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MATERNAL DEPRIVATION OF NEONATAL RATS INCREASED ANXIETY LEVELS AND PITUITARY-ADRENAL ACTIVITY OF PREWEANLING AND YOUNG RATS

(With 10 Figures and 3 Tables)

By

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الحرمان من الأمومة للفئران حديثة الولادة يزيد من مستويات القلق ونشاط الغدد النخامية والفوق كلوية للفئران الرضيعة والبالغة

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يهدف هذا البحث إلى التعرف على تأثير الحرمان من الأمومة للفئران حديثة الولادة عند عمر ٦ أيام على مستويات التوتر ونشاط الغدد النخامية والفوق كلوية لهذه الفئران أثناء فترة الرضاعة وعند البلوغ . وقد أجريت هذه الدراسة على الفئران من نوع ويستر واشتملت على تجربتين: **التجربة الأولى** تضمنت تأثير الحرمان من الأمومة لمدة ٢٤ ساعة للفئران حديثة الولادة عند عمر ٦ أيام وعند عمر ١٦ يوم تم حقن الفئران بمحلول فسيولوجي ثم أعيدت الفئران لمدة ساعة إما الى الأمهات الأصلية أو أمهات مخدرة أو أمهات ما بعد الرضاعة وتم قياس نشاط الغدد النخامية والفوق كلوية باستخدام التحليل المناعي الإشعاعي لقياس مستويات كل من الهرمون المنشط لهرمونات قشرة الغدة الفوق كلوية وكذلك هرمون الكورتيكوستيرون في الدم عند عمر ١٦يوم (قبل الفطام) وعمر ٤٥ يوم (بعد البلوغ). **التجربة الثانية** استخدمت هذه التجربة لقياس مستويات التوتر والقلق في مجموعة أخرى من الفئران التي سبق تعرضها للحرمان من الأمومة وبنفس طريقة العمل بالتجربة الأولى لكنها تركت حتى عمر أربعون يوما . أظهرت النتائج المتحصل عليها في هذه الدراسة أن الحرمان من الأمومة قد أدى إلى زيادة مؤكدة في كل من الهرمون المنشط لهرمونات قشرة الغدة الفوق كلوية وهرمون الكورتيكوستيرون في الفئران عند عمر ١٦ ، ٤٥ يوما كما أدى الحرمان من الأمومة أيضا إلى زيادة مؤكدة في معدلات التوتر والقلق في الفئران الناضجة (عمر ٤٠ يوما).

SUMMARY

Data in the literature demonstrate that a wide variety of stressful events, including inadequate maternal care during infancy may have deleterious effects on the physiological and emotional development. The present studies were designed to make a detailed investigation of this issue. In this study, the effect of prolonged (i.e. lasting for 24 h) maternal deprivation at postnatal day 6 (PND 6) on the pituitary-adrenal activity at postnatal days 16 and 45 (PND 16 and 45), was determined by measuring plasma levels of adrenocorticotrophic hormone (ACTH) and corticosterone (CORT). Over a period of one hour on PND 16, and after receiving an injection of saline administered intraperitoneally, i.p. (as a mild stressor), rats were allowed to remain with their mothers, or were placed with anaesthetized mothers or post-lactating dams to examine the importance of maternal contact as an effective inhibitor of adrenal response to stress for rat pups. A second group of neonatally deprived rats were weaned at PND 21, and then tested as adults (16 and 40 days of age) for their anxiety levels in the plus-maze. At PND 16, both male and female rat pups exhibited similar pattern of ACTH and CORT response. However, at 45 days of age, all female rats exhibited higher levels of ACTH and CORT from those found in other groups. Moreover, male and female rats which had been placed with post-lactating mothers showed significant increase ($P<0.05$) in anxiety levels as compared to any of the other treatment groups and control rats.Male and female rats (40 days of age), which have been placed with post-lactating mothers showed a significant increase ($P<0.05$) in anxiety levels as compared to corresponding control groups of animals. Taken together, the present study indicated that maternal deprivation during ontogeny produce sustained, long-lasting changes of CORT levels and the nature of these effects depended upon the gender of rat.

