

Effects of prenatal stress on male offspring sexual maturity

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ABSTRACT: Prenatal stimulations have been shown to have long-term effects on at reproductive activity. We evaluated the influence of the prenatal stress on the hypothalamic-pituitary-gonad (HPG) axis in male offsprings from mothers with high number of offsprings per litter (HNL) and low number of offsprings per litter (LNL) after hypothesizing that the number of offsprings per litter may modify the effect of the prenatal stress on the HPG of adult offsprings. Pregnant Wistar rats were used for this study. Immobilization (IMO) stress was used, 30 min, 3 times per week, from the 5th to 21st day of pregnancy. The weight of adrenal and gonads, and the corticosterone (COR), testosterone (TES) and luteinizing hormone (LH) plasmatic levels were analyzed in the male offspring at 30, 45 and 70 days of age. The offspring males coming from LNL showed a decrease in testicle weight and TES levels, without changes in the plasmatic LH levels. However, the offspring of HNL showed a decrease of LH levels. It is possible to conclude that in LNL prenatal stress would produce alterations to gonadal level, while in HNL the effect of stress would be evident at pituitary level.

Introduction

The survival of organisms depends on the maintenance of a constant internal body environment, or homeostasis. In several physiological conditions, the hypothalamic-pituitary-adrenal axis actively responds to both physical and psychological stress stimuli (Chousos *et al.*, 1988). In pregnant rats, the hypothalamic-pituitary-adrenal axis response is reduced during the last third of pregnancy. This response is manifested as a decrease in the levels of corticotropin-releasing-hormone (CRH), adrenocorticotropic (ACTH) and corticosterone (COR) hormones in animals exposed to prenatal stress (Fride and Weinstock, 1984). This attenuated response of the HPA axis during pregnancy may pro-

vide a protective mechanism during the development of the fetus axis (Neumann *et al.*, 1998). Plasmatic levels of progesterone and gonadotropins were shown to decrease in pregnant females exposed to restrain stress (Kinsley and Svare, 1986). Inhibition of progesterone secretion in rat during gestation could be due to the luteolytic effect of ACTH and COR, and it may be the cause of fetal loss during the first period of pregnancy. Maternal hormone imbalance affects the fetus, producing sexual changes in adult offsprings (Weisz and Ward, 1980; Kinsley and Svare, 1986). This effect can be blocked in pregnant rats with more than ten conceptus (Kato *et al.*, 1979). In fact, evidences indicate that rats with more than ten conceptus and high levels of progesterone are more resistant to stressful environmental conditions than those with only one conceptus (Sugino *et al.*, 1991).

As there is no direct neural connection between the fetus and its mother, the maternal hormones should mediate the changes in the neuroendocrine system of the fetus induced by maternal stress. These hormones

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are COR, catecholamines, ACTH, and β -endorphins, which reach the fetal brain through the placenta (Fisher and Brown, 1991).

Chronic immobilization (IMO) stress induces a quick fall of testosterone (TES) hormone and reduces the response of the Leydig cells to LH. This inhibiting effect induced by stress could be mediated by several substances, such as endorphins, glucocorticoid and catecholamines (Charpenet *et al.*, 1982). Other authors (Orr and Mann, 1990) observed that the greatest inhibiting capacity of chronic stress on the secretion of TES, without constant changes in the luteinizing (LH) hormone, would be due to an inhibition of the testicular steroidogenic, probably mediated by catecholamines (Collu *et al.*, 1984) or glucocorticoid (Closet *et al.*, 1986).

Sexual differentiation of the brain depends on the presence or absence of androgens during the early stages of the perinatal development. The levels of TES on days 18-19 of gestation (Ward and Weisz, 1980) are critical for sexual behavior and differentiation, gonadal secretion and several morphologic indexes. There is evidence that the sexual differentiation of the brain is also intimately related to the estrogen levels derived from the aromatization of the circulating TES (Chantal *et al.*, 1996).

