ORIGINAL ARTICLE

COMPARISON OF ESTRADIOL LEVELS IN RAT MODEL OF CHRONIC ALTERNATING STRESS

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Background: Stress is a prevalent factor in our daily lives and is affecting the reproductive system too. The objective of this study was to compare the effect of chronic alternating stress on two generation of rats by evaluating the corticosterone and estradiol levels. **Methods:** One hundred and thirty-six healthy wistar albino rats 11 weeks old were used in this experimental case-control study. They were distributed into case and control parent groups after assaying for baseline parameters. Case parents were given three weeks of chronic alternating stress while the control parents were not given any stress. Following mating within respective groups the offspring were placed into 5 groups each. One group was assayed at 5 weeks without any stress, the other was given 3 weeks of stress and assayed at 8 weeks, the third group was given stress at the age of 5 and 11 weeks, one group was given stress only at 11 weeks of age. There were two control groups which were offsprings that did not receive any stress and were assayed at the ages of 8 weeks and 11 weeks. Blood sera were assayed for corticosterone and estradiol using Enzyme Linked Immunosorbent Assay Techinique. In the offspring generation behavioural tests were done and analysed. **Results:** The rats given early life stress had raised corticosterone levels and decreased estradiol levels shown by significant *p*-values. **Conclusion:** The early life stressed rats did not fare well as compared to rats given repeated stress.

Keyword: Chronic Alternating Stress, Corticosterone, Estradiol

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INTRODUCTION

The concept of stress was introduced by Hans Selye who implied that stress could weaken the organism and lead to the development of diesease.[1] Stress can make an organism prone to a lot of diseases like diabetes[2], heart diseases[3], obesity[4] and psychiatric and psychological diseases.[5] Crohn's disease and ulcerative colitis symptoms are exacerbated by stress.[6]

Stress is now thought to be linked to diseases in early childhood. If the parent had been exposed to stress be it mother or father this affects the fetus and results in the fetus being vulnerable to many psychological disorders like autism spectrum dieseases and other diseases later in life. Prenatal stress has been proved now to change the hypothalamic-pituitary-adrenal axis activity.[7]

The hypothalamo-pituitary-adrenal axis (HPA axis) has an important role to play in homeostasis which is the maintenance of a balanced internal environment and is important in immunity, metabolism and cardiovascular functions. A hypothalamic nucleus called the paraventricular nucleus releases corticotropin-releasing factor (CRF) which causes the secretion of adrenocorticotropic hormone (ACTH). This causes the secretion of corticosterone (CORT) from the cortex of the adrenal gland which also feeds back negatively to the brain.CORT in rats and cortisol in humans is a glucocorticoid hormone which is a stress hormone and is released in stressful situations and also in low glucose levels in the blood. It is an immunosuppressant and increases the blood glucose concentration. It has an important role to play in the metabolism of fat, carbohydrate and protein.[8]

Stress response depends on the HPA axis activation which inhibits the release of Gonadotropin Releasing Hormone (GnRH) from the hypothalamus. This inhibition is mediated by the releasing factor for ACTH called the CRF. The glucocorticoids released during stress decrease the secretion of Leuteinising Hormone (LH) from the pituitary thereby inhibiting the release of estrogen and progesterone from the ovary. This causes stress-induced hypothalamic amenorrhea and this has been seen in anxiety, depression, malnutrition and excessive chronic exercise. Studies on animal model also suggest that stress can affect the menstrual cycle in a manner which is insufficient to cause amenorrhea but can cause infertility.[9]

Genes can be altered when stress is given to parents. The physiology of the offspring may be altered. If the same stress is given to the offspring, they can either succumb to the stress or become resilient to stress. We could not use humans to find this out, so we designed a rat model. Our objective was to find the effect of chronic unpredictable stress on parents (P) and offsprings (F-1) of stressed and non-stressed rats by comparison of corticosterone and estradiol levels.

MATERIAL AND METHODS

This was an experimental case control study carried out on rats which spanned for a duration of 2 years. Ethical approval was given by Ethical Committee of Khyber Medical University (Reference No. DIR-KMU-EB/HS/000675) and from Peshawar Medical College ethical committee (Reference No=Prime/IRB/2023-207). Sample size was calculated with the help of resource equation and there were 136 rats in the parent generation while in offspring generation there were 156 rats in the offspring generation which were distributed via random sampling into various groups. In the parent generation we included healthy stress free 11 weeks old rats while unhealthy, stressed or pregnant rats were excluded. There were two groups of parents, the case parents to whom we gave stress and the control parents to whom we did not give any stress. The offspring of case parents and control parents were kept separate from each other and were analysed at the age of 5, 8, 11 and 14 weeks.