Key Words: *Maternal deprivation, pituitary-adrenocortical activity.*

INTRODUCTION

Previous studies by Suchecki et al. (1995), have shown that prolonged maternal deprivation during early infancy increases basal and stress-induced adrenocorticotrophic hormone (ACTH) and corticosterone (CORT) levels and enhance hypothalamo-pituitary-adrenal (HPA) axis responsiveness to further stressors. However in the rat, from approximately postnatal day PND 6 till PND 14, the HPA activity is profoundly suppressed. This period is known as "stress hyporesponsive period" (SHRP) which is characterized by adrenal insensitivity to injection of ACTH and a failure of response to mild stressor (e.g. saline injection administered i.p.). There have been several reports stating that maternal factors are responsible, at least in part, for such adrenal insensitivity during the hyporesponsive period in the rats (Levine et al., 1991, Cirulli et al., 1992, and Suchecki et al., 1993a, b). Furthermore, Aria and Widmaier (1993), suggested that difference in rates of enzymes resposible for adrenal steroidogenesis might be also responsible for adrenal insensitivity during the (SHRP).

Recently, Darwish (1998) studied the effect of maternal deprivation lasting for 24 h of neonatal rats at PND 6, on the anxiety state of these animals at 60 days of age. In addition, the above author examined hypothalamo-pituitary-adrenal activity during the SHRP (at PND 10) and in adulthood (at PND 60). It is well known that, from about PND 6 until PND 14, the infant rat is hyporesponsive to stimuli which would normally result in a marked increase of adrenal activity (CORT secretion) beyond SHRP and in the adult rat. Moreover, it has been reported that CORT levels remain low and difficult to perturb during the SHRP in rats (Hennessy, 1997; Shoenfeld et al., 1980).

The present experiments were designed to address three issues; the first one was to explore the effect of early maternal deprivation (at PND 6) on the hypothalamo-pituitary-adrenal activity beyond the SHRP (at PND 16); the second one was to measure the anxiety levels of the deprived rat after puberty (45 days of age); and the third purpose was to support the concept that the adrenal response to stress can be regulated in an inhibitory manner by mother-infant interaction during the preweanling period of the development.

In the present study we have investigated in Experiment 1, the effect of 24 h maternal deprivation at PND 6 on pituitary-adrenal activity by measuring the plasma levels of ACTH and ACTH at PND 16 and 45. These ages were chosen because elevations of CORT are small and often variable prior to 14 days of age in rats (Hennessy, 1997; Shoenfeld et al., 1980; Stanton et al., 1987; 1988a,b). In

Experiment 2, the behaviour of rats at PND 40 was examined by measuring the anxiety level using plus-maze test.

MATERIALS and METHODS

Experimental animals

Rat pups (n = 40) of Wistar strain were used in the study. The date of birth was designated as day 0, at which rat litters were culled to 5 vivid male and 5 female pups. The dams and the litters were housed in a transparent plastic cages, in a temperature controlled room maintained on a 12: 12 h light and dark cycle. Food and water were available *ad lib.* The mothers and the pups were untouched until day 5.

Experiment 1:

Maternal deprivation studies were commenced at PND 6.This experiment was performed on four groups of rat pups (n = 10 per experimental group).The deprivation procedure (3 groups of rats) involved separating the pups from their mothers and placed them in a separate groups without food and water. They were kept in the animal-room under the same temperature, humidity and lighting conditions as the main colony. The fourth, the control group of pups was neither deprived nor injected. After 24 h of deprivation, the rat pups were returned to the mothers and were left undisturbed until the test time.

At PND 16, male and female pup rats were identified and marked, then injected intraperitoneally (i.p.) with 0.9 % saline solution in a volume of 0.1 ml/100 g body weight (as a mild type of stress). Pups of the group 1 were placed with their own mother while, animals of the group 2 were placed to an anaesthetized lactating dam (lactating dam has pups of the same age as the experimental subjects, was injected i.p. with urethane 1.1g/kg, 30 min. before placing pups with her), and the group 3 placed with a post-lactating mother. These pups were left with foster mothers for 1 hour, then all groups of pup rats were sacrificed by decapitation between 13:00 and 14:00 h. Trunk blood was collected in EDTA-treated precooled 1.5 ml tubes. The blood samples were centrifuged at 3000 rpm for 20 min. at 2 °C. Plasma was separated, placed in precooled sample vials and kept frozen (-20 °C) until radioimmunoassay was performed to measure ACTH and CORT.

