International Journal of Psychological Studies; Vol. 4, No. 3; 2012
ISSN 1918-7211 E-ISSN 1918-722X
Published by Canadian Center of Science and Education

Effect of Early Stimulation on Some Immune Parameters in a Model of Prenatally Stressed Rats

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Received: March 15, 2012 Accepted: June 1, 2012 Online Published: August 22, 2012

doi:10.5539/ijps.v4n3p73 URL: http://dx.doi.org/10.5539/ijps.v4n3p73

Abstract

The aim of this research was to investigate the effects of prenatal stress and handling on immune system cell distribution and lymphocyte T proliferation in adult Albino Wistar male rats. Prenatal stressed (PS) offspring by immobilization (IMO) were handled during the first week of life. Animals of both treatments were acute IMO stressed. Blood was extracted from 0 to 330 min, and counting of white blood cells, leucocyte subpopulations and levels of corticosterone (COR) were made. Lymphocyte T spleen proliferation was determined. COR, leucocyte, lymphocyte and neutrophil profiles and lymphocyte T proliferation were significant different between prenatal stress and non-handling group and prenatal control and non-handling group, however these responses were attenuated when animals were handled. In conclusion, early handling revert the effects of PS with re-exposure to the same postnatal stressor on the activity of hypothalamo-pituitary-adrenal axis, the dynamic of leucocyte distribution and the mitogenic response of T lymphocytes.

Keywords: handling, prenatal stress, immunity, rats

1. Introduction

Maternal stress during pregnancy may have long-lasting adverse effects on physical development and behaviour of the offspring (Rodriguez et al., 2007). In rodents and primates it increases the incidence of attention deficits, impairs coping behaviour in novel and intimidating situations (Weinstock, 1997) and induces learned helplessness and anhedonia (Keshet & Weinstock, 1995). It has previously been shown that prenatally stressed rats exhibit hyperanxiety (Salomon et al., 2011; Nachum Biala et al, 2006) depressive–like behaviour and learning deficits in their youth and adulthood (Morley-Fletcher et al., 2003; Poltyrev et al., 2005).

Prenatal chronic stress by immobilization produced basal hyperactivity of the HPA axis and its reponse decreased (habituation) to the same stress applied postnatally (Mayer et al., 2011). Others authors (Rabasa et al., 2011) applying the same chronic stress during adulthood also found habituation.

Moreover, this prenatal chronic stress, especially during pregnancy as well as in the post natal period (Seckl, 2004), modifies the functional status of the immune system and the vulnerability of offsprings to immunotoxicants effects or immune mediated diseases (Dhabhar et al., 1996; Wright, 2010). Kohman et al. support these findings and describe a direct connection with high levels of cytokines and exaggerated cognitive deficits. Previous works have shown that stress stimuli in pregnant monkeys diminished the in vitro mitogen-induced lymphocyte proliferation (Halper et al, 1991; Jessop et al., 1987)

Another effect is a redistribution of the absolute and relative number of leukocytes and of lymphocytes and neutrophils, which could be compatible with the reduction of the possibility of these cells to access organs in contact with antigens when subjected to the same prenatal stress in adult life (Dhabhar et al., 1994, 1995, 1996). This alteration may be due, at least in part, to the habituation in the functionality of the HPA axis (Mayer et al, 2011).

Early handling (H) may antagonize the stress consequences. Meaney et al. (2000) demonstrated that not

prenatally stressed rats neonatally handled, had a permanent increase in concentrations of receptors for glucocorticoids in the hippocampus. Thus, at all ages tested, rats that were not handled secreted more glucocorticoids in response to stress than did H rats. Moreover, some research has confirmed that, adult H rats responded to stressors with more modest increases in corticosterone and adrenocorticotrophic hormone (ACTH) and a faster return to basal plasma concentrations (Meaney et al., 1996). H rats had significantly greater (30–40%) glucocorticoid receptor (GR) binding capacity in the hippocampus compared with non-handled (NH) rats (Meaney et al., 1988, 2000). This change in hippocampal GR binding capacity resulted in enhanced negative-feedback effects of corticosterone (Lemaire et al., 2006; Meaney et al., 2000). Most recently, an elevated GR mRNA containing the hippocampus-specific exon 1_7 was founded (Mc Cormick et al., 2000). Furthermore, animals that were early handled demonstrated low anxiety-like behavior, expressed as high exploratory behavior compared to non handled individuals (Vallée et al., 1997, Chapillon et al., 2002).

