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Comparison Of Testosterone Levels In Chronic Alternating Stress Model Of Wistar Albino Rats And Their Offspring

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Abstract:
Background: Stress has become a part of our everyday life. Stress has an adverse effect on all systems of the body including the reproductive system. The percentage of infertility is increasing day by day which can be due to stress in the reproductive years which are also the career-building years of young couples. With the passage of time stress has increased due to a more competitive lifestyle. This project was designed to study the transgenerational effect of stress. How do the offsprings of parents exposed to stress react to stress when they grow up? This study was carried out on rats because they get mature and reproduce much more quickly.
Objective: To find the effect of chronic alternating stress on parents and then study the effect of the same stress on their offspring by comparison of
1. ACTH,
2. Corticosterone,
3. Testosterone.
Methods: This was an experimental case-control study. We took 130 healthy wistar albino rats. They were 11 weeks old and we assayed them at the start of the experiment. Then we divided them into two groups. The case parents and the control parents. To the case parents we gave 3 weeks of chronic alternating stress and to the controls we did not give any stress. The genders were kept separate before and during the stress period after which they were allowed to mate. The offsprings of case and control parents were divided into groups. One group was given early life stress starting at 5 weeks and ending at 8 weeks. One group was given late-life stress starting at 11 weeks and ending at 14 weeks. One group received both early and late life stress. Then there were controls that did not receive any stress but belonged to the same group of parents. The protocol of chronic alternating stress was the same as that of the parents.
Results: The early life stressed rats had increased Corticosterone levels ($P \leq 0.05$) while testosterone ($P \leq 0.05$) was decreased in early life stressed rats as compared to the late life stressed rats. ACTH was increased in the late life stressed rats ($P \leq 0.05$).
Conclusion: The rats given both early and late life stress fared better than the rats given only early life stress.

Key Words: Adrenocorticotropic Hormone (ACTH), Corticosterone, Testosterone, Chronic Alternating Stress, Wistar albino rats.

Introduction:

Hans Selye a pioneering Hungarian-Canadian endocrinologist was considered by many as the first to demonstrate the existence of biological stress. Selye was aware of the role of glucocorticoids in the stress response. His student Walter B. Cannon focused on the function of the sympathetic nervous system in adaptation and created the terms "fight-or-flight reactions" and "homeostasis".[1]

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 and other diseases like diabetes, cardiovascular diseases, cancers etc. Prenatal stress has been proved now to change the hypothalamic-pituitary-adrenal axis activity.[6]

It is generally recognised that the hypothalamic-pituitary-adrenal axis contributes to the maintenance of the neurological system's basal and stress-related homeostasis as well as of its immunological, metabolic, and cardiovascular systems. The paraventricular nuclei (PVN) of the hypothalamus play a major role in the regulation of the HPA axis' circadian and stress-related activity. The bulk of these neurons release CRH and vasopressin (VP), which together boost the pituitary

corticotrophic cells' ability to generate ACTH. Once in the systemic circulation, ACTH promotes the release of corticosterone from the adrenal cortex and the synthesis of corticosterone from cholesterol, which sends an inhibitory feedback signal to the system.[2]

The hypothalamic-pituitary-gonadal (HPG) axis is inhibited by stressors. Stress has been shown to alter the GnRH pulse sensitivity and GnRH inturn cannot sectrete the LH and FSH in the required amount or at the required time. It is widely acknowledged that LH has a significant regulatory effect on testicular steroidogenesis. Membrane receptors on the surface of Leydig cells are responsible for LH binding.[3] These Leydig cells secrete testosterone. The epithelium of seminiferous tubule Sertoli cells is the main location of FSH activity. These Sertoli cells contain cytoplasmic receptors that testosterone can bind to and testosterone is crucial for spermatogenesis. The hypothalamus and pituitary are negatively affected by both testosterone and oestradiol.[4]

Rationale: Stress to the parents can alter the expression of genes. The offsprings of such parents may have altered physiology. These offsprings might be more vulnerable if they are exposed to the same stress as their parents or they can also be more fit for the same stress compared to control group who is not exposed to stress. Since this study could not be done on humans so we devised a rat model of chronic stress to study this trans- generational effect.

2. MATERIAL AND METHODS:

Study Setting Animal house, Peshawar Medical College. Animal research lab, Agriculture University, Peshawar. Khyber Medical University, Peshawar.

Study Design: Experimental Case Control Study.