It has been hypothesized that prenatal stress disrupts the normal maternal-fetal hormonal environmental and suppresses the fetal TES peak on gestational days 18 and 19, a peak necessary for later expression and maintenance of male sexual behavior (Menéndez-Patterson *et al.*, 1982; Ward and Weisz, 1984).

Considering that the number of offsprings per litter could modify the effects induced by stress on the hypothalamic-pituitary-gonad axis, the objective of the present work was to determine the influence of prenatal stress on the male's sexual maturity evaluating the gonadal-somatic index (IGS) and the activity of the hypothalamic-pituitary-gonadal axis through TES and LH levels in male offsprings from mothers with low number of offsprings per litter (LNL) and high number of offsprings per litter (HNL).

Materials and Methods

Albino Wistar rats of about 300 g body weight and four month of age were housed with *ad libitum* access to food and water, in constant light/dark cycle (lights on at 7 hr, and off at 19 hr), temperature ($22 \pm 2^\circ\text{C}$), and humidity. Male and virgin female pairs were housed together overnight. The next morning, a vaginal smear was obtained and examined microscopically for the pres-

ence of sperm (day 1 of pregnancy). This process was repeated until sperm was found in the animal's vagina.

Pregnant rats were separated into two groups: a control group without stress, and an experimental group subjected to chronic IMO stress (Michajlovskij *et al.*, 1988) for 30 minutes at 10 a.m. from day 7 to day 21 of pregnancy, three times a week.

The litters, experimental: (E) and control: (C), were separated into groups according to the number of offsprings per litter: HNL (E = 12.2 ± 0.46 ; C = 11.3 ± 0.37) and LNL (E = 6.30 ± 0.75 ; C = 7.1 ± 0.70). The offsprings were weaned 21 days after birth, and two offsprings (male and female) were housed together for the rest of the study. This method contributes to the sexual development of the male. Both animals were separated before sexual maturity.

Animals

The number of offsprings was recorded at birth. All the determinations were performed in male offsprings at 30, 45 and 70 days of age, considering the litter size from which each offspring came from. COR, TES and LH levels were determined in heparin plasma samples. Control and stressed animals were sacrificed by decapitation. Plasma was stored at -20°C .

Corticosterone assay

Corticosterone concentrations were measured by radioimmunoassay (Krey *et al.*, 1975) using a sheep antibody with high specificity (TECNOLAB). The corticosterone standards were supplied by Sigma Chemical CO. The radioactive steroids were (0.25mCi) corticosterone, [1,2,6,7- ^3H (N)] - 3.0 TBq/mmol (80.00 Ci/mmol). 0.25 ml of ethanol. Sciences, Inc. Boston, MA. The intra and interassay coefficients of variation were 8.8%.

Testosterone assay

Determinations of testosterone levels were carried out using the Coated Tube ^{125}I RIA Kit, COAT-A-COUNT Total Testosterone. DPC-Diagnostic Products Corporation, Los Angeles CA.

Luteinizing hormone assay

Plasma LH concentration were measured using the RIA Kit for rat LH elaborated by Dr. A. F. Parlow, Director of Pituitary Hormone & Antisera Center, Harbor-UCLA Medical Center. National Institute of Dia-

betes & Digestive & Kidney Diseases (NIDDK). The kit provided the following reagents:

1) RAT LUTEINIZING HORMONE ANTIGEN, highly purified, for iodination, NIDDK-rLH-I-9 (AFP-10250C) 100 µg-ampoule, lyophilized.

2) RAT LUTEINIZING HORMONE ANTISERUM (rabbit), NIDDK-anti-rLH-S-11 1.0 ml, 1:70 dilm., lyophilized, in 2% normal rabbit serum (NRS) in phosphosaline buffer (PBS).

3) RAT LUTEINIZING HORMONE REFERENCE PREPARATION, NIDDK-rLH-RP-3 (AFP-7187B) for use as “cold standard” only. 10 µg-ampoule, lyophilized in 1 ml of 1% Bovine serum Albumin (BSA) in PBS.