However, the influence of postnatal handling on the immune system remains unclear: Thus, the aim of the present study is to analyze the effects of prenatal stress and handling on immune system cell distribution and mononuclear spleen cells proliferation at basal levels and after the same postnatal acute stress in adult male rats.

2. Materials and Methods

Animals: Albino Wistar rats (280-300g), were housed in individual plastic cages under standard laboratory conditions (12 hours light/12 hours dark, 22 °C, constant humidity, water and food available *"ad libitum"*). The first day of pregnancy was determined by the presence of sperm plug.

During the last two weeks of pregnancy, females in the stressed group were exposed for 30 min. to chronic and unpredictable stress by plate immobilization (IMO) according to the method described by Michajlovskij et al. (1988), three times a week. Control female rats were left undisturbed in the cages. The offspring males from these two groups were referred to as prenatal stress (PS) and control (PC). The offspring were submitted to postnatal handling as described by Meaney et al. (2000). This manipulation was performed daily from postnatal day 1 until postnatal day 3. Briefly, the pups were picked up and transferred from their home cage to another one containing paper toweling. Separate cages were used for each litter throughout in the cage for 1 min (between 9 to 11 a.m. every day) before being returned to their home cage. The mother was taken out of the home cage before the pups, kept alone in another cage for the 1 min, and then returned to the home cage after the pups. Handling sessions were always performed in the same room by the same experimenter. The stimulation was performed daily (between 9 to 11 A.M.) from postnatal day 1 until 3.

Immediately litters were culled to eight pups to prevent the influence of number of pups on the parameters. Offspring were weaned 21 d after birth and housed in groups of four males by litter, and left undisturbed until testing at 90 d of age. Only two male siblings per litter from each group were tested in adult life.

The PS group was assessed to two groups: prenatal stress and handling (PSH) and prenatal stress and non-handling (PSNH). The PC group was divided into two groups: prenatal control and handling (PCH) and prenatal control and non-handling (PCNH).

2.1 First Experiment

Plasma analysis: Blood samples (200-300 µl) of all groups were collected by the tail clip method (Tulli et al., 1995) before treatment for baseline measurements (0 min), immediately after the acute IMO session (20 min), and at 60, 90, 120, 150 and 330 min). COR levels of the blood plasma (Armario & Castellanos, 1984) were measured by radioimmunoassay using highly specific rabbit antiserum to COR from Bioclin (Cardiff, UK) Assay sensitivity was 10 pg of COR; the inter and intra-assay coefficient of variability was <10%.

For leukocyte cells analysis, the relative leukocyte formula was determined by examining 200 stained cells with the method of May Grünwald-Giemsa.

2.2 Second Experiment

Animals of alls groups were sacrificed by decapitation immediately after IMO. For lymphocyte T cell proliferation, spleen was extracted and cell proliferation was determined using the [^{3}H] thymidine assay. The spleen cells were seeded in microplate (2 x10^5/well) and cultured in RPMI 1640 from 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, under 5% CO_2 atmosphere, stimulated with Concanavalin A (2,5 µg/ml, Sigma) during 72 hours (Mayer et al., 2011).

The statistical comparisons in the *First Experiment* were analysed using a three-way 2x2x7 repeated measure MANOVA, between: mother treatment (prenatal stress), and offspring (postnatal handling); within: time

treatment (T=0, T=20, T=60, T=90, T=120, T=150, T=330). In *Second Experiment* were analysed using two-way 2x2 ANOVA, between: mother treatment (prenatal stress) and offspring (postnatal handling). Post-hoc comparisons were made using Duncan's test.