Inclusion Criteria Healthy Wistar Albino rats of appropriate age.

Exclusion Criteria: Unhealthy rats, not of the required age group or having any observable disease, or were pregnant were excluded.

Sampling Technique: Lottery Method.

Study Duration: Two years from November 2019 to December 2021.

Ethical Approval

Ethical Committee of Khyber Medical University gave the ethical approval (Reference No = DIR-KMU-EB/HS/000675). We also took ethical approval from Peshawar Medical College (Reference No=Prime/IRB/2023-207).

Sample Size Calculation:

There were around 130 rats in the parents group.
Sample size of offspring was calculated by resource equation,
E=Total number of rats – Total number of groups
E is degree of freedom of analysis of variance, E should lie between 10 and 20,
Assume 14 rats in a group and there are 12 groups.

$$E = (14 \times 12) - 12$$
$$= 168 - 12$$
$$= 156$$

In each group, number of rats=156/12=13 which is rounded off to 14.[5]

In each group, 12 rats were required for behavioral tests due to high variability in results of behavioral tests, 10 for blood sampling and 4 for histology. For histology of gonads, 4 male and 4 female samples were taken. Keeping an attrition rate of 10 percent so we took 14 rats in each group.

Experimental Procedure:

A total of 130 adult healthy Wistar rats weighing, 280-300 g; age 11 weeks that were raised at the Peshawar Medical College Animal House. The rats were housed in cages with 6 rats per cage at 25±2°C temperature in a humidity of 40-60% under a 12-hour light/dark cycle. There was free availability of food (standard laboratory diet and water). Preliminary behavioural tests were carried out on all rats. The rats that were already stressed were removed. 10 out of remaining rats were sacrificed after giving isoflurane anaesthesia through open drop method[6] for baseline values for control parents, P1B group. Blood specimens were collected for blood markers, corticosterone, ACTH, Testosterone and a few other hormones through intracardiac puncture.[7] Also, gonadal tissue sections were made for histological evaluation from 4 males and 4 females. The remaining rats in the parent generation were divided into case parents; P1A (exposed to chronic stress) and control parents; P1B (unexposed to chronic stress). The case parents group were exposed to chronic stress at 11 weeks of age for three weeks i.e, from 11 to 14 weeks of age. The chronic stressors applied were alteration in circadian rhythm, cold water immersion stress, followed by restraint stress on the third day. We gave one stressor per day and the cycle was repeated for 21 days. The stress protocol is explained in another paper by Khattak *et al.*,[8] and the behavioural tests are explained by Usman *et al.*,[9] in another research article. We alternated the stressors to avoid adaptation. Behavioural tests (open field and hole board) were carried out to ensure induction of stress and the rats that were not stressed were removed. In the remaining

rats, 10 were sacrificed for the baseline values of case parents, P1A group. The remaining case parents left were allowed to mate. The control parents group were also allowed to mate. We took 14 offsprings in each group. There were 6 case and 6 control offsprings groups respectively so required number of rats in case and control offsprings groups were 14X6=84 respectively and the total number of offsprings required were 84+84=168.
This research work is part of the PhD thesis of the first author and the rest of the hormones and histology results will be published later.

RESULTS:

Data Analysis:

The data was analysed by spss software version 25. Normality of the data was checked using tests of normality, Kolmogorov-Smirnov and Shapiro-Wilk test and came out to be a non-normal distribution. Kruskal Wallis test was applied for comparison between all the groups and Mann Whitney U test was applied for comparison between any two groups. P≤ 0.05 was taken as a cutoff point for significance. Graphs were made through Graph Pad Prism version 9.1.0 for all the blood markers.

Hormone Levels

We used ELISA kits for measuring the hormone levels. The graphs for the hormone levels were made by Graph Pad Prism and followed the following color coding for each group. There were two groups of parents and there were six groups of case offsprings and six groups of control offsprings the details of which are explained in the below figure**.**