The sample was counted in automatic gamma, WIZARD WALLAC, Mod 1470. The sensibility of the LH assay was 0.3 pg/tube, with 4.2% intra assay variability.

The adrenal-somatic and the gonadal-somatic indexes were calculates as follows:

$$\text{Adrenal-somatic index} = \frac{\text{Adrenal weight}}{\text{Corporeal weight}} \times 1000$$

$$\text{Gonadal-Somatic Index} = \frac{\text{Gonadal weight}}{\text{Corporeal weight}} \times 1000$$

Statistical analysis

The results were analyzed by three-way ANOVA; prenatal treatment (Stress vs Control), days of pregnancy and number of offsprings (LNL vs HNL). A $p < 0.05$ were considered. Was accepted as statistically signifi-

cant. Student-Newman-Keuls post hoc test was performed as required.

Results

Our studies showed that, in rat, body weight at birth and 70 days of age were not affected by prenatal stress (Table 1).

ANOVA demonstrated a significant effect in the following parameters: offspring number ($F_{(1,84)} = 4.55, p = 0.035$); treatment ($F_{(1,84)} = 38.08, p = 0.001$) and time ($F_{(2,84)} = 12.75, p = 0.001$). A significant interaction between offspring number vs. treatment was observed ($F_{(1,84)} = 6.04, p = 0.015$). In the LNL group the adrenal-somatic index was increased in prenatal stressed offspring compared with their controls, while differences were not significant in the HNL group (Fig. 1).

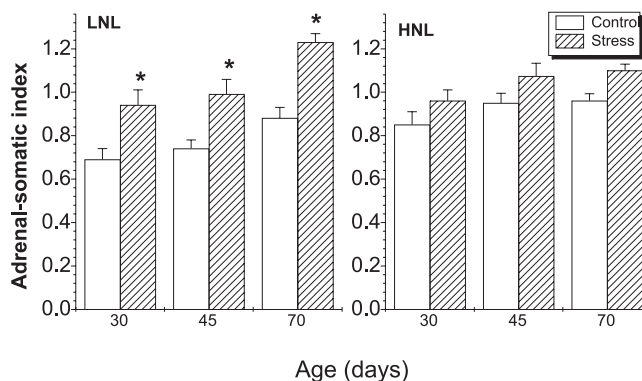


FIGURE 1. Adrenal-somatic index of prenatal stressed and controls offspring rats from LNL and HNL. The results are expressed as mean ± SEM, with an n = 8 / bar. * $p < 0.05$: Stress vs. Control.

TABLE 1.

Body weight at birth and 70 days of age

Age (day)	0	70
Body weight (g)	Mean ± SEM	Mean ± SEM
LNL - C	7.00 ± 0.26	141.67 ± 2.34
HNL - C	6.54 ± 0.28	150.87 ± 1.27
LNL - E	6.72 ± 0.17	150.21 ± 2.75
HNL - E	6.29 ± 0.30	146.01 ± 2.37

LNL = low number of offsprings per litter – C = control – E = stress
 HNL = high number of offsprings per litter – C = control – E = stress

The statistical analysis also revealed significant effects of the treatment ($F_{(1,60)} = 7.003$, $p = 0.0103$) and time ($F_{(1,60)} = 4.90$, $p = 0.0106$) on the plasma corticosterone, as well as significant interaction between these factors.

Differences of COR levels between stressed and control animals were significant in 70-day offsprings in LNL group. The same tendency was observed in HNL; the differences however, were not significant (Fig. 2).

ANOVA showed, in addition, significant effects of offspring number ($F_{(1,84)} = 4.45$, $p = 0.037$), treatment ($F_{(1,854)} = 24.69$, $p = 0.001$) and time ($F_{(2,84)} = 79.12$, $p = 0.001$) on gonadal-somatic index, as well as interaction of the three factors ($F_{(2,84)} = 10.74$, $p = 0.001$).

