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The impact of prefertilization chronic mild stress on postnatal morphometric development

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Abstract

Objective. The aim of the present study was to examine the effects of chronic mild stress (CMS) induced before the fertilization on gestational maternal weight, length of gestation, and postnatal morphometric development.

Material and method. Study is carried out on 18 Wistar albino rats; six females in the stress group, six females in the control group, and six males to be used for mating. CMS was induced in rats of the stress group for 4 weeks, followed by a post-CMS waiting period of 5-weeks. Rats were left for mating at the end of the post-CMS period. Weight gain in pregnant rats was monitored and length of gestation and litter size were recorded in the stress and control groups. Growth parameters of pups pertaining to the body, cranium, thorax, and limbs were measured until week 11.

Findings. Weight gained by pregnant rats in the stress group was less than the control group. Increases in mean morphometric parameters from day 0 to week 11 in the stress group were less than the control group ($p < 0.05$). Furthermore, when developmental parameters at weeks 7, 9, and 11 were compared between genders, females in the stress group were found more affected than males ($p < 0.05$).

Conclusion. CMS sustained before fertilization has a negative effect on gestational maternal weight and postnatal morphometric development of pups, more prominently in females.

Keywords: *Chronic mild stress, morphometry, developmental anatomy, rat, gestation*

Introduction

Organisms exert a common biological reaction in response to unfavorable sensorial and physiological events. First system that is activated in response to stress is the hypothalamus and it starts to secrete hormones. Among these hormones are cortisol and beta-endorphins. Secondarily, epinephrine and norepinephrine are secreted from the adrenal medulla via sympathetic nervous system. If the source of stress persists or occurs frequently, secretion period continues [1].

Uncontrollable or unavoidable sources of stress during gestation may affect fetal development, resulting in unwanted consequences [1]. Animal studies showed that stress, experienced during gestation, causes premature birth, low birthweight, small head circumference (HC), small pups, structural malformations, growth retardation, and change in male/female ratio [2–4]. Furthermore, prenatal stress was also shown to induce changes in psychosocial behavior and decrease cerebral cellular proliferation and hippocampal volume significantly in the postnatal period [3,5–14]. Prenatal stress also lowers the testosterone level in pups and delays or reduces sexual performance [15]. Since autonomic nervous system of the fetus is developed by the last stages of gestation, stress experienced by the mother would inflict changes in the autonomic system of the fetus. In such cases, problems related to the gastro-intestinal and cardiovascular systems may also be observed [5]. Certain psychiatric and neurological disorders of adults have been argued to be a result of stress of mother during gestation [5].

Although numerous publications on stress during pregnancy exist in the literature, they do not include any reports on postnatal morphometric development in pups of rats that experienced chronic mild stress (CMS) before gestation. Previous studies argued that biological effects of stress would be observed not only at the time of exposure to the stressor but also after the stressor is removed [5]. In the present study, we aimed to uncover the effects of CMS before fertilization on postnatal morphometric development. Previous studies addressed the effects of stress during gestation on prenatal morphometric development of fetus and postnatal development of offspring. We planned to conduct a more detailed study by examining more parameters in addition to the previously examined ones.

Material and method

This study is organized in three periods, namely, CMS induction, post-CMS, and postnatal period. Approval from the Ethics Committee of Süleyman Demirel University Faculty of Medicine was obtained prior to the commencement of the study. A total of 18 Wistar albino rats, 6 males