Table 1. Color Coding for Various Groups For Graphs of Hormone Levels

	P1B	Negative Control Group or parents not given stress
	P1A	Case Parents given stress
	F1A	Preliminary tests of 5 week old offsprings of case parents
	F1A1	Early life stressed offsprings of case parents who were started on stress protocol at 5 weeks and were sacrificed at 8 weeks.
	F1A2	Both early and late life stressed offsprings of stressed parents who were given three week stress twice, one started at 5 weeks and ended at 8 weeks while the other started at 11 weeks and ended with sacrifice at 14 weeks.
	F1A3	Late life stressed offsprings of case parents These were given stress at 11 weeks and were sacrificed at 14 weeks.
	CtF1A1	Control for early life stressed offsprings of case parents who were not given stress and were sacrificed at 8 weeks.
	CtF1A2	Control for late life stressed offsprings of case parents who were not given any stress and were sacrificed at 14 weeks.
	F1B	Preliminary tests of 5 week old offsprings of control parents
	F1B1	Early life stressed offsprings of control parents who were started on stress protocol at 5 weeks and were sacrificed at 8 weeks.
	F1B2	Both early and late life stressed offsprings of control parents who were given the three week stress twice, one started at 5 weeks and ended at 8 weeks while the other started at 11 weeks and ended with sacrifice at 14 weeks.
	F1B3	Late life stressed offsprings of control parents. These were given stress at 11 weeks and were sacrificed at 14 weeks.
	CtF1B1	Control for early life stressed offsprings of control parents who were not given any stress and were sacrificed at 8 weeks.
	CtF1B2	Control for late life stressed offsprings of control parents who were not given any stress and were sacrificed at 14 weeks.

Adrenocorticotropic Hormone (ACTH)

Table 2. Significant Differences (p-value) among Groups in ACTH levels

Groups	P1A	P1B	F1A	F1A1	F1A2	F1A3	CtF1A1	CtF1A2	F1B	F1B1	F1B2	F1B3	CtF1B1	CtF1B2
P1A		0.004	0.023					0.004						
P1B	0.004				0.000	0.000	0.008		0.034	0.002	0.005	0.001	0.049	0.016
F1A	0.023				0.003					0.028	0.023	0.016		
F1A1					0.034	0.003		0.049						
F1A2		0.000	0.003	0.034			0.059	0.000	0.05					
F1A3		0.000		0.003				0.000						
CtF1A1		0.008			0.059			0.04						
CtF1A2	0.004			0.049	0.000	0.000	0.04			0.002	0.004	0.001		0.035

F1B		0.034			0.05									
F1B1		0.002	0.028					0.002			0.049			
F1B2		0.005	0.023					0.004		0.049			0.05	
F1B3		0.001	0.016					0.001						
CtF1B1		0.049									0.05			
CtF1B2		0.016						0.035						

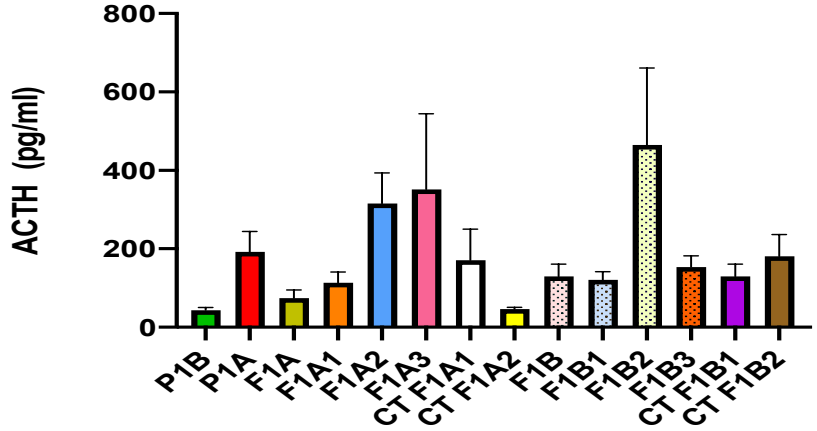

Figure 1: Comparison of ACTH Levels in pg/ml (Mean with SEM) Between all The Groups

ACTH levels results: The stressed parent group had increased ACTH levels. F1B and F1A had decreased ACTH levels showing little stress. Among the offsprings of stressed parents F1A2 and F1A3 had increased ACTH levels. Among the offsprings of control parents F1B2 had increased ACTH levels. The controls had significantly decreased ACTH levels. F1A1, F1B1 and F1B3 had comparatively decreased ACTH levels. F1B2 had significantly raised ACTH levels as compared to all the offsprings exposed to stress followed by F1A3 and F1A2. Thus, showing that both early and late life stressed offsprings of control parent fared the worst when exposed to stress. F1A1 and F1B1 had decreased ACTH levels along with F1B3. So ACTH was most raised in F1B2 followed by F1A2 and F1A3.