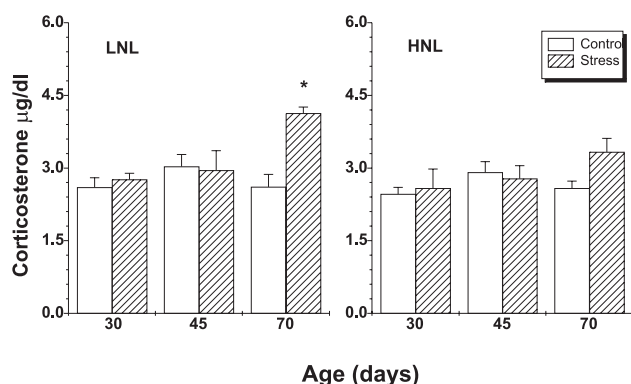


FIGURE 2. Plasma corticosterone levels of prenatal stressed and controls offspring rats from LNL and HNL. The results are expressed as mean \pm SEM, with an $n = 6$ / bar. * $p < 0.05$: Stress vs. Control.

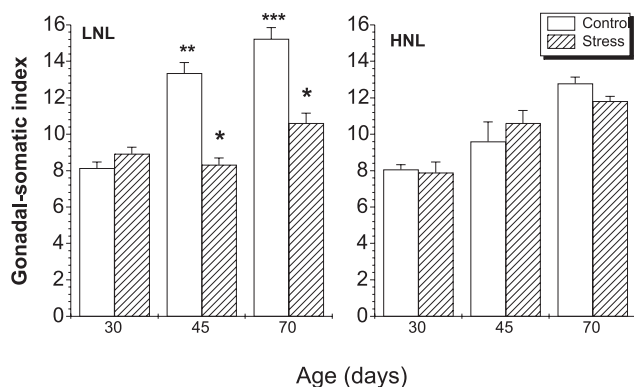


FIGURE 3. Gonadal-somatic index of prenatal stressed and control offsprings from LNL and HNL groups. The results are expressed as mean \pm SEM, with an $n = 8$ /bar. * $p < 0.05$: Stress vs. Control. ** $p < 0.05$: Control LNL vs. Control HNL at 45 days. *** $p < 0.05$: Control, LNL vs. Control HNL at 70 days.

In control animals, a significant increase in the gonadal-somatic index was observed in offsprings from LNL group in comparison with HNL offsprings, at 45 and 70 days of age (Fig. 3).

In addition, prenatally stressed male offspring belonging to LNL had a smaller gonadal-somatic index than controls at 45 and 70 days of age; differences in HNL were not significant.

The ANOVA of TES levels revealed significant effects of offspring number ($F_{(1,85)} = 16.31$, $p = 0.001$); treatment ($F_{(1,84)} = 57.34$, $p = 0.001$) and time ($F_{(2,84)} = 588.72$, $p = 0.001$), as well as significant interaction between the three factors ($F_{(2,84)} = 56.76$, $p = 0.001$).

Plasma TES levels in control male LNL offsprings were higher at 70 days of age than the concentrations observed in control HNL animals. However, the TES concentrations in stressed LNL offsprings at 70 days of age were significantly lower than the concentrations in stressed HNL offsprings (Fig. 4).

On the other hand, when comparing the levels of TES between treatments within a litter we observed that the plasmatic levels of TES were significantly lower in the stressed animals in comparison with their controls at 70 days of age in LNL groups. The same tendency was observed in HNL offsprings, however, differences were not significant (Fig. 4).