Experiment 2:

This experiment was similarly done as mentioned before except that all groups of rat pups were weaned at PND 21, and housed in groups of 5 according to their sex. Animals were tested for anxiety level at 40 days of age. The anxiety levels were measured in the plus-maze apparatus. At 45 days of age, rats were sacrificed by decapitation between 13:00 and 14:00 h. Blood was collected in EDTA- treated precooled sample vials, centrifuged and kept frozen until radioimmunoassay for ACTH and CORT.

The plus-maze apparatus:

The elevated plus-maze test has been in use as a rodent model of anxiety for a decade, and is representative of those tests that are based upon the study of spontaneous behaviour patterns and which have high ethological validity (Dawson and Tricklebank, 1995; Rodgers and Dalvi, 1997). The elevated plus-maze test probably is the most popular of all currently available animal models of anxiety, and affords an excellent example of a model based on the study of unconditioned, or spontaneous behaviour (File, 1992; Handley and McBlane, 1993; Rodgers and Cole, 1994). Commenting on the advantages of the plus-maze, Pellow et al., (1985) stated that: (1) the test is fast and simple, and does not involve expensive equipment; (2) it is based on spontaneous behaviour and thereby avoids lengthy training, the need for food or water deprivation, and the use of noxious stimulation; (3) it is able to identify acute anxiolytic effects of benzodiazepine (an anxiolytic); and (4) it is bidirectionally sensitive to manipulations of anxiety. Given this profile, the plus maze seems to offer many advantages both in routine drug- screening and in the study of the mechanisms of anxiety.

The plus-maze used in this experiment was made of smooth, gray opaque Perspex, with two open arms (50X10 cm) and two closed arms of the same size; the walls of this chamber were 40 cm high, and the whole apparatus was elevated 50 cm above the ground. The open and closed arms of the maze were identical except for the addition of a Perspex ledge, 0.5 m high, around the perimeter. A video camera was mounted vertically above the maze, and the behaviour was scored by means of a monitor and computer keyboard in an adjacent room. Each rat was placed in the central square (10X10 cm), facing the closed arm, and was allowed to explore freely the maze for 5 min. At the end of each trial, the maze was wiped clean with a damp cloth, to remove excreta and individual odours that might have affected the behaviour of the animal tested subsequently. The times spent in the open and closed arms were computed. The criterion for arm entry was '4 paws in one of the arms', while the criterion for exit was '2 paws out of the arm'. In addition, the percentage of time spent in the open arms was calculated [open time: (open + closed time) x 100]. By convention, an increase in the percentage of time spent in the open arms was interpreted as an anxiolytic response, whereas the number of entries into closed arms was taken as a measure of general activity, (e.g. anxiogenic response).

Blood sampling and hormone assays:

Blood samples were collected in EDTA-coated, precooled 1.5 ml tubes. The samples were kept cold until and throughout centrifugation (3,000 rpm for 20 min at 2°C). Plasma was separated and placed in pre-cooled vials and kept frozen (at -20 °C) until assayed for adrenocorticotropic hormone (ACTH) and corticosterone (CORT). Specific radioimmunoassays (RIA; ICN Biomedical, Costa Mesa, CA) were used to assay the hormones and the manufacturer's instructions were followed.

Statistical analysis:

In all illustrations of the data, mean ± standard error of the mean (SEM) are depicted. Statistical analysis was based on raw data, and performed using a software package (SigmaStat; Jandell Scientific). Data were subjected to one-way anaylsis of variance (ANOVA), followed by parametric or non-parametric pairwise comparisons, depending on whether the data passed a normality test or not. The level of significance in all tests was preset at $p \leq 0.05$.

RESULTS

Experiment 1:

The results of Experiment 1 are shown in Figs.1-8, and describe the effect of maternal deprivation on the pituitary-adrenal activity at PND 16 and 45. Data were analyzed separately for each sex and at each age.

<u>At 16 days of age</u> (PND 16), over a period of one hour and after the administration of an i.p. injection of saline solution (mild stress), the rats were allowed to remain with their conscious mother, or were placed with an anaesthetized mother, or post-

lactating mother. This experiment was performed to examine whether maternal contact is sufficient to inhibit corticoid response of preweanling rats to stressor (novelty, saline solution injection).

