi *et al* 2003) on a 20 x 20 cm wood board by taping their limbs to metal mounts as described by Michajloskij *et al* (1988). Each female was subjected to 30 min stress by IMO three times each week at different times in the mornings of different days (PS). Control female rats were left undisturbed in their home cages (PC). Each mother had an average of 10 live neonates. The male pups from these PS and PC groups were used for subsequent experimentation.

POSTNATAL HANDLING

Half of litters of stressed and control mothers (n: 36) were handled daily from postnatal day 1 until postnatal day 3 as described by Meaney *et al* (1987). Briefly, the mother was removed from the home cage before the pups and kept alone in a separate cage. The pups were then picked up and transferred from their home cage to another one containing paper toweling. Separate cages were used for each litter, pups were kept away from the mother for 1 min (between 9-11 a.m. every day) and then, they were returned to their home cage; finally the mother was reunited with pups. This period of separation stimulates maternal handling, which refers to arc-backed nursing, licking and grooming that rat pups receive from their mothers when they were reunited. All handling sessions were conducted in the same room and were supervised by the same researcher.

EXPERIMENTAL DESIGN

Pregnant rats were randomly allocated to either stressed (PS) or non-stressed control (PC) group (figure 1). Each litter from PS and PC groups was assigned to either handled (PSH or PCH) or non-handled control (PSNH or PCNH). Due to all mother had more than 8 pups per litter, within 24 h of birth, the litters were culled to eight per mother to minimize the influence of number of offspring on the parameters measured. All groups were housed in the same animal room and pups were kept with their mothers. The offspring were weaned 21 days after birth and housed in groups of four males by litter and left undisturbed until testing. Two 90 day-old male siblings per litter from each group were tested.

CORTICOSTERONE LEVELS

Blood samples (200-300 µl) were collected from control and stressed mothers using the tail clip method (Tuli *et al* 1995). Samples were taken at 6 and 21 days of pregnancy. For stressed mothers, blood sampling was performed immediately after the acute IMO session (30 min). COR levels in plasma were measured by radioimmunoassay (RIA, Armario and Castellanos 1984), using high specificity rabbit antiserum to COR from Bioclin (Cardiff, UK). Assay sensitivity was 10 pg of COR and inter and intra assay coefficients of variability were <10 per cent.

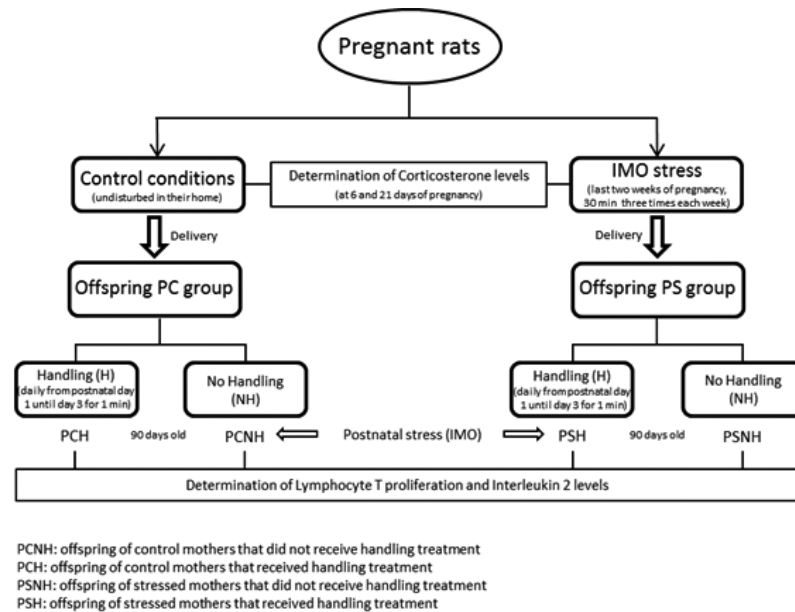


Figure 1. Schematic diagram of the experimental groups used in this research.