Table 3. ACTH Levels in various Experimental Groups

Groups	ACTH (pg/ml)
P1B	43.69 ± 20.53
P1A	192.16 ± 165.24
F1A	74.23 ± 67.76
F1A1	113.39 ± 87.01
F1A2	315.95 ± 244.76
F1A3	351.75 ± 609.99
CtF1A1	170.92 ± 251.75
CtF1A2	46.32 ± 15.32
F1B	128.71 ± 100.07
F1B1	121.19 ± 65.71
F1B2	465.11 ± 618.50
F1B3	153.92 ± 90.28
CtF1B1	129.07 ± 98.7
CtF1B2	180.97 ± 174.98

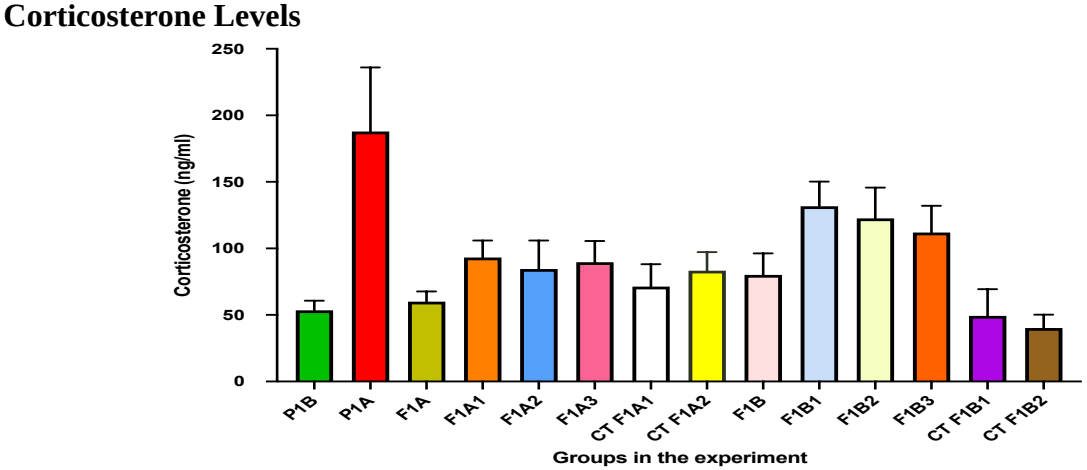

Figure 2. Comparison of Corticosterone Levels in ng/ml (Mean With SEM) Between all The Groups

Corticosterone levels Results:

P1A had increased corticosterone levels as compared to P1B and was more stressed as compared to all its offsprings. Among the offsprings of case parents F1A1 had significantly decreased corticosterone level as compared to F1A showing that early life stressed offsprings fared worst against stress. P1B the control parents were significantly less stressed as compared to their early life stressed offsprings and both early and late life stressed offsprings. Among the offsprings of control parents F1B1 was the most stressed as shown by increased corticosterone levels followed by F1B2 and F1B3 while both the controls had significantly decreased corticosterone levels. Thus the early life stressed offsprings fared the worst as shown by the above figure and the below table.

Table 4: Significant Differences (p-value) among Groups in Corticosterone Levels

Groups	P1A	P1B	F1A	F1A1	F1A2	F1A3	CtF1A1	CtF1A2	F1B	F1B1	F1B2	F1B3	CtF1B1	CtF1B2
P1A		0.000	0.001	0.028	0.05		0.005	0.006	0.01				0.002	0.000
P1B	0.000			0.019						0.000		0.023	0.070	
F1A	0.001			0.034						0.01		0.041	0.034	
F1A1	0.028	0.019	0.034							0.01			0.013	0.003
F1A2	0.05													
F1A3													0.023	0.016
CtF1A1	0.005									0.041			0.028	0.05
CtF1A2	0.006													0.013
F1B	0.01									0.05			0.028	
F1B1		0.000	0.01	0.01			0.041		0.05				0.013	0.002
F1B2														0.034
F1B3		0.023	0.041										0.049	0.016
CtF1B1	0.002	0.070	0.034	0.013			0.028		0.028	0.013		0.049		
CtF1B2	0.000			0.003			0.05	0.013		0.020	0.034	0.016		