A total of 136 wistar albino rats aged 11 weeks were included in the parent generation and were all checked for anxiety by behavioural tests. The rats which were showing stressed behaviour on the behavioural tests were removed. The remaining rats were grouped into the control group and the case group. We subjected the case parents to three weeks of stress and then checked them with the help of behavioural tests for induction of stress. We removed those which were not stressed. The stressed parents were allowed to mate and their offsprings were called the case offsprings who were divided into 6 groups. To the control group of parents we did not give any stress and were allowed to mate at the same time as the case parents. Their offsprings were then divided into 6 groups. Both the groups of offsprings were given stress, some were given early life stress, some were given late life stress while some were given both. There were also offsprings who were not given any stress and served as controls. Blood sampling was done from both the parent groups and their offsprings and we took blood from 10 rats in each group and extracted blood via intracardiac puncture.[10] Blood sera were assayed for stress hormones using Enzyme Linked Immunosorbent Assay Techinique. In the offspring generation we recorded and analysed behavioural tests from 12 rats.

The stress protocol comprised of 3 stressors alternating with each other. On day 1 there was alteration of circadian rhythm when lights were turned on at night time and it was dark during the day time.[11] On day 2 there was immersion in cold water at 15 to 18 °C for 5 minutes with the height of water column of 15 Cm.[12] On day 3 we subjected them to restraint stress for 2 hours where they were kept in a cylindrical tube having ventilation holes but they could not move inside the tubes.[13] After 3 weeks of alternating stressors the rats were subjected to behavioural tests on 22nd day. Two behavioural tests were used, one was hole board test and the other was open field test.[14] The statistical analysis was done on SPSS-25. The data were not normally distributed. Kruskal Wallis test and Mann Whitney U test were used to find out differences between the groups, and $p \leq 0.05$ was taken as significant.

RESULTS

The offspring and parent groups are explained in Table-1. 'P1' stand for parent generation and 'F1' stands for first filial generation. 'A' stand for the case parents or the offsprings of case parents while 'B' stands for control parents or offsprings of control parents. In a rat study the offsprings of the same parents like the case parents are considered as the same and the point of assaying of an offspring can be generalised to all the offsprings of the same parents so the early life stressed offspring of the case parent is taken as representative of the early life stress given rats in F1A2 which was the group given stress two times except that the rat in F1A2 after early life stress was not assayed and was assayed after late life stress, at the age of 14 weeks.

Table-1: Description of various experimental groups

Abbreviation	Group description
P1B	Control group of parents to whom no stress was given
P1A	Case parent group to which stress was given
F1A	5 wks old rat who were the offspring of case parents and from whom preliminary parameters were collected
F1A1	Offspring of case parents given early life stress from 5 wks to 8 wks and were assayed at 8 wks
F1A2	Offsprings of case parents given stress twice once at 5 wks and the other at 11 wks and they were assayed at 14 wks age
F1A3	Offsprings of case parents given late life stress at 11 wks and were assayed at 14 wks
CtF1A1	Case parent offsprings not given stress and assayed at 8 wks
CtF1A2	Case parent offspring not given stress and assayed at 14 wks
F1B	5 wks old rat who were the offspring of control parents and from whom preliminary parameters were collected
F1B1	Offspring of control parents given early life stress from 5 wks to 8 wks and were assayed at 8 wks
F1B2	Offsprings of control parents given stress twice once at 5 wks and the other at 11 wks and they were assayed at 14 wks age
F1B3	Offsprings of control parents given late life stress at 11 wks and were assayed at 14 wks
CtF1B1	Control parent offsprings not given stress and assayed at 8 wks
CtF1B2	Control parent offsprings not given stress and assayed at 14 wks

The corticosterone levels were increased in the case parents P1A when compared with P1B ($p<0.01$) and to its offsprings. F1A1 had significantly raised corticosterone levels as compared to the F1A offspring who were assayed at 5 weeks before starting early life stress ($p=0.03$). F1B1 had significantly raised corticosterone levels as compared to F1B ($p=0.05$), CtF1B1 ($p=0.01$) and Ct F1B2 ($p<0.01$). The control group offsprings had decreased stress levels as shown by significantly decreased corticosterone levels as compared to the offspring given stress. Early life stressed offspring were more stressed as compared to the rest of the offsprings. (Table-2, 3).