LYMPHOCYTE T PROLIFERATION

Adult animals of all groups were sacrificed by decapitation immediately after IMO. For lymphocyte T cell proliferation, spleen was excised and cell proliferation was determined using the [³H] thymidine assay. The spleen cells were seeded in microplate (2 x 10⁵ cells/well) and cultured in RPMI 1640 (Sigma, St. Louis, MO, USA) complete medium (10% FBS, 25 mM HEPES, 2 mM L-glutamine, 50 μM 2-4 mercaptoethanol, 100 U/ml penicillin, 100 μM streptomycin), at 37°C a 5% CO₂ in air and 95 % humidity. Twenty four hours later, cells were stimulated with concanavalin A (2.5 μg/ml, Sigma) during 24 h (Mayer *et al* 2011). Lymphocyte T proliferation was measured by radioimmunoassay and the counts per million (cpm) was the unit that represent this assay.

INTERLEUKIN 2 LEVELS

Interleukin 2 (IL-2) concentration in the supernatant of lymphocyte T cultures stimulated during 24 h with concanavalin A was determined. IL-2 was measured in culture supernatant by a commercially available enzyme immunoassay kit (ELISA) specific for rat (Bendermeds Systems, San Diego, CA) according to the manufacturer specifications. The absorbance was measured in a microplate ELISA CPD reader. Sample concentrations (pg/ml) were determined by extrapolation of data from a standard curve.

STATISTICAL ANALYSIS

Data were analysed using Statistical Software (Tulsa, OK). Differences between prenatally stressed offspring

that were handled and not handled were analysed using two-way 2 x 2 ANOVA: the factors were prenatal stress and postnatal handling. Post-hoc comparisons were performed using Duncan's test. The values are expressed as means ± SEM and *P*<0.05 was considered statistically significant.

RESULTS

CORTICOSTERONE LEVELS DETERMINATIONS

We have demonstrated in a previous work (Chen *et al* 2013) that basal plasma corticosteroid levels in adult prenatally stressed offspring (PSNH and PSH) were higher than in control (PCNH and PCH) groups. With the aim of elucidating if this observation correlated with stress-associated mother COR alterations, we determined plasma COR levels in pregnant rats subjected to stress compared with non-stressed counterparts. As shown in figure 2, COR concentration in plasma of control animals on the 6th day of pregnancy was lower than the same group on the 21st. In contrast, 30 min after stressor application, the hormone concentration markedly increased, being this effect markedly in PS animals on the 6th day.

LYMPHOCYTE T PROLIFERATION

In our previous work we demonstrated that handling treatment attenuated the observed reduction of T proliferation associated with prenatal stress in non-stressed rats (Liaudat *et al* 2012). In this study we further investigated the effect of handling treatment on T lymphocyte proliferation in animals subjected to postnatal stress. Figure 3 shows that acute stress is associated with a reduction of T

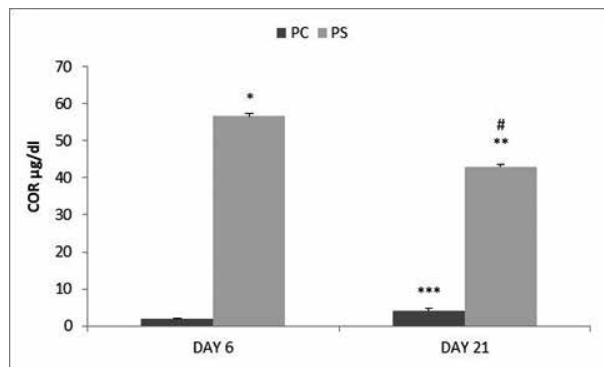


Figure 2. Effect of stress and no stress treatment on pregnant rats COR level. PC day 6 (n: 5), PC day 21 (n: 5), PS day 6 (n: 5), PS day 21 (n: 5). Each bar represents the mean \pm SE. * $P < 0.05$ vs. PC day 6, # $P < 0.05$ vs. PC day 21, ** $P < 0.05$ vs. PS day 6, *** $P < 0.05$ vs. PC day 6.

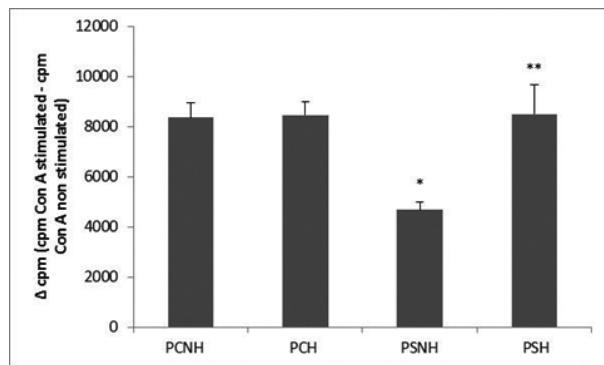


Figure 3. Effects of prenatal stress and postnatal handling on spleen lymphocytes T proliferation in animals subjected to postnatal stress: PCNH (n: 8), PCH (n: 8), PSNH (n: 8) and PSH (n: 8). Each bar represents the mean \pm SE. cpm: counts per million. * $P < 0.05$ vs. PCNH, ** $P < 0.05$ vs. PSNH.

lymphocyte proliferation in PSNH animals compared with proliferation values of control PCNH group. In contrast, handling treatment (PSH) restituted T cells proliferation to values comparable to those in non-handled group (PSNH).