Table 5. Corticosterone Levels in various Experimental Groups

Groups	Corticosterone (ng/ml)
P1B	53.57±22.55
P1A	187.82 ± 152.53
F1A	60.06 ± 24.21
F1A1	93.16 ±40.55
F1A2	84.58 ± 67.53
F1A3	89.67 ± 50.36
Ct F1A1	71.33 ± 53.16
Ct F1A2	83.25 ± 43.97
F1B	80.11 ±51.16
F1B1	131.70 ± 58.42
F1B2	122.57 ±73.25
F1B3	111.90 ± 63.46
Ct F1B1	49.33 ± 63.17
CtF1B2	40.20 ± 31.86

Values are in Mean ± Std. Deviation

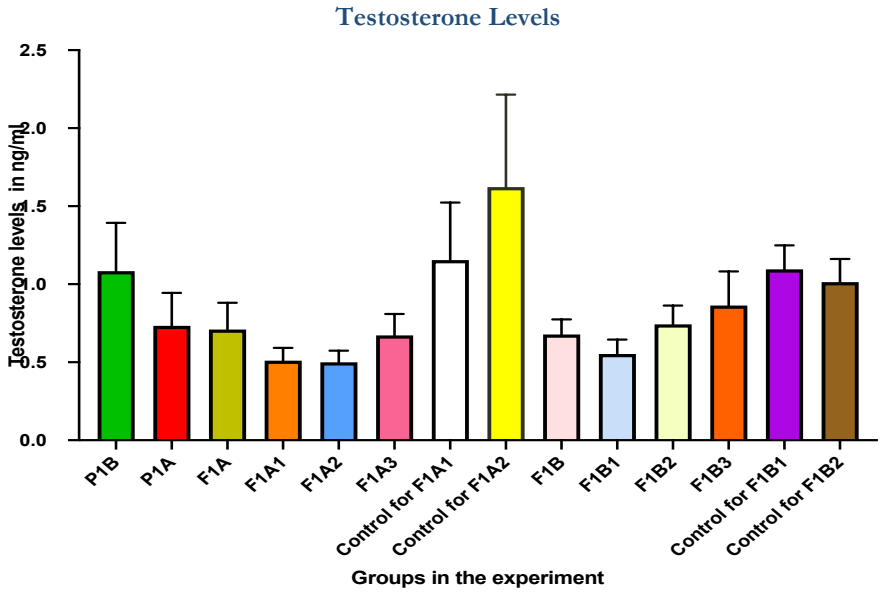

Figure 3. Comparison of Testosterone levels (mean with SEM) in ng/ml Between all The Groups.

Testosterone Levels Results:
Among the Group of offspring's given early life stress testosterone was the least in F1A1 and F1B1 which showed that early life stress was not good for reproductive function. In F1A3 and F1B3 the testosterone was comparatively increased showing that late life stress effects can be coped with better. The controls had increased testosterone levels as shown by the above figure and the below table.

Table 6. Significant Differences (p-value) among Groups in Testosterone Levels.

Groups	P1A	P1B	F1A	F1A1	F1A2	F1A3	CtF1A1	CtF1A2	F1B	F1B1	F1B2	F1B3	CtF1B1	CtF1B2
P1A													0.049	
P1B				0.019	0.016					0.041				
F1A														
F1A1		0.019											0.007	0.019
F1A2		0.016												
F1A3													0.034	0.049
CtF1A1														
CtF1A2														
F1B													0.041	0.049
F1B1		0.041											0.023	0.028
F1B2														
F1B3														
CtF1B1	0.049			0.007		0.034			0.041	0.023				
CtF1B2				0.019		0.049			0.049	0.028				

Table 7. Testosterone Levels in various groups.

Groups	Testosterone in Both Gender (ng/ml)
P1B	1.08 ± 0.98
P1A	0.73 ± 0.67
F1A	0.71 ± 0.54
F1A1	0.51 ± 0.26
F1A2	0.49 ± 0.23
F1A3	0.67 ± 0.43
Ct F1A1	1.15 ± 1.17
Ct F1A2	1.62 ± 1.87
F1B	0.68 ± 0.31
F1B1	0.55 ± 0.29
F1B2	0.74 ± 0.38
F1B3	0.86 ± 0.69
Ct F1B1	1.09 ± 0.48
Ct F1B2	1.01 ± 0.47