3. Results

3.1 First Experiment

When controls plasma COR levels with or without handling treatment were analyzed, no statistical differences were observed at the evaluated times.

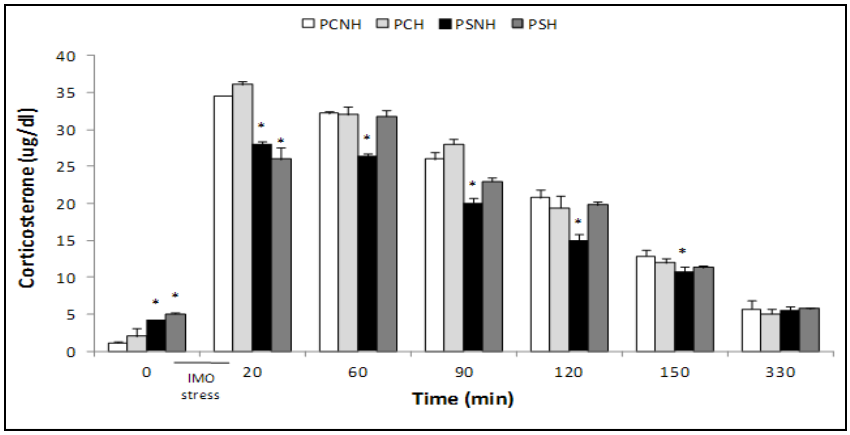

Figure 1. Effects of prenatal stress and postnatal handling on corticosterone plasma levels

Note. Effects of prenatal stress and postnatal handling on corticosterone plasma levels of PCNH (n = 10), PSNH (n = 8) and PSH (n = 8) adults male offspring rats after exposure to acute postnatal stress for 20 min. Each bar represents the mean ± S.E.M. *p<0.05 *vs*. PCNH and PCH groups.

As revealed in Figure 1, COR plasma analysis showed that PSNH basal levels of plasma COR were significantly higher than the levels of the PCNH group of rats showed significant effects of the prenatal stress treatment ($F_{(3,30)}$ =10.72; p = 0.001) and time ($F_{(6,180)}$ = 130.02; p = 0.021). The interaction between both factors was also significant ($F_{(18,180)}$=18.89; p = 0.001). PSH basal levels of plasma COR were also significantly higher than the levels of the PCNH group of rats, showing similar levels of COR between PSNH and PSH. As expected, all groups of animals had a higher increment of COR levels after IMO postnatal stress until 150 minutes. PSNH animals showed significantly lower COR levels than the control group at 20, 60, 90 and 120 min post stress. However, the PSH values were not different from those of the PCNH group from 60 min. At 150 and 330 min plasma COR levels were the same for all groups of animals.

It was observed that total leukocyte number (Figure 2) showed significant effects of the prenatal stress treatment ($F_{(3,30)}$ = 6.96; p = 0.021) and time ($F_{(6,180)}$ = 65.32; p = 0.0032). The interaction between both factors was also significant ($F_{(18,180)}$ = 1.39; p = 0.025). After 60 min of the acute postnatal stress stimuli, it was possible to observe a decrease in the number of leucocytes in the peripheral blood with respect to basal levels both in PSNH and in PCNH animals; the decrease continued until 150 min. The number of leucocytes was higher in PSNH animals than in their respective controls PCNH and PCH at 120, 150 and 330 min. This difference disappeared when the prenatal stress animals were submitted to postnatal handling PSH.