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used for mating and 12 females 8-weeks-old weighing 184 to 213 g were used. Two groups with six female rats each were formed. In the CMS group, previously reported methods to induce CMS were employed [16–18]. The temperature for both groups was maintained at 22 to 25°C. Rats in the control group were kept under a standard 12/12 light–dark cycle and supplied with sufficient food and water. Procedures related to CMS are presented in Table I. Rats in the CMS and control groups had free access to food, water, and 1% sucrose solution for a week to acclimate to the surrounding and adapt to the taste of sucrose. Sucrose preference test was utilized in rats in the CMS and control groups before CMS was induced. Sucrose preference test is used to define 'anhedonia' [18]. Anhedonia is defined as reduction in sucrose intake or preference in comparison to the baseline values of sucrose preference test. Anhedonia which is checked by sucrose preference test is suggested as a depression model and it also has etiological reliability [19]. After this period, the sensitivity of rats was determined to be decreased [20]. Sucrose preference test is accepted as the corresponding of anhedonia experimentally. In the sucrose preference test, rats were not allowed to have any food or drink for 20 h as described in the related article [18]. At the end, rats were allowed to drink as much water and sucrose as possible for an hour. Bottles filled with water and sucrose solution were weighed before and after the test to calculate their consumption. Results were recorded as baseline values. Sucrose preference test was conducted in both groups on every Wednesday at 12.00 am throughout the CMS period of 4 weeks and results were recorded.

To check for the efficacy of the CMS method, as reported in the relevant literature, sucrose preference test was carried out for another 2 weeks in both groups after the CMS induction was terminated in the CMS group [18]. Because it was reported that preference deficits for sucrose were maintained for more than 2 weeks after termination of the stress regime [16], there was not anything else certain about the duration of the effects of sucrose preference test and that is why we handled these 2 weeks as a touchstone and performed this application only the first 2 weeks of the post-CMS period. In the post-CMS period, the effects of CMS were allowed to recover for 5 weeks.

Following 5 weeks of post-CMS period, two female rats were placed in a cage with a male rat, all rats in both groups at the same time, and left for 24 h for mating [13–18]. Males and females were separated after 24 h. Pregnancies of the female rats were controlled by vaginal smears. Five rats in the CMS group and six rats in the control group were pregnant. Pregnant rats were placed in cages in groups of two. The day female and male rats were separated was designated 'Embryonic day 1, E1.' Weights of rats in the CMS and control groups were measured every other day for the duration of the gestation. All animals were maintained in standard conditions throughout the gestation [13–18]. Each pregnant rat was transferred to a separate cage on day 18 of gestation. Length of gestation was determined in the CMS and control groups and any deviation from normal (21 days) duration was noted (premature or postmature birth). Litter size in the CMS and control groups were also determined. Sucking/rooting reflex, movement, color, presence of anal and urethral openings, eye and ear opening times, and presence of any malformations were examined.

Morphometric growth parameters were measured in each pup born to rats in the CMS and control groups. We used standard anthropometric points for morphometric measurements [9,13–16]. Morphometric reference points were used for the extra parameters that were measured in the present study only [21].

Parameters in pups were measured once a week until week 6 and once a fortnight thereafter until week 12.

Parameters measured in the present study were:

(a) Pup weight: Measured using DENSI DS-05 electronic scale.
(b) HC: The distance around the widest part of the skull passing from the glabella of the frontal bone, parietal tuber, and posterior-most point of the occipital bone.
(c) Bi-parietal diameter (BPD): Transverse distance between the parietal tubers.
(d) Skull length: Sagittal distance between glabella and the posterior-most point of the occipital bone.
(e) Face length: Distance between glabella and the anterior-most point of the mandible.
(f) Bi-orbital diameter: Transverse distance between the lateral rims of the orbits.
(g) Thorax circumference: Distance measured at the widest part of the thorax.

Table I. One week of chronic mild stress (CMS) protocol carried out for 4 weeks before fertilization.