As shown in Fig. 1-4 & Table 1, male and female pups exhibited similar pattern of ACTH and CORT response. The plasma hormone levels in the pups placed with their own mother and anaesthetized mother did not differ significantly from those found in control rats (not deprived, not stressed). Moreover, male and female pups placed with post-lactating mothers showed a significant increase in their plasma levels of ACTH and CORT as compared to all other groups of rats ($P<0.05$).

At 45 days of age, males of all experimental groups showed a significant increase ($P<0.05$) in the plasma levels of ACTH and CORT as compared to those at PND 16 and control non-deprived rats (Figs.1, 2, 5 & 6 and Table 1, 2). Moreover, males placed with post-lactating mothers showed a significant elevation of their plasma levels of ACTH and CORT from those found in all other groups ($P<0.05$). However, males placed with their own mother and anaesthetized mother showed significantly elevated levels of ACTH and CORT as compared to control rats. Female rats placed with post-lactating mothers showed a significant increase ($P< 0.05$) in plasma levels of ACTH and CORT as compared to other treated and/or control groups (Fig.7, 8 & Table 2). However, female rats belonging to own mothers, and anaesthetized mothers did not differ from control females in their hormonal parameters. It is clearly demonstrated that all female rats including those non-deprived ones (control) exhibited higher values (about 4 folds) of plasma levels of CORT than those observed in male rats.

Experiment 2:

Each rat (40 days of age) was placed on the center of the maze, facing toward one closed arm. During the 5 min test period, the percent of time spent in open arms relative to total time spent in the maze (open/open + closed) was recorded. A rat was considered to have entered an arm if all four limbs had left the central area of the maze. The maze was wiped thoroughly after each trial.

Males: as shown on Fig. 9& Table 3,the percentage of time spent in open arms was measured in mature male rats (40 days old) that had been exposed to two stimuli: an initial 24 h maternal deprivation at PND 6 (then left undisturbed with their mother until PND 16) and then 1 h exposure to either their own mother, or an anaesthetized mother or a post-lactating mother. Rats which were placed with their own mother showed a reduced aversion for the open arms, because (Newman-Keuls test) revealed a significant increase in the percentage of time spent in the open arms as compared with those placed with a post-lactating dam ($P<0.05$). In other words, these rats revealed a low level of anxiety reflected by the long time spent in the open arms. The same rats which were placed with their own mother tended to spend a greater percentage of time on open arms, although this measure did not reach a significant difference compared with those placed with an anaesthetized lactating rat. young male rats previously placed with an anaesthetized mother showed significantly increased in time spent on the open arms ($P<0.05$) when compared to those rats which were placed with a post-lactating dam.

Females: as can be seen on Fig. 10& Table 3, the percent of time spent in open arms was measured in adult female rats (40 days of age) that had been exposed to two stimuli: an initial 24 h maternal deprivation at PND 6 and then 1 h exposure to either their own mother, an anaesthetized mother and a post-lactating mother at PND 16. Female rats which were either placed with their own mother, or an anaesthetized lactating dams showed significant difference in time spent on the open arms as compared to those placed with post-lactating mothers.

Taken as a whole, the results of experiment 2, specifically distinguish the relationship between maternal deprivation at early infancy (PND 6) and the anxiety levels of young rats (40 days of age). On the basis of these results however, it could predicted that male and female rats which had been placed with post-lactating mothers showed significant increase ($P<0.05$) in anxiety levels as compared to those previously placed with own mothers, anaesthetized mothers or control rats. Those animals have increase anxiety levels as they spent significantly ($P<0.05$) less time in the open arms to the total time spent in (open and closed arms) the plus-maze test.

DISCUSSION

The present study clearly indicate the presence of a strong maternal component in the ontogeny of the neuroendocrine regulation of the stress response. Thus infant rat maternally deprived during "stress hyporesponsive period" responded with significant increases in plasma levels of ACTH and CORT when exposed to mild challanges (e.g. intraperitoneally administered saline solution) during the preweanling period.

In the rat, from about postnatal day (PND) 4 to 14, the hypothalamo-pituitary-adrenal activity is profoundly suppressed: plasma corticosterone levels are low, and stressors or even injection of adrenocorticotrophic hormone (ACTH) do not induce a large increase in corticosterone secretion. This period (PND 4-14) is referred as the stress hyporesponsive period (SHRP), (Rosenfeld et al., 1992a,b; Levine 1994; Suchecki et al., 1995). After such period CORT levels gradually increases to reach the adult responsiveness levels. Evidence for this SHRP is apparent in the present study because at 16-days of age (beyond SHRP), the plasma level of corticosterone in non-deprived pups was significantly higher values if compared to those reported in rat pups at PND 10 (see Darwish, 1998).