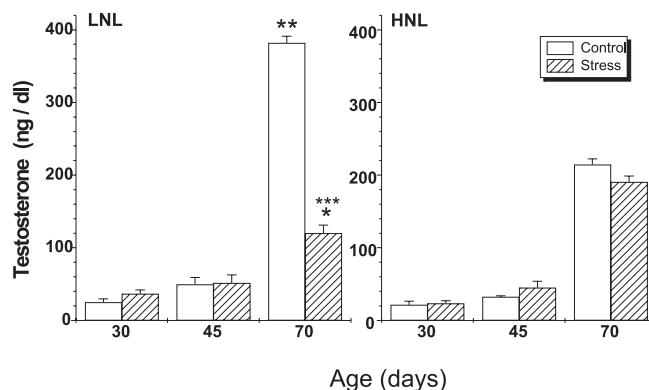


FIGURE 4. Plasma testosterone levels in LNL and HNL groups. The results are expressed as mean \pm SEM, with an $n = 8$ /bar. * $p < 0.05$: Stressed vs. Control. ** $p < 0.05$: Control LNL vs. Control HNL at 70 days. *** $p < 0.05$: Stress LNL vs. Stress HNL at 70 days.

The ANOVA test indicated a significant effect on LH levels of the treatment ($F_{(1,49)} = 11.83$, $p = 0.0011$), as well as an interaction between treatment and time ($F_{(1,49)} = 4.35$, $p = 0.042$).

When analyzing plasma LH levels in 70 days old control animals from both litters, it was observed that LH levels were higher in HNL than in LNL (Fig. 5). However, LH levels of stressed animals from both litters were similar. Stress had a significant effect on LH concentrations in HNL animals.

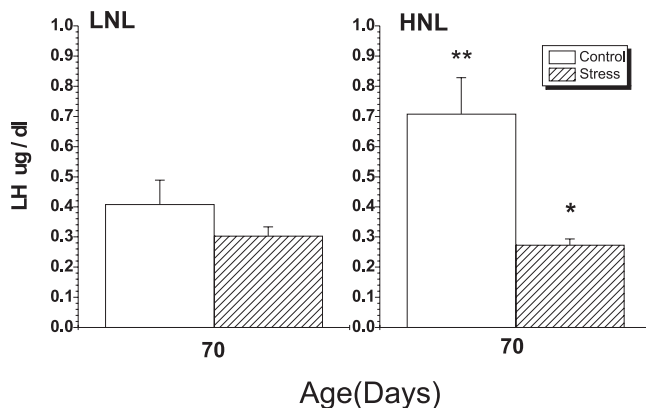


FIGURE 5: Plasma LH levels (mg/dl) in prenatally stressed and control offsprings from LNL and HNL groups. The results are expressed as mean \pm SEM, n = 13/bar for LNL and HNL stressed animals. n=12 and n=15 for LNL and HNL controls, respectively. * p < 0.05: Stressed vs. Control at 70 days. ** p < 0.05: Control LNL vs. Control HNL at 70 days.

Discussion

Our results show that the corporal weight was not significantly affected by prenatal stress. Prenatal stress increased the adrenal-somatic index significantly only in male LNL offsprings. The higher adrenal-somatic index in LNL animals could be due to the fact that these animals probably had higher adrenal gland weight at the moment of birth, as a consequence of higher levels of maternal ACTH. The decrease in serum progesterone levels, which in gestation are proportional to the number of fetuses, could be the cause of the increase of hypothalamic-pituitary-adrenal axis sensitivity, with higher secretion of ACTH during stress. (Viau and Meaney, 1991; Sugino *et al.*, 1994). Sugino *et al.* (1991) suggested that the corpora lutea of pregnant rats with more than ten conceptus, which were sufficiently exposed to placental hormones before day 12 of pregnancy may not be influenced by the pituitary-adrenal system. In addition progesterone may inhibit ACTH secretion during stress (Viau and Meaney, 1991). The higher concentration of serum progesterone in HNL than in

LNL groups may block the effect of stress by inhibiting ACTH release from the pituitary.

This effect could persist during the postnatal life, without necessarily implying hormonal differences, generating a probable morfofunctional dissociation. This seems to happen in the male offsprings of smaller litters.

These results agree with those observed in adult animals exposed to repeated social or immobilizations stress, which induces an increase of the weight of the adrenal gland (Lemaire *et al.*, 1997; Martí *et al.*, 1994) that persists through time without generating morpho-functional dissociation.