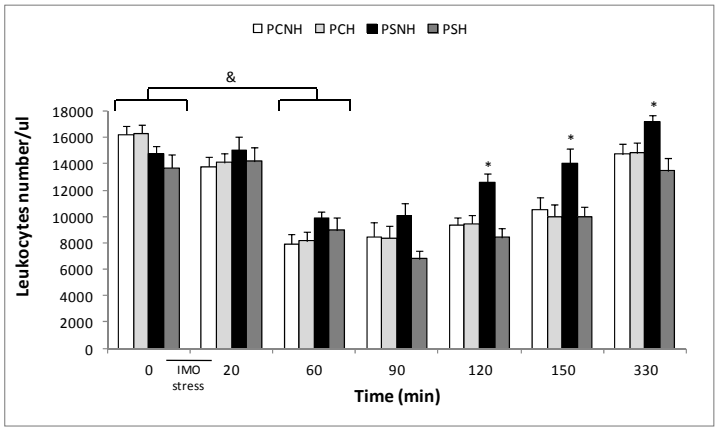

Figure 2. Effects of prenatal stress and postnatal handling on kinetic profile of the number of leukocytes in peripheral blood

Note. Effects of prenatal stress and postnatal handling on kinetic profile of the number of leukocytes in peripheral blood of PCNH (n = 10), PSNH (n = 8) and PSH (n = 8) adult male offspring after exposure to acute postnatal stress for 20 min. Each bar represents the mean ± S.E.M. & $p<0.05$ all grups T=0 *vs*. all groups T=60. *$p<0.05$ vs. PCNH and PSH groups.

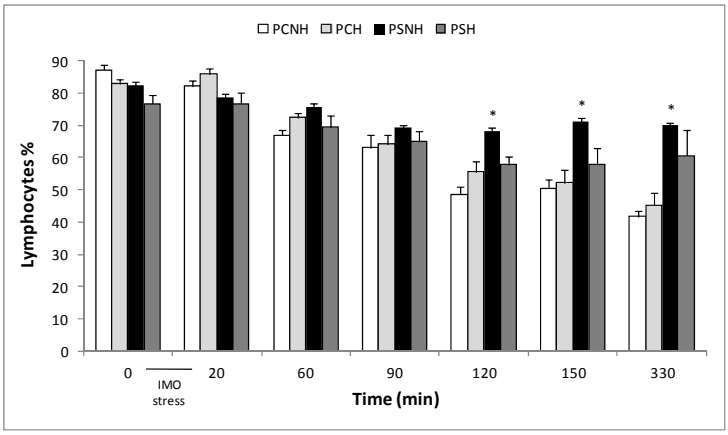

Figure 3. Effects of prenatal stress and postnatal handling on kinetic profiles of lymphocyte percentages in peripheral blood

Note. Effects of prenatal stress and postnatal handling on kinetic profiles of lymphocyte percentages in peripheral blood of PCNH (n = 10), PSNH (n = 8) and PSH (n = 8) adult male offspring after exposure to acute postnatal stress for 20 min. Each bar represents the mean ± S.E.M. *$p<0.05$ *vs*. PCNH and PCH groups.

Significant effects in percentages of lymphocytes were observed between prenatal stressed rats and their controls (Figure 3). The acute postnatal stress stimuli decreased the lymphocyte percentage in all experimental groups after 120 min, but PSNH animals showed significantly increased values ($F_{(3,30)}$=4,37; p=0.0091) in comparison to PCNH and PCH groups at time ($F_{(6,180)}$ =78,80; p=0.001) 120, 150, 330 min. The interaction between both factors was also significant ($F_{(18,180)}$=2.59; p = 0.0005). This difference disappeared when the prenatal stress animals were submitted to postnatal handling PSH.