It appears that there are several mechanisms in place during the SHRP that maintain stable and low corticosteroid levels which serve to prevent endogenous elevation of the steroid which appear to have long-term effects on the brain. However, a growing body of evidence indicating that specific forms of maternal stimuli (i.e providing nutrients, warmth, protection, tactile stimulation, licking and nursing) regulate some aspects of the infant rat's HPA axis to enforce the stress hyporesponsive period, and that removal of these forms of maternal stimuli results in a gradual dysregulation (i.e. potentiation) of HPA activity (Cirulli et al., 1992; Levine et al., 1991, 1992; Pihoker et al., 1993; Suchecki et al., 1993a; Avishai-Eliner et al., 1995; Sutanto et al., 1996; Rots et al., 1996; Vázquez et al., 1996; Hennessy, 1997; Suchecki and Tufik 1997).

The present study represents an important confirmation and extension of our and other studies (Darwish, 1998; Stanton et al., 1987, 1988a,b) indicating that the hypothalamo-pituitary adrenal activity is subjected to maternal regulation.

In the present study, both maternal deprivation (separation of pups for 24 h) and reunion paradigm were employed; i.e. maternally deprived pups returned to the mother, that anaesthetized mother or post-lactating dams. As far as it is possible to determine that the effects of reunion are complementary to those of separation. Similarly, our studies of reunion have indicated that anaesthetized lactating dams are effective inhibitors of stress, particularly for 16-days old pups. In other respect, the present data evidently, confirm that maternal presence plays an important stress-reducing role for the rat pups, i.e. the HPA axis can be regulated in an inhibitory manner during the preweanling period of development in the rat, such evidence, convincingly demonstrate that maternal factors are important in the regulation of the neonatal HPA axis. Similarly, Levine, (1994); Levine et al. (1994) found that contact with a lactating dam suppressed the CORT response in maternally deprived pups who were either exposed to novelty or injected with saline solution. Furthermore, Cirulli et al., (1992) provided the evidence that maternal contact in the absence of suckling and/or feeding was capable of down regulation the infant HPA axis.

Data presented in this study showed clearly that maternal deprivation induces long-term alterations in the HPA. Neonatally deprived animals (placed with a post-lactating mothers) showed elevated plasma levels of ACTH and CORT as compared with rats placed with their own mothers. These findings suggested that deprived rats displayed mild hypercorticalism after puberty. Similarly, Rots et al., (1996) and Darwish (1998), reported that maternal deprivation of neonatal rats for 24 h at PND 3 or 6 enhances the adrenocortical response to stress and /or ACTH stimulation during SHRP and in later life at 60 days of age. They also found that maternally deprived neonatal rats displayed elevated plasma ACTH associated with reduced corticotrophin releasing hormone (CRH) transcript level in the hypothalamic paraventricular nucleus (PVN).

Another interesting finding in the work here is that at 45 days of age, differences in CORT response to stress were attenuated in males than in females placed with a post-lactating dams. Moreover, CORT levels in females showed higher levels in response to stress than males. The present findings clearly demonstrate the existence of gender differences in ACTH and CORT response to stress in rats at 45 days of age. Furthermore, maternal deprivation which occurred during early ontogeny (PND 6) caused distinct sex-dependent effect at 45 days of age. An explanation of such gender differences in CORT response to stress may come from a recent report in which 24 h of maternal deprivation on PND 3 was shown to differentially alter the population of corticosteroid receptor in the hippocampus of 48-day-old males and females, whereas males showed a down-regulation of both glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs), females exhibited an up-regulation of GRs at PND 48 (Sutanto et al., 1996; Suchecki and Tufick, 1997).

It is important to note that maternal deprivation during ontogeny (PND 6) produced sustained (long-lasting) effects on corticosterone-response to stress and the nature of the effect depended upon the gender (sex) of the pups. The results of the present investigation implicate plasma levels of corticosterone in the modulation of anxiety levels in young rats at 45 days of age.