INTERLEUKIN 2 LEVELS

IL-2 is crucial for the maintenance of regulatory T cells and for the differentiation of CD4⁺ T cells into a defined effector T cell (Boyman and Sprent 2012). A decrease in IL-2 concentration was observed when the offspring was subjected to prenatal stress by IMO. Handling treatment reverted this effect in animals that were in control conditions (figure 4a) or with postnatal stress (figure 4b).

DISCUSSION

Environmental influences during pregnancy and early postnatal period may modulate neurobehavioral activity

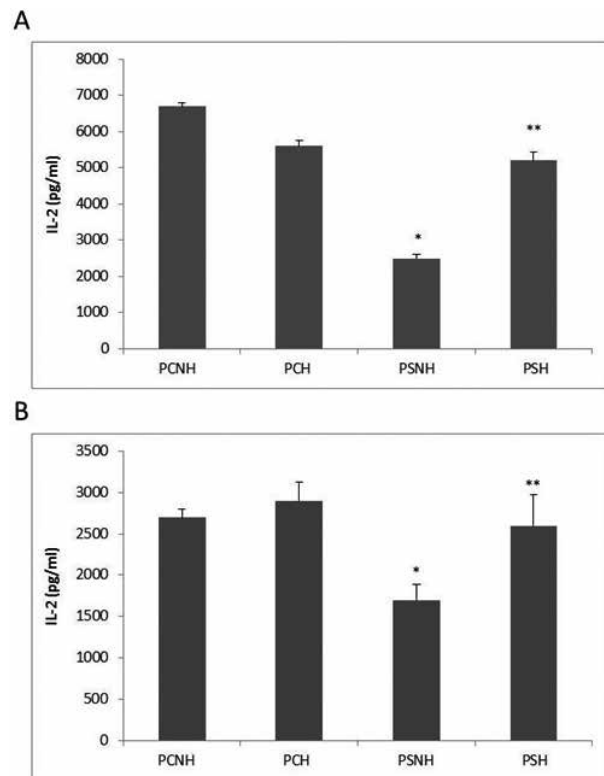


Figure 4. Effects of prenatal stress and postnatal handling on IL-2 level of animals subjected to control (a) and stress (b) postnatal treatment: PCNH (n: 5), PCH (n: 5), PSNH (n: 5) and PSH (n: 5). Each bar represents the mean \pm SE. * $P < 0.05$ vs. PCNH, ** $P < 0.05$ vs. PSNH.

in exposed individuals, effects that can last to adulthood. Early life is characterised by fast growth and execution of a precise developmental program based on epigenetic modifications, therefore it is a period particularly vulnerable to surrounding signals, such as psychosocial stimulation and maternal behavior (Desplats *et al* 2015). Animals exposed to prenatal stress or postnatal maternal deprivation (suboptimal maternal care, long time of separation) have enhanced neuroendocrine response to stress, increased levels of anxiety, depression-like behaviours and cognitive problems (Maccari *et al* 2014). Lemaire *et al* (2006) demonstrated that postnatal stimulations after delivery reduced the stress response in adult individuals. Additionally, Zhang *et al* (2012) indicated that neonatal handling and environmental enrichment enhance learning and memory of prenatally stressed offspring.