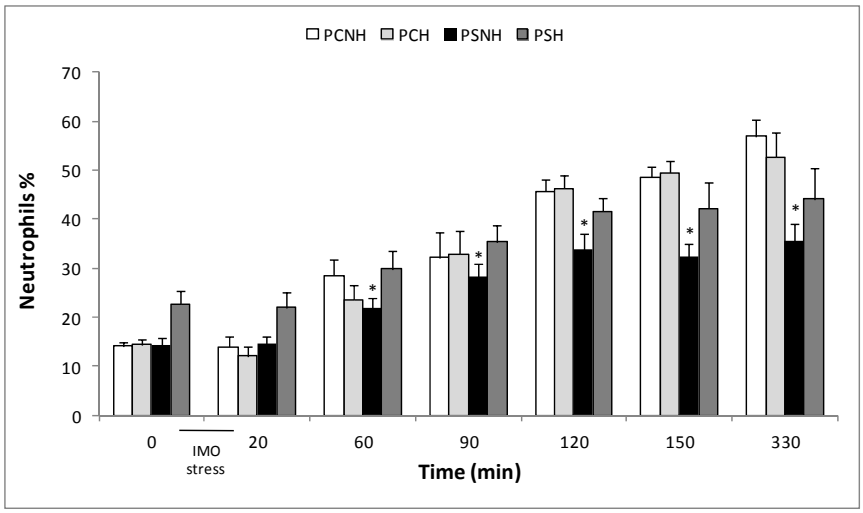

Figure 4. Effects of prenatal stress and postnatal handling on kinetic profile of percentage of neutrophils in peripheral blood

Note. Effects of prenatal stress and postnatal handling on kinetic profile of percentage of neutrophils in peripheral blood of PCNH (n = 10), PSNH (n = 8) and PSH (n=8) adult male offspring after exposure to acute postnatal stress for 20 min. PC: animals housed in standard conditions, PSNH: animals with prenatal stress treatment and without handling, PSH: offspring prenatally stressed and with postnatal handling. Each bar represents the mean ± S.E.M. *p<0.05 *vs*. PCNH and PCH groups.

As shown in Figure 4, after stress acute stimuli, the PSNH percentages of neutrophils were lower than the values of PCNH and PSH groups ($F_{(3,30)}$ = 6,92; p= 0.006) after 60 min. ($F_{(6,180)}$= 66,21; p=0.001). The interaction between both factors was also significant ($F_{(18,180)}$= 3.15; p = 0.0028). These results show that postnatal handling attenuated the effects of prenatal stress. No significant difference was found between PCNH or PCH and PSH groups.

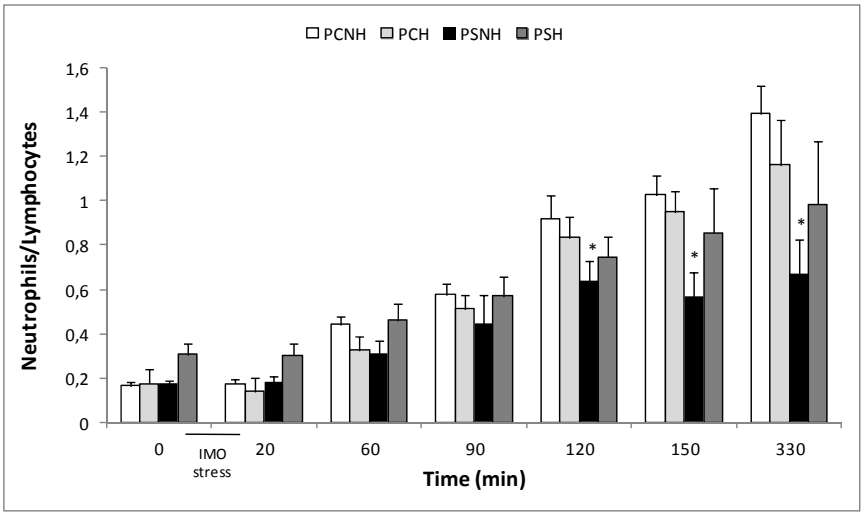

Figure 5. Effects of prenatal stress and postnatal handling on kinetic profile of the neutrophil-lymphocyte relationship in peripheral blood

Note. Effects of prenatal stress and postnatal handling on kinetic profile of the neutrophil-lymphocyte relationship in peripheral blood of PC (n: 10), PSNH (n: 8) and PSH (n: 8) adult male offspring after exposure to acute postnatal stress for 20 min. PC: animals housed in standard conditions, PSNH: animals with prenatal stress treatment and without handling, PSH: offspring prenatally stressed and with postnatal handling. Each bar represents the mean ± S.E.M. *p<0.05 *vs*. PCNH and PCH groups.