Table-2: Corticosterone in groups showing significant *p* shown in bold in various groups

Groups	P1A	P1B	F1A	F1A1	F1A2	F1A3	CtF1A1	CtF1A2	F1B	F1B1	F1B2	F1B3	CtF1B1	CtF1B2
P1A		**<0.01***	**<0.01***	**0.03***	**0.05***	**0.02***	**<0.01***	**<0.01***	**<0.01***	0.32	0.49	0.08	**<0.01***	**<0.01***
P1B			0.59	**<0.01***	0.71	0.08	0.49	0.08	0.29	**<0.01***	0.08	**0.02***	0.07	0.19
F1A				**0.03***	0.88	0.14	0.82	0.13	0.73	**0.01***	0.11	**0.04***	**0.03***	0.11
F1A1					0.59	0.62	0.13	0.73	0.29	0.13	0.25	0.49	**0.01***	**<0.01***
F1A2						0.65	0.82	0.76	0.71	0.08	0.22	0.36	0.19	0.17
F1A3							0.23	0.88	0.41	0.17	0.32	0.36	**0.02***	**0.01***
CtF1A1								0.13	0.88	**0.04***	0.29	0.15	**0.03***	**0.05***
CtF1A2									0.49	0.08	0.33	0.25	**0.02***	**0.01***
F1B										**0.05***	0.29	0.17	**0.03***	0.07
F1B1											0.82	0.52	**0.01***	**0.00***
F1B2												0.571	0.820	**0.03***
F1B3													**0.04***	**0.02***
CtF1B1														0.59
CtF1B2														

*Significant

Table-3: Levels of Corticosterone in various experimental groups (Mean±SEM)

Groups	Corticosterone (ng/mL)
P1B	53.57±7.13
P1A	187.82±48.24
F1A	60.06±7.66
F1A1	93.16±12.82
F1A2	84.58±21.36
F1A3	89.67±15.93
Ct F1A1	71.33±16.81
Ct F1A2	83.25±13.90
F1B	80.11±16.18
F1B1	131.70±18.47
F1B2	122.57±23.16
F1B3	111.90±20.07
Ct F1B1	49.33±19.98
CtF1B2	40.20±10.08

Estradiol levels were significantly decreased in stressed parents P1A compared to control offspring CtF1B1 (p=0.01) CtF1B2 (p<0.01). F1A1 had significantly decreased estradiol levels compared to F1A (p=0.04), F1A2 (p=0.04), F1A3 (p=0.03) and Ct F1A2 (p=0.03). Thus late life stressed rat fared better than the early life stress given rat. The stress given offspring of control parents showed that F1B2 had the highest estradiol level followed by F1B3 and the early life stress given rat fared the worst. Offspring of control parents F1B1 which were early life stress given offspring had decreased estradiol levels compared to F1B2 (p=0.03), CtF1B1 (p=0.02), and CtF1B2 (p=0.01). The offsprings who were given stress at early and at late life seemed resilient than the rest (Table-4, 5).

Table-4: Table showing significant *p* in levels of estradiol in different groups

Groups	P1A	P1B	F1A	F1A1	F1A2	F1A3	CtF1A1	CtF1A2	F1B	F1B1	F1B2	F1B3	CtF1B1	CtF1B2
P1A		0.09	0.71	**0.05**	0.88	0.59	0.82	0.59	0.45	0.71	0.08	1.0	**0.01**	**<0.01**
P1B			**0.05**	**0.01**	0.09	0.29	**0.05**	0.49	**0.04**	0.22	0.82	0.15	0.94	0.32
F1A				**0.04**	0.65	0.45	0.94	0.29	0.54	0.36	**0.04**	1.0	**0.01**	**<0.01**
F1A1					**0.04**	**0.03**	0.08	**0.03**	0.17	**0.04**	**<0.01**	0.17	**<0.01**	**<0.01**
F1A2						0.49	0.76	0.54	0.23	0.36	**<0.01**	0.94	**<0.01**	**<0.01**
F1A3							0.55	0.76	0.19	0.82	0.17	0.76	**0.05**	**0.02**
CtF1A1								**<0.01**	0.71	0.45	0.11	0.88	**<0.01**	**<0.01**
CtF1A2									0.23	0.76	0.49	0.45	0.26	0.13
F1B										0.19	**0.019**	0.94	**0.01**	**<0.01**
F1B1											**0.03**	0.94	**0.02**	**0.01**
F1B2												0.76	0.36	0.23
F1B3													0.41	0.08
CtF1B1														0.41
CtF1B2														

*Significant

Table-5: Levels of Estradiol in pg/mL in various experimental groups (Mean±SEM)

Groups	Estradiol (pg/mL)
P1B	7.97±2.86
P1A	1.75±0.57
F1A	2.002±1.02
F1A1	0.71±0.35
F1A2	0.88±0.17
F1A3	3.06±1.35
Ct F1A1	2.29±1.01
Ct F1A2	13.66±9.40
F1B	1.72±1.13
F1B1	1.94±0.90
F1B2	4.49±1.21
F1B3	4.39±1.57
Ct F1B1	5.32±1.26
Ct F1B2	16.36±8.60

DISCUSSION

The lifestyle has dramatically changed in the past few decades and so has the incidence of various diseases. Epidemiological studies provide compelling evidence that stressful early life events such as famine or war may significantly influence the likelihood of developing diseases in future generations. Stress during pregnancy is closely linked to a higher likelihood of neurodevelopmental problems, such as attention deficit hyperactivity disorder[15] and schizophrenia[16]. Both the stress experienced by the mother and father may be transmitted to their kids either directly during pregnancy or via epigenetic modifications in the germ cells.[17]

Parents who are exposed to stressors during their reproductive years can have offsprings who are either resilient to stress or become more vulnerable to stress when they grow up. In this research project we compared the effect of stress across two generations of rats and we found different responses that were dependent on the age at exposure and the number of times the stress was given.