Several authors have demonstrated that prenatal stress is associated with increased plasma COR levels (D'mello *et al* 2006, Mayer *et al* 2011, Abdul Aziz *et al* 2012) and handling treatment reduces the hyperactivity of HPA axis produced by the first treatment (Liaudat *et al* 2012, Desplats *et al* 2015). Experimental evidence indicates that changes at epigenetic level are responsible for the reversion effects that postnatal tactile stimulation have on prenatal stressors consequences (Murgatroyd

et al 2015). Recently published data showed that COR can pass through the placental barrier from the blood of a stressed pregnant female to the fetal circulation. In our study, close to the end of pregnancy (day 21), COR levels were higher in control pregnant rats compared with those in control animals at day 6; similar findings were reported by Brazao *et al* (2015). This result could be explained, at least in part, due to corticosterone concentration in the maternal plasma, the major circulating glucocorticoids in rats, which also increased as pregnancy progressed and are correlated with the number of live foetuses during the last 3 days of gestation (Dupouy *et al* 1975, Atkinson and Waddell 1995, Brázao *et al* 2015). This association was also observed by Atkinson and Waddell (1995), when stressor was applied to pregnant females at the beginning of pregnancy (day 6), COR response to the stressor was more intense than that in pregnant rats close to the parturition (day 21), that could be explained, at least partially, by the documented hyporesponsiveness of HPA axis to stress in late pregnancy (Barlow *et al* 1973, Douglas *et al* 2003). High levels of this hormone in stressed pregnant rats can lead to a reduction in the number of mineralocorticoid receptors (MRs) but not glucocorticoid receptors (GRs) present in the hippocampus of the pups (Maccari *et al* 1995, Barbazanges *et al* 1996, Maccari *et al* 2003). Furthermore, MRs are usually the main substrates that exerts negative feedback control of adrenal glucocorticoid secretion under basal conditions (De Kloet and Reul 1987).

The consequences of prenatal stress and postnatal handling on the development of fetal immune system are not completely clarified. Immune cells, such as seen in other organs, express hormones and neurotransmitters receptors (Elenkov *et al* 2000). Hunk *et al* (1991) showed that activation of hormone receptors produces modulation of immune responses. An example is the constitutive expression of CD25, the IL-2 receptor α -chain, which defines a distinct population of CD4⁺ T cells with suppressive activity *in vitro* and *in vivo* (de la Rosa 2004). Moreover, there is strong evidence indicating the involvement of the autonomic sympathetic system in modulating the activity of lymphocytes through specific receptors, which in turn trigger intracellular signaling mechanisms (Elenkov *et al* 2000). It has been suggested that inadequate communication between the neuroendocrine and immune system induce physiopathologies and immune disorders (Bakker *et al* 1995).

It has been found, in murine species, that the response of the immune system to conditions of chronic stress depends on increased COR secretion (Elenkov *et al* 2000). Our experimental analyses show that high basal COR concentrations in prenatal mothers produce immunosuppression in the offspring, indicated by impairment in T-cell mitogenesis. A similar effect was also found by Tuchscherer *et al* in 2002. Furthermore, results previously obtained in our laboratory indicate an inverse association between COR level and the activity of the immune

system (Liaudat *et al* 2012). However, animal handling can restore T cell proliferation values to those observed in non-stressed controls.

High GC values not only influence T cells proliferation but also modify the secretion of IL-2, since a decrease in both parameters was seen in prenatal animals. IL-2 is crucial for the maintenance of regulatory T cells and differentiation of CD4⁺ T cells into defined effector T cell subsets following antigen-mediated activation (Boyman and Sprent 2003). For CD8⁺ T cells, IL-2 signals both effector T cell generation and differentiation into memory cells. Our results agree with those previously reported by Fillion *et al* 1994. In accordance to this knowledge, epinephrine and corticosterone significantly altered mitogen-induced T-cell proliferation in response to different stress situations (Silberman *et al* 2003).

When prenatally stressed animals received postnatal stimulations, the immune parameters studied improved considerably. This effect is shown through the increase of T cell proliferation and IL-2 secretion in animals with handling treatment. Similar results were found by Barabanova *et al* (2006), who showed that adaptation of animals to experimental conditions prevented the decrease in the number of IL-2 positive cells in the supraoptic nucleus after intranasal administration of Epithalon. Normalization of lymphocyte proliferation leads to adequate cellular immune response, which in conjunction with the mediating action of IL-2 would result in better protection against infections.

It is concluded that chronic prenatal exposure to a severe stressor such as IMO alters HPA axis activity as shown by the increased maternal COR concentration. Due to the relationship between neuroendocrine and immune system, prenatal stressed animals showed lower T cell proliferation and decreased IL-2 release. On the contrary, and being the novelty of this research, early postnatal stimulation reverses the effects of prenatal stress on the HPA axis, the proliferation of T lymphocytes and the concentration of IL-2 in response to the same stress condition.

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