The present data revealed that control male rats exhibit slightly higher levels of anxiety, if compared to control female rats at 45 days of age. Moreover, rats which were subjected to maternal deprivation (PND 6) and placed with post-lactating mothers at PND 16 showed significantly increased levels of anxiety at 45 days of age. Therefore, it seems that corticosterone binds with glucocorticoid and/or mineralocorticoid receptors in certain brain areas that are involved in modulating the levels of anxiety and fear in rats (Korte et al., 1996; Sutanto et al., 1996).

In conclusion, maternal deprivation during ontogeny produce sustained changes in corticorticosterone levels and the nature of these effects depend upon the gender of the pups. These data also support the recognition that early life events have long-lasting effect on stress response system and behavioral adaptation.

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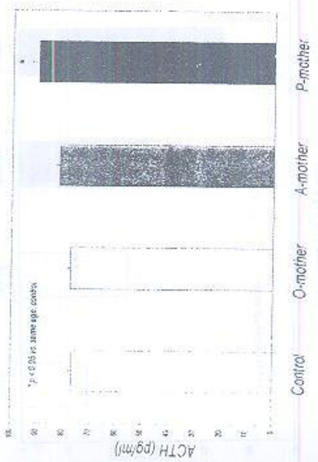


Fig. 1 Males (16 days old)

Fig. 2 Males (16 days old)

O: own mother A: anaesthetized mother P: post-lactating mother

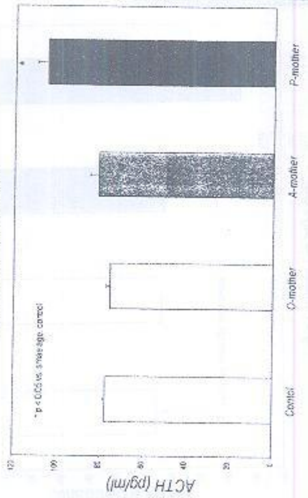


Fig.3 Females (16 days old)

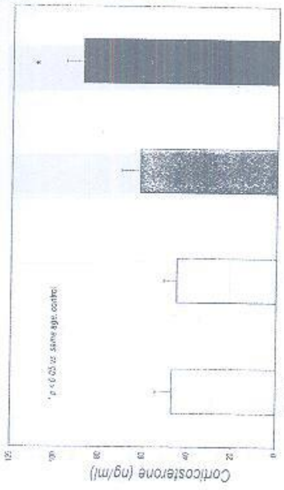


Fig.4 Females (16 days old)

O: own mother A: anaesthetized mother P: post-lactating mother

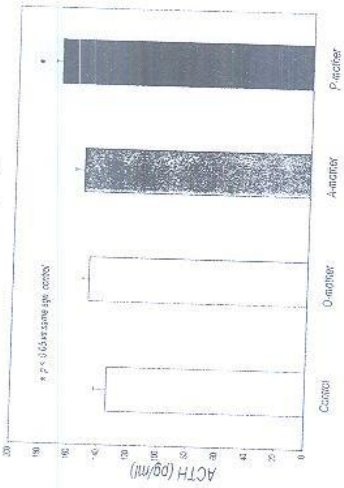


Fig. 7 Females (45 days old)

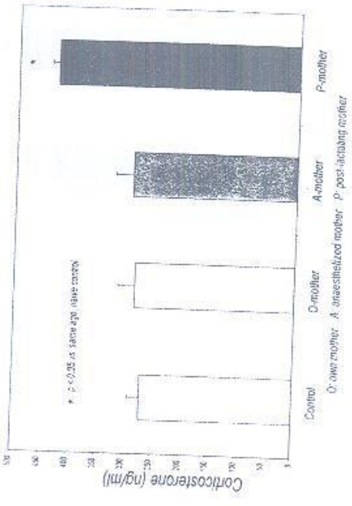


Fig. 8 Females (45 days old)

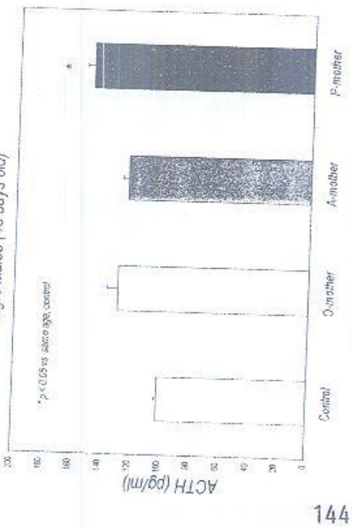


Fig. 5 Males (45 days old)