	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
Depriving of water	04:00 pm →	08:00 am					
Placing empty bottles		08:00 am–09:00 am					
Illuminating continuously	04:00 pm →	08:00 am			05:00 pm →	10:00 am	
Beveling the cages		11:00 am–05:00 pm					
Separating the cages	→ → →	08:00 am		06:00 pm →	02:00 pm	10:00 am	→ → →
Spilling into the bedding (300 cc)					05:00 pm →	10:00 am	
90 DB nois						10:00 am–01:00 pm	
Stroboscopic illumination	11:00 am–04:00 pm			01:00 pm–03:00 pm			

One period of the experiment, which lasted for a week, started on Sunday morning at 8:00 am and ended on next Sunday at 8:00 am. Animals were deprived of water between 4:00 pm on Sunday and 8:00 am on Monday, followed by placing empty water bottles in cages for an hour between 8:00 and 9:00 am. Cages were illuminated continuously between 4:00 pm on Sunday and 8:00 am on Monday and between 5:00 pm on Thursday and 10:00 am on Friday. Each animal was placed in a separate cage between 8.00 am on Sunday and 8:00 am on Monday, 6:00 pm on Wednesday and 2:00 pm on Thursday, and 10:00 am on Friday and 8:00 am on Sunday. At room temperature, 300 ml of water was spilled into the bedding between 5:00 pm on Thursday and 10:00 am on Friday. Animals were exposed to 90 dB noise between 10:00 am and 1:00 pm on Friday and stroboscopic illumination between 11:00 am-4:00 pm on Sunday and between 1:00 pm-3.00 pm on Wednesday [18]. This cycle was repeated for a period of 4 weeks.

(h) Thorax width: Transverse distance between two vertical planes passing through the outermost points of the thorax.
(i) Crown-rump length (CRL): Distance between the vertex and the point where the tail started.
(j) Naso-anal length: The distance between the tip of the nose and the midpoint of the anus.
(k) Forearm length: Distance between the midpoint of the elbow joint and the tip of the longest digit on forelimb.
(l) Leg length: Distance between the midpoint of the knee joint and the tip of the longest digit on hindlimb.
(m) Bi-acetabular distance: Transverse distance between the greater trochanters.
(n) Ano-genital distance: Distance between the midpoint of the anus and the external urethral orifice.

Data obtained was pooled in three groups and assessed separately for:

(a) Parameters pertaining to newborn period (day 0)
(b) Parameters pertaining to Lactation period (days 7, 14, and 21)
(c) Parameters pertaining to puberty and adulthood (weeks 5, 7, 9, and 11)

Arithmetic means of all parameters in CMS and control groups and standard deviations associated with these means were calculated for each week. Arithmetic means and standard deviations of these parameters with respect to gender were also determined from week 7 and onwards. Student's *t*-test and nonparametric Mann-Whitney *U* test were used to compare the parameters between the CMS and control groups. The relations between age and all parameters obtained between newborn period and adulthood were tested by Pearson's correlation test. The level of statistical significance was set at 0.05.

Findings

In the first phase of this study, sucrose preference test was administered to the rats in both groups, as explained in the Material and Method section. As expected, there was a decrease in mean sucrose consumption in six rats in the CMS group in comparison to the baseline values and the control group (Figure 2). After the experiment was terminated (31 days), there was a post-CMS waiting period of 5 weeks during which nothing was performed on the CMS or control groups.

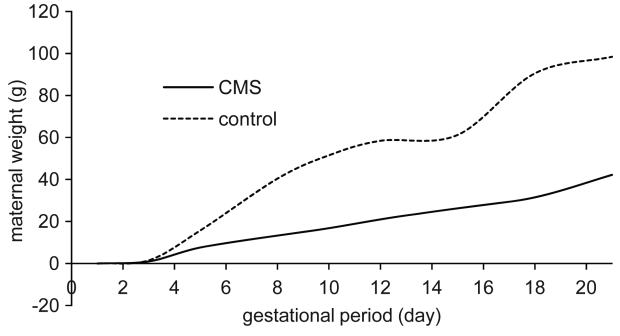

Figure 1. Changes in mean maternal weight gained by pregnant rats in the chronic mild stres (CMS) and control groups throughout the pregnancy.