The neutrophil-lymphocyte relationship, a stress marker, was determined (Figure 5). These results revealed a significant decrease in the values concerning to PSNH in comparison to PCNH and PCH animals ($F_{(3,30)}$ = 5,47; p = 0.0027) at 120, 150 y 330 min. ($F_{(6,180)}$ = 36,55; p = 0.001). The interaction between both factors was also significant ($F_{(18,180)}$= 2.35; p = 0.0021). The prenatal effect observed in PSNH group was attenuated when the prenatal stress animals were handled PSH.

3.2 Second Experimen

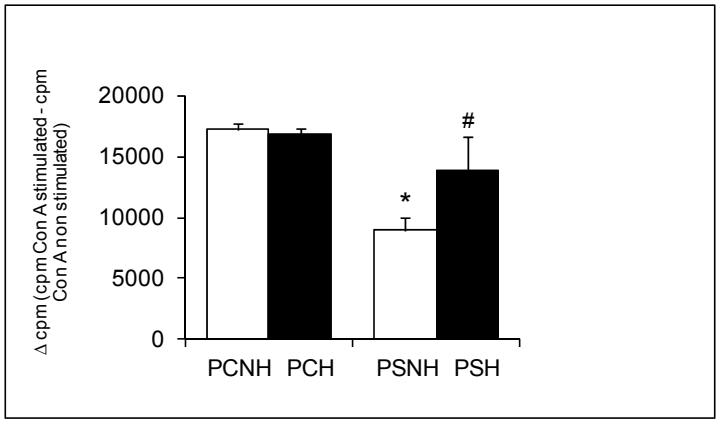

Figure 6. Effects of prenatal stress and postnatal handling on spleen lymphocytes T proliferation

Note. Effects of prenatal stress and postnatal handling on spleen lymphocytes T proliferation in control conditions: PCNH (n = 8), PCH (n:5), PSNH (n:5) and PSH (n:5).Each bar represents the mean ± S.E.M. *p<0,05 PSNH vs. PCNH and PCH grups, #p<0.05 PSH vs. PSNH

Figure 6 describes a low immune cell proliferation in animals that were submitted to prenatal stress situation in comparison to the PCNH group. The lowest lymphocyte T proliferation events occurred in the PSNH group, but when animals with the same treatment were handled this effect disappeared and the level of cell proliferation was similar to PCNH. Showed significant effects of the prenatal stress treatment ($F_{(1,27)}$= 7.14; p = 0.012) and postnatal handling ($F_{(1,27)}$ = 5.98; p = 0.021). The interaction between both factors was also significant ($F_{(1,27)}$= 7.36; p = 0.011).

4. Discussion

We found that the basal level of COR of prenatal stress and non-handling group as well as prenatal stress and handling animals was higher than the control group level. These data suggest that the HPA axis of the experimental groups was hyperactive. These results are in agreement with previous findings (Bauer et al., 2001; Sterlemann et al., 2008). This hyperactivity may be due to a lower GR mRNA and MR mRNA expression (Sterlemann et al., 2008; Maccari et al., 2003). A consequence of the decreasing levels of corticoid receptors are the up-regulation of COR production for the homeostasis maintenance. It is important to note that the stress consequences produced in the offspring may have been produced by direct stress effects, due to the fact that the COR is a steroid hormone and it can cross the placental barrier (Zarrow et al., 1970).

Our results demonstrated that prenatal stress and non-handling group plasma COR levels after acute postnatal stress condition was significantly lower than the prenatal control non-handling group until 150 minutes. This effect was reverted by handling treatment since 60 minutes after postnatal stress. These findings could be explained by the increase of GC receptor gene expression in hippocampus cells of all the handled animals, as described by O'Donnell et al. (1994). Furthermore, Meaney et al. (1988) demonstrated that the pituitary-thyroid system mediated the effect of postnatal handling on hippocampal GR expression, and in 2000 the same author suggests that via cAMP-PKA, postnatal handling could alter glucocorticoid receptor expression (Meaney et al., 2000).