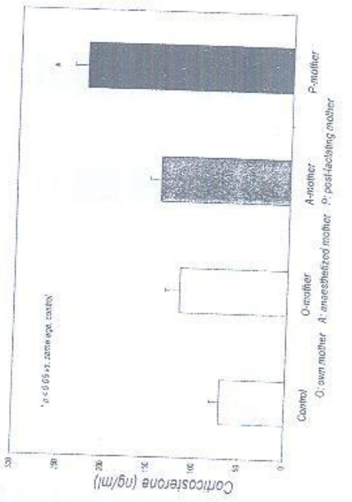
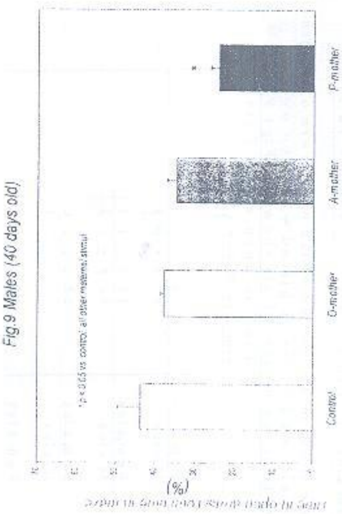


Fig. 6 Males (45 days old)



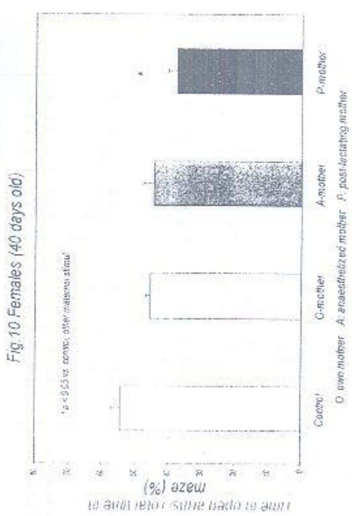


Table (1): Showing the plasma levels of ACTH and CORT in control male and female rats and these placed with their own mothers, anaesthetized mothers and post-lactating mothers at 16 days of age (Data shown in Figs. 1,2,3,4).

16 days old		ACTH	CORT
Control	Males	76.6 ± 0.924	49.4 ± 8.6
	Females	77.4 ± 1.0	47.3 ± 7.1
Own mother	Males	77.3 ± 0.927	50.7 ± 6.2
	Females	75.5 ± 1.7	45.1 ± 6.0
Anaesthetized mother	Males	81.9 ± 1.014	63.4 ± 9.9
	Females	81.2 ± 3.7	61.9 ± 8.7
Post-lactating mother	Males	90.4 ± 2.263	121.2 ± 7.3
	Females	105.3 ± 4.8	88.1 ± 7.8

Table (2): Showing the plasma levels of ACTH and CORT in control male and female rats and those placed with their own mothers, anaesthetized mothers and post-lactating mothers at 45 days of age (Data shown in Figs. 5,6,7,8).

45 days old		ACTH	CORT
Control	Males	101.6 ± 1.9	72.3 ± 10.6
	Females	134.7 ±8.3	272.7 ± 20.3
Own mother	Males	129.7 ± 6.8	118.7 ± 15.7
	Females	148.5 ± 3.8	284.0 ± 30.4
Anaesthetized mother	Males	124.2 ± 3.4	142.5 ± 11.9
	Females	153.4 ± 5.8	291.3 ± 31.4
Post-lactating mother	Males	149.4 ± 4.6	226.4 ± 14.4
	Females	170.6 ± 4.2	430.0 ± 10.5

Table (3): Showing the percentage of time spent in open arms of the plus-maze of control and stressed adult (40 days) male and female rats previously placed with their own mothers, amaesthetized mothers and post-lactating mother (Data shown in Figs 9,10).

40 days old		Time in open arms/ Total time in maze (%)
Control	Males	43.7± 5.7
	Females	54.2 ± 2.9
Own mother	Males	37.9 ± 0.8
	Females	45.5 ± 1.3
Anaesthetized mother	Males	34.8 ± 2.3
	Females	44.5 ± 3.0
Post-lactating mother	Males	24.1 ± 2.1
	Females	37.5 ± 2.6