In the first two weeks of the post-CMS period, sucrose preference test was readministered to both groups. The reason for a second administration was to determine whether there was an increase in sucrose consumption, in other words, to determine whether rats were out of depression. In the post-CMS period, mean sucrose consumption of six rats in the CMS group was higher than the baseline values (Figure 3), as was reported in relevant literature [18]. There was no statistically significant difference in sucrose consumptions between CMS and control groups in this period.

Mean weight gained during gestation by female rats in the CMS and control groups in the 3rd period was measured and mean weight gained by rats in CMS group was less than the control group (Figure 3). While mean weight gained during gestation by control rats was 98.4 g and that gained by stressed rats was found as 42.2 g.

When we compared the length of gestation in both groups, we found that, with the exception of one animal in the CMS group with 20 days of gestation, length of gestation was 21 days in all rats.

Following the gestation, one female in the CMS group died on postnatal day 1, along with all of her pups. In the CMS group, 36 pups were born to 4 rats while 57 pups were born to 6 rats in the control group.

There were no anomalies or pathologies in any of the pups in the CMS or control groups with regard to sucking/rooting reflex, movement, color, anal and urethral openings, or eye and ear opening times. No complications such as spontaneous abortion were noted in either group.

Growth parameters of pups in the CMS and control groups pertaining to the body, cranium, thorax, and limbs were measured between day 0 and week 11, in newborn and lactation periods and adulthood (Tables II–IV). Tables II–IV show the arithmetic means and standard deviations of all

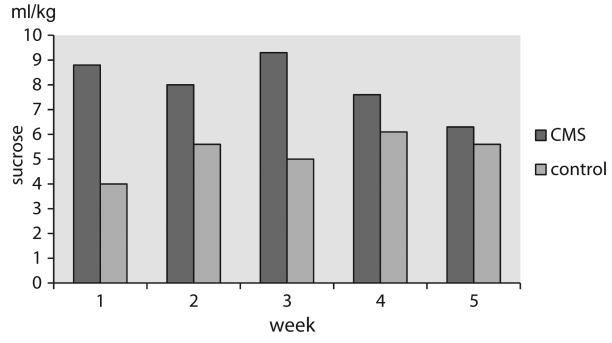

Figure 2. The change of the sucrose consumption of the CMS and control groups during the CMS period.

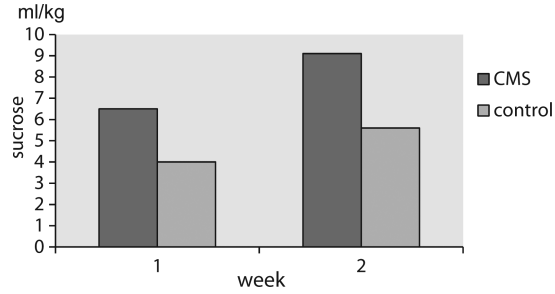

Figure 3. The change of the sucrose consumption of the CMS and control groups during the two weeks of post-CMS period.

Table II. Arithmetic means (mm) and standard deviations of general morphometric parameters of chronic mild stress (CMS) and control pups measured between newborn period and adulthood.