Previous studies describe a strong relationship between endocrine and immune systems in animals submitted to stress-induced alterations (Bowers et al., 2008; Couret et al., 2009). Significant correlations were observed between high levels of COR and leukocyte distribution (Dhabhar et al., 1996). Bauer et al. (2001) demonstrated that the COR levels produced by stress could modify the cell adhesion molecule (CAMs) expression and promote cellular transmigration. In fact, in our previous experiments, prenatally stressed animals re-exposed to

the same stressor postnatally did not demonstrate effects on circulating lymphocyte percentages, suggesting an endocrine-immune habituated response to stress stimuli (Mayer et al., 2011).

When in this study we evaluated the leukocyte total numbers and the lymphocyte and neutrophil percentages, we observed that the prenatal stress and non-handling group showed significant differences of kinetic profiles for prenatal control non-handling group. The postnatal handling treatment reversed the effect of prenatal stress over leukocyte total numbers and lymphocyte and neutrophil percentages. The leukocyte numbers decreased in the four experimental groups after acute stress stimuli since 60 min. This effect could have been produced by the increased immune cell migration to the immune system compartment, in concordance with Dhabhar et al. (1996). These data suggest that the normalized levels of glucocorticoids influence the integrin expression and, as a consequence, the leukocyte migration. However, the decreased in leukocyte number is minor in prenatal stressed due to habituation. The neutrophil-lymphocyte relationship, a stress marker, showed that postnatal handling in prenatally stressed animals return to normal values since 120 min.

Another possible explanation for the similar results observed in prenatal control non-handling group and prenatal stress handling group may be due, at least in part, to the decrease in the pro-inflammatory cytokine levels, restoring the immune cell distribution.

The lower lymphocyte T proliferation described in prenatal stress non-handling group is in agreement with Silberman et al. (2004). When prenatally stressed animals were postnatally handled, the lymphocyte T proliferation is reverted to control levels. These results are difficult to explain because the underlying mechanisms are not clear. In our studies, the results are independent of plasma COR levels at that time but it could be expressed via monoaminergic (Meaney et al., 2000).

There are many evidences from various years ago demonstrating in human that exists effects from prenatal stress of psychophysic nature as mental retard, dream disturbs on child (Stott D. N., 1973; Schell L. M., 1981), cognitive deficits (Koehl 2002 ,Weinstock, 2001), addiction to drugs (Deminière et al., 1992), sexual function altered (Ward, 1972, 1984; Papaioannou A, et al. 2002), increase attention déficit with hyperactivity (Clements A. D., 1992) and hyperanxiety (Vallée et al., 1997; Ward, 1991). Also, prenatal stress impairs coping behaviour in novel and intimidating situations, induces depressive-like behaviour and learned helplessness and anhedonia (Papaioannou et al. 2002). These processes could be prevented by early handling treatment.(Nachum-Biala et al, 2006).

A considerable array of manipulations in early development have been shown to permantely modify the development and subsequent function of HPA (Kapoor et al, 2006; Vallée et al, 1997; Armario et al, 2011). There is additional evidence for the involvement of associative processes since adaptation of the ACTH and the adrenaline responses to repeated handling was lost when the person who handled the animals was changed (Dobrakovova et al, 1993). In both human and in rodents there is a critical neonatal period in which the disruption of maternal care and possibly the presence of other stressful situations can reprogram the HPA axis (Seckl & Meaney 2004). These events may culminate in early potential damage of brain function because the brain's exposure to corticosteroids is increased

5. Conclusion

We demonstrated that early postnatal handling regulates immune responses through immune cell distribution and lymphocyte T proliferation induced by prenatal stress.

Psychological stress interacts to increase vulnerability and put the human being at the greatest risk for disease. This is important because immune dysregulation in human is more frequently and seriously associated with clinical impairment (Bellinger et al., 2008).

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