This could also explain the absence of significant differences in the adrenal-somatic index between control and stressed HNL offsprings, because the stressed mothers' axis could be desensitized (lower levels of ACTH and COR) due to the protective effect of gestation hormone levels (Viau and Meaney, 1991; Sugino *et al.*, 1994). Significant differences were also observed in plasma COR levels in prenatally stressed LNL animals.

These results suggest that the hypothalamic-pituitary-adrenal axis of prenatal stress rats is hyperactive, as the levels of basal plasmatic COR are higher than their corresponding controls. These findings agree with those of Clarke *et al.* (1994), Fride *et al.* (1986) and Peters (1982). The hyperactivity observed was manifested with an increase of the total size of the adrenal gland in prenatally stressed LNL animals. However, only a tendency was observed in HNL males at the same age. This result could be due to the protective effect of high levels of maternal progesterone (Viau and Meaney, 1991). Those results are consistent with those from Weinstock (1997) that indicated that prenatally stressed male offspring did not show an increase of the basal COR levels.

The effects of pregnant female stress on the hypothalamic-pituitary-adrenal offsprings axis are possible since the maternal COR crosses the placenta and reaches the fetus, probably affecting adrenal development and generating a stronger cortex-marrow relationship, as observed by Mayer (2004) and Klemcke *et al.* (1995).

The hyperactivity of the hypothalamic-pituitary-adrenal axis may have been generated by the decrease in the number of COR receptors present in the hippocampus of the offsprings (Maccari *et al.*, 1995; Maccari *et al.*, 2003), which are the main control of COR adrenal secretion by negative feedback. Thus, a decrease of these corticosteroid receptors would be followed by an increase of COR secretion.

With regard to the gonadal-somatic index, it was observed that the prenatally stressed male offspring belonging to LNL had a lower gonadal-somatic index

than their controls at 45 and 70 days of age. There is evidence that prenatal stress reduces testicular weight in recently born males (Dahlof *et al.*, 1978) similar results were observed in males of 105 and 115 days of age (Meisel *et al.*, 1979; McLeod and Brown, 1988). In the HNL groups, differences between stressed animals and their controls were not significant.

Plasmatic TES levels showed a clear reduction in stressed LNL males of 70 days of age. However, only a tendency was observed in the HNL males of the same age. Other authors have reported similar results (Gerardin *et al.*, 2005). In control animals, the plasmatic TES values in 70-day-old LNL rats were higher than those in HNL males.

The number of offsprings per litter might affect the TES values in control animals. When comparing the TES values with the gonadal-somatic index, a relationship was observed between the gonad size and hormone levels, it is well known that the TES concentrations are intimately related with the degree of testicular development. The same considerations that explain differences in gonadal-somatic index between litters can be applied to TES data. A additional stress during the last time of gestation might be a possible explanation (Ward *et al.*, 2003; Marques Pereira *et al.*, 2006).

It is known that fetuses exposed to environmental factors or pharmacological agents suffer alterations that inhibit the normal gonadal activity, which in turn affects sexual development and behavior in adulthood (Taché *et al.*, 1980; Ward *et al.*, 1994). Also is known that chronic immobilizations applied in mature males produces a drastic fall of the levels of plasma TES until 18 hs after stress (Tsuchiya and Horii, 1995). This may explain the results, related to the gonadal-somatic index and the testicular TES production in LNL offsprings. HNL animals showed the same the tendency.

These are few studies regarding the mechanisms through which the prenatal stress exercises its action on TES secretion. It is known that administration of pharmacological doses of catecholamines reduces the plasmatic TES levels in male rats (Damber and Janson, 1978; Levin *et al.*, 1967; Stahl *et al.*, 1984), while these levels increase after adrenalectomy (Frankel and Ryan, 1981). On the other hand, perinatal administration of antagonists of the GABAergic receptors, such as picrotoxina, in the dorsomedial hypothalamus, which induces an increase of the plasma COR and hypothalamic noradrenergic, produces a decrease of the plasma TES in mature animals (Silva *et al.*, 1998). Therefore, it is possible that stress exercises its effect in part, through this mechanism.