The stressed parents had increased CORT levels as compared to the control parents. The early life stressed offspring of case parents as well as the control parents had raised CORT levels. Our research project was akin to another research in which 6 to 7 week rats were given unpredictable stress and CORT levels showed a significant increase.[18] In a similar research by Zhang *et al*, CORT levels were raised in rats given chronic unpredictable mild stress.[19] However, Toth E *et al*, took two groups of rats, one group had young 30 days old rats and the other group had 60 days old rats and subjected them to chronic mild stress. They did not observe any difference in circadian CORT levels in the young rats and suggested that it was because of development of resilience to stress however the older rats did have increased corticosterone levels.[20] They used a different stress protocol for 4 weeks and used different behavioural tests like sucrose consumption test. Furthermore they took the blood sample in the middle of the stress protocol and after 2 weeks of the completion of the stress protocol. The age of sampling was younger than our rats so that may be a reason why corticosterone levels were not raised in their younger rats.

In a research by Henry *et al*, pregnant rats were given stress and when the litters were born they were subjected to stress alongside pups who were offspring of control rats to whom no stress was given. Prenatally stressed pups aged 3 and 21 days showed increased plasma CORT levels and altered HPA axis reactivity. In the adult control rats the CORT levels dropped after 2 hours while they did not do so in the rats given prenatal stress. This may be because they were exposed to high corticosterone levels of the mother who was given stress during pregnancy.[21] This corticosterone hormone can cross the placental tissue into the fetal circulation and can also reach the brain by crossing the blood brain barrier.[22] This research had similar findings to our research however they gave prenatal stress while we gave stress before mating. In our research the rats given stress at an early age were more stressed as evidenced by stressful behaviour and increased corticosterone levels as compared to the rats given late life stress.

The CORT levels were increased in the early life stressed rats but as the offspring rats were given the same stress again a second time in late life the CORT levels decreased which showed that the rats had developed some degree of resilience to the stress.

Estradiol levels were decreased in stressed parents P1A and in the offsprings given stress. Among the offsprings who were given stress F1B2 had the highest estradiol levels followed by F1B3 and F1A3. Thus, as the rat matured its estradiol levels increased and it helped the rat to cope with stress.

The hypothalamo-pituitary-adrenal (HPA) axis has an inverse relationship with the hypothalamo-pituitary-gonadal (HPG) axis. When one is activated it impacts the other axis. Stress activates the HPA axis which inhibits the HPG axis. Estrogen and Testosterone secretion is inhibited by activation of HPA axis due to stress. When an adult female rat is given stress the sympathetic axis is activated which releases noradrenaline into the ovary resulting in a noncyling ovary with the development of cysts.[23]

There was one human research which showed that stress decreased the estradiol levels in saliva on days that the patient perceived depressed mood in the same cycle.[24]

Stressors given experimentally such as isolation stress, keeping the rats blind folded or the odors of predators like fox urine increased the CORT levels while decreasing the gonadotropin levels, lowered luteal phase progesterone levels and reduced the occurrence of estrogen-dependent sexual behaviour.[24] Increasing evidence suggests that stress inhibits reproductive neuroendocrine function by hypothalamic mechanisms that reduce pulsatile GnRH release.[22] This could be thae reason why estradiol levels were decreased in the early life stress given rats.. The rats with decreased estradiol levels after stress showed increased occurance of stressful behaviour as compared to the rats with increased estradiol levels. It was also observed in this study that the rats given stress two times did not have reduced estradiol levels after the second stress exposure which made the rat resilient.

CONCLUSION

The early life stressed offsprings had increased corticosterone and reduced estradiol as compared to the offsprings given late life stress especially the offspring given both early and late life stress. Thus some stress within physiological levels is beneficial during the growing age so as to develop resilience in adulthood.

LIMITATIONS OF THE STUDY

Our study did not investigate the molecular mechanisms underlying the stress responses observed.

RECOMMENDATIONS

Future research should focus on elucidating the molecular mechanisms underlying the observed changes in this study. Understanding these mechanisms could provide critical insights into developing strategies for better stress management.

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MK: Conception, acquisition, analysis of data and drafting manuscript
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