	General parameters															
	N		Weight (g)		Crown-rump length (CRL)		Thorax circumference		Thorax width		Naso-anal length		Ano-genital distance	
	CMS	Control	CMS	Control	CMS	Control	CMS	Control	CMS	Control	CMS	Control	CMS	Control
Newborn														
First day	36	57	5.5 ± 0.5	5.9 ± 0.4	40.0 ± 3.4	41.9 ± 2.8	41.5 ± 2.3	43.6 ± 3.7	14.4 ± 1.1	16.0 ± 1.2	42.7 ± 3.7	45.1 ± 3.3	2.4 ± 0.9	2.3 ± 0.7
Lactation														
1st week	33	53	11.3 ± 1.2	12.0 ± 1.6	52.0 ± 2.6	53.3 ± 2.9	51.3 ± 4.4	53.1 ± 3.1	17.6 ± 1.1	19.0 ± 2.2	55.5 ± 3.3	57.8 ± 4.4	3.6 ± 1.2	3.8 ± 1.1
2nd week	26	52	17.5 ± 0.5	21.1 ± 2.3	63.2 ± 3.1	68.9 ± 5.5	58.4 ± 5.3	62.9 ± 5.3	20.9 ± 2.8	25.0 ± 2.1	71.0 ± 2.6	80.3 ± 9.3	6.5 ± 0.5	8.1 ± 1.4
3rd week	26	47	22.5 ± 3.9	25.5 ± 3.5	74.6 ± 7.9	77.7 ± 3.9	64.8 ± 8.3	70.0 ± 3.2	29.5 ± 3.1	32.5 ± 3.0	76.5 ± 13.2	80.0 ± 21.8	10.3 ± 2.4	9.7 ± 1.8
Adulthood														
5th week	24	37	59.3 ± 8.8	65.5 ± 7.7	105.5 ± 5.9	109.7 ± 4.8	89.2 ± 6.5	93.7 ± 5.9	34.0 ± 3.7	36.1 ± 2.0	117.7 ± 9.3	123.5 ± 6.0	14.8 ± 3.3	14.6 ± 3.9
7th week	24	33	86.2 ± 4.6	97.5 ± 12.9	122.2 ± 5.7	130.4 ± 7.2	103.5 ± 5.5	111.7 ± 4.0	42.6 ± 2.1	45.0 ± 2.9	140.5 ± 11.6	150.7 ± 8.7	15.6 ± 3.5	20.3 ± 6.1
9th week	24	33	117.8 ± 11.7	156.1 ± 6.4	131.7 ± 6.0	141.7 ± 5.5	109.7 ± 5.5	126.8 ± 8.1	45.7 ± 2.5	50.0 ± 3.8	144.7 ± 7.4	156.4 ± 9.9	16.4 ± 3.9	18.7 ± 4.0
11th week	24	33	135.5 ± 18.2	152.4 ± 2.3	143.9 ± 5.8	151.8 ± 4.6	112.9 ± 3.2	119.8 ± 8.2	45.6 ± 2.6	49.2 ± 4.3	158.3 ± 8.8	163.8 ± 11.5	17.0 ± 5.9	19.1 ± 6.3

$p < 0.05$. Difference in all parameters between CMS and control groups.

parameters measured in pups. At weeks 7, 9, and 11, measurements were performed separately for males and females and arithmetic means and standard deviations are given in Tables V–VII. Comparison of parameters between the CMS and control groups revealed that there were small but significant differences in all periods and all parameters, with all parameters being smaller in the CMS group ($p < 0.05$, Tables II–IV). When we compared the parameters of males in the CMS group with the parameters of males in the control group and repeated the similar comparison for females (male–male, female–female), we noted that 83% of the parameters were significantly larger in females of the control group and 23% of the parameters were significantly larger in males of the control group ($p < 0.05$, Tables V–VII). Very significant correlations appeared between age and all parameters between newborn period and adulthood ($p < 0.001$).

Discussion and conclusion

A literature search revealed no studies that investigated the effects of exposure to CMS before fertilization despite studies in which CMS was performed during gestation. The present study is important since it is the first study that addressed the impact of CMS before fertilization on changes in maternal weight during gestation, prenatal development of the fetus, and postnatal growth of pups.

It is a well-established fact that stress alters the eating behavior [22]. Gestation, in the meantime, causes positive and negative stresses. Adequate weight gain, but not excessive, is necessary during gestation for the fetus and dam alike [23].