Values are in Mean ± Standard Deviation

Discussion:-
Stress is known as one of the most important reasons of several diseases. Physiological restructuring that happens in reaction to unfamiliar or threatening stimuli is what defines stress. Repeated stress has unfavourable impacts, such as increased risk of depression, panic attacks, post-traumatic stress disorder, drug dependence, and cognitive decline.[12] Below is the discussion of the effect of stress on the hormones that we checked:-

Adrenal Corticotropic Hormone (ACTH) levels:
The stressed parent group had increased ACTH levels. The controls had significantly decreased ACTH levels. Among the offsprings exposed to stress ACTH was most raised in F1B2 followed by F1A2 and F1A3 showing that the late life stress factor was a common factor among the offsprings who were stressed. In a study by Bomhalt *et al.,* in 2005 the ACTH levels increased after a stresser had been given.[13] One age group in our study was the juvenile age group which was F1A1 and F1B1. This age group had decreased ACTH levels as compared to the rats sacrificed at 14 weeks. Fuentes S *et al.,* reported that the peak ACTH levels were decreased in juvenile rats exposed to stress.[14] Another research showed increased levels of corticosterone and ACTH to acute stressers in the First Filial generation of prenatally stressed rats.[15]
An adverse environment in early life is often associated with dysregulation of the hypothalamo pituitary adrenal (HPA) axis and higher rates of mood disorders in adulthood. In rats, exposure to social stress during pregnancy results in hyperactive HPA axis responses to stress in the adult offsprings and heightened anxiety behaviour in the males, but not the females. Heightened anxiety in the male rats in the second filial generation of prenatally stressed rats was associated with greater CRH mRNA expression in the central nucleus of the amygdala compared with controls.[15]
During periods of prolonged stress, it's critical to maintain healthy levels of the HPA axis response. Depending on the kind of stress, there are three fundamental patterns of reaction that may be found:
(a) Desensitisation of ACTH responses to a sustained stimulus, but hyperresponsiveness to a novel stress despite elevated plasma glucocorticoid levels.[16]
(b) No Desensitisation of ACTH responses to a repeated stimulus and hyperresponsiveness to a novel stress, as seen during repeated painful stress and insulin hypoglycemia; and

(c) Small brief increases in ACTH but long-lasting increases in plasma corticosterone and diminished ACTH responses.[16]
In the case offsprings who were given stress both the ACTH and the Corticosterone levels were increased which follows the second of the above three scenarios while the stressed offsprings of the control parents had decreased ACTH levels while their Corticosterone levels were considerably high which follow the third of the above three scenario.
The half-life of ACTH is less than the half-life of Corticosterone thus this can be the reason why ACTH is decreased in the offsprings F1B and F1B1 and F1B3 whereas their CORT levels were increased. So, at the time of sampling the ACTH might have been decreased due to a short half-life while Corticosterone might have remained the same. The half-life of ACTH is around 5-10 min with some study saying 2-3 min[17] while the half-life of corticosterone is 60 minutes.[18]