Charpenet *et al.* (1981) demonstrated that Sertoli cells express β_2 -adrenergic receptors and that, through these receptors, catecholamines stimulates estradiol secretion. High estradiol levels act on Leydig cells and inhibit TES production. Although this mechanism has been described only in mature animals, it is likely that a similar process takes place during fetal development at the mother's catecholamines expense. Similar results were reported by Ward *et al.* (2003). This might probably produce a lower liberation of TES in adult life as a result of a modification of the gonad functionality.

Differences observed in the levels of LH plasma in control animals of 70 days of age could be due to a modification in the negative feedback mechanism of TES on hypothalamic-pituitary-gonadal axis. Indeed, in LNL animals, plasma TES levels were elevated in post-natal life, and this could inhibit the hypothalamic-pituitary-gonadal axis activity, producing a significant decrease in LH secretion. In the HNL rats, as TES levels at 70 days of age were lower, the inhibitory effect on the hypothalamic-pituitary-gonadal axis could be lower, which might explain the higher plasma LH levels.

When analyzing the plasma LH levels in stressed males, we observed a significant decrease in the HNL groups at 70 days of age. In LNL, there were no significant differences.

Stress effects on the hypothalamic-pituitary-gonadal axis hormones are relatively complex. It is known that stress stimuli of short duration, independently of their intensity, cause an increase in LH secretion, while LH levels decrease when the stimuli last several hours and are of high intensity (Lopez-Calderon *et al.*, 1990; Silva *et al.*, 1998). Some studies attempted to elucidate the mechanisms implied in the inhibition of LH secretion by longer and more severe stress situations. The role of the monoamines is not known and the experimental data point to an inhibitor effect of the CRH, acting directly or indirectly through the opiate endogenous activation that play a predominantly inhibiting role in the gonadotropins secretion (Rivier and Rivest, 1991; Rivier and Vale, 1986).

In conclusion, maternal COR seems to affect the hypothalamic-pituitary-gonadal axis of the fetus diminishing the testicular development represented by the gonadal-somatic index. As consequence, Leydig cells TES production is affected in LNL animals when reaching sexual maturity. A another probable mechanism would be mediated by catecholamines, inhibiting TES synthesis through the adrenergic receptors present in Sertoli cells.

Effects of prenatal stress in the LNL rats are manifested mainly at the gonadal level without apparent modifications at pituitary level, no changes were observed in offspring LH levels.

On the contrary, in the HNL animals the impact of stress might be specifically at pituitary level. The absence of changes in the parameters measured at gonadal level in these litters could have at least two explanations. On the one hand, the effects of stress at gonadal level seem to have a lower sensitivity in HNL animals after both litter's comparisons. In this respect, it is well known that there is a direct relationship between the number of fetuses in the uterus of pregnant rats and circulating progesterone levels (Kato *et al.*, 1979), could enhance the inhibitory effect on the sensibility of the mother's hypothalamic-pituitary-adrenal axis, exercising a lower stress effect on the litter. These antecedents indicate that the rats belonging to litters with a high number of offsprings might be more resistant to stress than those belonging to litters with few offsprings.

On the other hand, it is probable that the effects of stress were not manifested in HNL animals in the measured times, since it seems to exist a certain delay in the gonad growth sexual development, both in control and experimental animals. This might produce a weaker negative feedback of the hypothalamic-pituitary-gonadal axis.

Our data suggest that HNL animals are more sensitive to the effects of prenatal stress evaluated through the plasmatic LH levels. However, the absence of significant differences in the LH levels between stressed and control animals in LNL suggest that stress affects the levels of LH differently depending on litter size. The effect of stress on LH levels in LNL rats could be masked by the negative feedback that TES exerts on the HPG axis.

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