Maternal weight loss is a crucial parameter in experimental studies carried out during the gestation with exposure to stress [2]. Insufficient weight gain, on the other hand, brings with itself complications such as prematurity and low birthweight [24–27]. Picone and colleagues' study on pregnant women demonstrated a negative correlation between psychological stress during pregnancy and maternal weight gain by showing high stress scores being associated with low weight gain [26]. Previous studies reported that rats exposed to stressors during gestation gained less weight than the controls 27–30. Osorio et al. [27] induced pregnant rats to prenatal stress and showed that they gained less weight during gestation. In a study by Hougaard et al. [29], authors reported that pregnant rats in the control group gained 100.7 g while rats exposed to CMS and pharmacological stressor (dexamethasone) gained 98.2 g and 64.4 g, respectively. Kinsley et al. [31] reported that maternal weights gained during pregnancy were 33.01 g, 36.84 g, and 36.15 g for the stress, nonstress, and control groups, respectively. Authors noted that maternal weight gain in the stress group was lower than the other two groups (nonstress and control) and this difference was found statistically significant. The present study demonstrated that female rats exposed to CMS before fertilization gained less weight during gestation than the controls (Figure 1). We found that pregnant rats in the CMS group gained 42.2 g during gestation while those in the control group gained 98.4 g. It should be noted that rats were exposed to CMS before the fertilization. Although the timing of exposure to stress was different from other studies, findings of the present study are in agreement with their results showing unfavorable effects of stress on maternal weight [29,31]. Sufficient and satisfactory data on how long the effects of stress or depression last in humans or animals is not available. Sucrose preference test may not be sufficient and reliable in that respect. Rats in

Table III. Arithmetic means (mm) and standard deviations of morphometric parameters pertaining to the cranium and thorax of chronic mild stress (CMS) and control pups measured between newborn period and adulthood.

	Cranium and thorax parameters									
	Skull length		Face length		Bi-orbital diameter		Head circumference		Bi-parietal diameter	
	CMS	Control	CMS	Control	CMS	Control	CMS	Control	CMS	Control
Newborn										
First day	14.9 ± 2.0	16.7 ± 1.1	8.5 ± 1.2	9.6 ± 1.0	9.1±1.7	10.2 ± 0.9	34.5 ± 2.2	36.0 ± 3.5	8.8 ± 1.1	9.5 ± 1.7
Lactation										
1st week	22.2 ± 1.3	23.0 ± 2.1	12.1 ± 1.1	13.5 ± 1.5	10.5 ± 0.9	12.5 ± 1.8	41.5 ± 3.8	44.5 ± 4.8	13.4 ± 2.2	14.7 ± 2.3
2nd week	28.8 ± 4.2	32.7 ± 3.0	17.2 ± 1.8	18.6 ± 2.1	14.9 ± 1.9	16.7 ± 1.4	58.0 ± 3.4	55.4 ± 2.8	18.2 ± 2.2	19.5 ± 1.5
3rd week	30.6 ± 3.3	33.4 ± 9.1	19.4 ± 1.8	20.5 ± 5.6	15.9 ± 1.4	17.8 ± 1.8	56.4 ± 3.7	59.7 ± 4.0	20.6 ± 1.5	23.1 ± 2.4
Adulthood										
5th week	41.7 ± 2.1	43.5 ± 4.0	21.2 ± 2.4	23.5 ± 2.8	17.9 ± 1.8	19.6 ± 2.6	65.2 ± 2.4	63.1 ± 3.9	21.5 ± 1.1	22.8 ± 1.9
7th week	43.6 ± 7.5	51.8 ± 2.6	23.3 ± 1.3	24.7 ± 1.4	18.4 ± 2.9	20.6 ± 0.8	65.6 ± 4.3	68.9 ± 3.2	23.1 ± 1.5	24.0 ± 0.9
9th week	38.2 ± 4.4	44.6 ± 2.6	25.5 ± 1.9	27.6 ± 2.6	19.6 ± 1.1	21.0 ± 1.5	72.9 ± 2.7	80.9 ± 4.5	28.5 ± 2.5	28.9 ± 3.0
11th week	36.5 ± 3.3	41.1 ± 4.7	25.5 ± 1.0	31.5 ± 3.4	18.2 ± 0.8	21.0 ± 1.7	71.9 ± 2.7	74.4 ± 3.8	28.8 ± 1.5	29.6 ± 1.2

$p < 0.05$. Difference in all parameters between CMS and control groups.