Corticosterone levels:
The corticosterone is secreted from adrenal cortex in response to adrenocorticotropic hormone released from anterior pituitary gland. Under stressful situations, the secretion of corticosterone is increased. The secretion of corticosterone is highly regulated by its own negative-feedback mechanism.[19]
P1A had significantly increased corticosterone levels as compared to P1B. Among the offsprings of case parents F1A1 had significantly increased corticosterone level as compared to F1A showing that early life stressed offsprings fared worst against stress. Among the offsprings of control parents F1B1 was the most stressed as shown by increased corticosterone levels followed by F1B2 and F1B3 while both the controls had significantly decreased corticosterone levels. Thus, here the early life stressed offsprings had the worst results. Our research was similar to another study which took 6-7 week rats and subjected them to Chronic Unpredictable Mild Stress and reported raised corticosterone levels.[20]
Another research compared the effect of an anti-depressant drug on different rat groups subjected to Chronic Uncontrolled Mild Stress and found that Corticosterone was significantly raised in the untreated CUMS group.[21]Another study by Zhang also reported increased corticosterone levels in rats subjected to chronic unpredictable mild stress in rats.[22]
In contrast to our finding of significantly raised corticosterone in younger age group exposed to early life stress, study done by Toth E et al., did not observe any significant difference between the effects of chronic mild stress (CMS) on circadian corticosterone levels in the different age groups suggesting the development of resilience.[23]
The same age group was researched in a different study by Henry et al., but in that study, stress was administered to mothers during pregnancy, and once the pubs were born, they were also exposed to stress alongside pubs from control rats. According to the study, maternal stress during pregnancy affects the offsprings's HPA axis reactivity in both the short- and long-term. Male 3- and 21-day-old rats showed that prenatal stress substantially increased plasma corticosterone levels in response to stress. Corticosterone levels in adult control rats started to fall after 120 minutes, while they remained high in prenatally stressed rats at the same time. Corticosterone released by the mother under stress may be a key factor in this behaviour. This hormone could communicate with the growing fetus's central nervous system since it can penetrate the placental and hematoencephalic barriers.[16]
When evaluated in adulthood (70 days of age), adolescent male and female rats who have been socially isolated for three weeks (between 30 and 50 days of age) exhibit sex-specific variations in HPA reactivity. According to Weintraub et al., adult females who had previously experienced isolation during adolescence exhibit enhanced restraint-induced corticosterone responses compared to control females, while adult males who had previously experienced isolation during adolescence exhibit reduced corticosterone responses to restraint stress compared to socially raised controls. It's interesting to note that these effects on hormonal reactivity were only observed in plasma corticosterone levels and not ACTH levels suggesting that changes in adrenal sensitivity rather than changes in hypothalamic or pituitary function were responsible for these long-term and sex-specific effects of social isolation during adolescence.[24] In contrast to controls, social isolation during the adolescent development of male mice (from 21 to 70 days of age) resulted in lower basal corticosterone levels but higher corticosterone reactivity to a novel environment.[25]
The corticosterone levels were increased in the early life stressed offsprings as compared to the late life stressed offspring. Another study by Zhang also reported increased Corticosterone levels in rats subjected to chronic unpredictable mild stress in rats. [22]
In contrast to our finding of significantly raised corticosterone in younger age group exposed to early life stress, study done by Toth E et al did not observe any significant difference between the effects of chronic mild stress (CMS) on circadian corticosterone levels in the different age groups. Toth E et al in their study related to the substantially different neurochemical effects chronic stress exerts in young and adult animals, observed resilience in behaviour on exposure to chronic stress in young animals.[23]

Testosterone levels:
Among the Group of offsprings given early life stress testosterone was the least in F1A1 and F1B1 which showed that early life stress was not good for reproductive function. In F1A3 and F1B3 the testosterone was comparatively increased showing that late life stress effects can be coped with better. The controls had increased testosterone levels. In a study it was reported that the levels of serum testosterone changed with age in rats. Serum Testosterone levels begin to increase after 25 days of age which is 3.5 weeks, and it reaches its peak at 60 to 110 days which is 8.5 weeks to 15 weeks of age.[26]
The testosterone levels were in the lower range in all the groups. Testosterone levels are decreased in stressed rats. In a study by buwalda et al there was a reduction in testosterone level in rats of social defeat. [27] Xiaofan Xiong et al., also reported that 21 days of chronic stress decreased the testosterone levels in male rats.[28]Testosterone treatment in elderly men leads to enhancements in working memory, verbal memory and spatial cognition. A key contributor to the development of male reproductive failure is psychological stress. Stressful situations exacerbate mental disease and hasten its progression

including male infertility. Prolonged stress significantly reduced testosterone levels, and proteomics research revealed alterations in testicular gene expression patterns.[28] Thus increased testosterone levels are beneficial in stress.

Conclusion:
ACTH was increased in the late life stressed rats as compared to the early life stressed rats. The early life stressed rats had decrreased Testosterone levels while their corticosterone levels were increased. The late life stressed rats had increased testosterone levels while their corticosterone levels were decreased. Thus repeated stress can help in development of resilience in later life when the offspring is exposed to the same stress. Furthermore stress at an early age is more detrimental as compared stress at an older age.

Limitations of the study: We did not look into the molecular mechanisms underlying the changes in hormone levels.

Future Recommendations: This study should be performed on more advanced primates like monkeys. The molecular mechanisms underlying stress should be determined.

Conflict of interest: The authors declare that there is no conflict of interests.

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Authors Contributions

1.Dr Madiha Khattak
Study Design, lab work, data collection and analysis, manuscript writeup.

2.Dr Omar Malik (corresponding author)
Manuscript writeup, Idea of study, statistics, critical reading.

3.Dr. Robina Usman
Study design, Research work. statistics, critical thinking.

4.Dr.Hamid Habib
Idea of study, critical reading.

5.Dr Umar Saddique Khattak
Lab work, Critical reading.

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