Table IV. Arithmetic means (mm) and standard deviations of morphometric parameters pertaining to the limbs of chronic mild stress (CMS) and control pups measured between newborn period and adulthood.

	Limb parameters					
	Forearm length		Leg length		Bi-acetabular distance	
	CMS	Control	CMS	Control	CMS	Control
Newborn						
First day	10.2 ± 1.1	11.5 ± 0.9	7.8±1.0	8.5 ± 0.7	12.2+2.1	13.1 ± 1.9
Lactation						
1st week	16.9 ± 1.8	18.7 ± 1.7	13.9 ± 2.1	14.8 ± 1.5	17.5 ± 2.0	19.9 ± 4.1
2nd week	22.9 ± 2.4	25.8 ± 2.2	21.3 ± 1.2	22.4 ± 2.1	21.5 ± 3.4	25.2 ± 3.2
3rd week	28.7 ± 1.6	31.1 ± 2.1	26.0 ± 2.0	28.7 ± 1.6	31.5 ± 3.5	34.1 ± 3.7
Adulthood						
5th week	32.2 ± 1.7	34.4 ± 2.0	30.6 ± 1.5	31.8 ± 1.7	36.9 ± 3.8	38.7 ± 2.9
7th week	34.5 ± 2.9	36.8 ± 1.7	33.4 ± 1.2	34.4 ± 1.4	41.2 ± 3.2	43.0 ± 2.2
9th week	40.4 ± 1.5	41.9 ± 2.7	36.2 ± 4.6	39.2 ± 2.7	53.5 ± 2.6	55.3 ± 6.6
11th week	41.0 ± 4.2	43.6 ± 1.8	36.1 ± 4.0	39.7 ± 2.7	54.5 ± 4.0	57.2 ± 4.5

$p < 0.05$. Difference in all parameters between CMS and control groups.

the CMS group were maintained for 5 weeks after the CMS (post-CMS waiting period), as described in relevant publications. Gradual increase in sucrose consumption during this period indicates that the effects of CMS were resolving. The fact that maternal weight gain by the CMS group during gestation was less than the control group led us to think that the effects of CMS persisted even if it was induced before the fertilization. Our results raise questions regarding the reliability of sucrose preference test results and suggest that a 5-week waiting period may not be enough. Hence, more studies are needed to show when all effects of CMS clear, whether it be induced before fertilization or during gestation. Furthermore, differences between the effects on maternal weight gain of CMS induced before fertilization and during gestation should also be elucidated.

Prenatal stress causes changes during gestation as well. Engel et al. [4] reported that prenatal stress increased the length of gestation. Our results contradict their finding such that there was no significant change in the length of gestation in the CMS or control group. This result was interpreted as prefertilization CMS not having an impact on the length of gestation.

Stress experienced by mother during gestation adversely affecting prenatal development has been demonstrated by various studies [32]. Prenatal stress is also closely related to biometric parameters [32]. High levels of anxiety and depression, especially during the first 3 months of pregnancy, cause low birthweight and smaller HC [3]. Symptoms of posttraumatic stress following prenatal stress have also been argued to be related to the HC of the newborn [4]. In an ultrasound study, researchers showed that daily stress, depression and state anxiety scores correlated negatively with fetal biometric data between 16 and 29 weeks of gestation [32]. Animal studies on prenatal stress showed that anogenital distance was smaller in male pups born to rats exposed to stress in the last week of gestation [33]. However, we did not find any abnormalities or pathologies in sucking/rooting reflex, movement, color, anal and urethral openings, and eye and ear opening times in any of the newborn pups of CMS and control groups. This result suggests that CMS before fertilization did not have any effect on newborn features and morphology.

Studies that examined the effects of prenatal maternal stress on offspring established that prenatal stress caused

Table V. Arithmetic means (mm) and standard deviations of general morphometric parameters of male and female pups in the chronic mild stress (CMS) and control groups measured at weeks 7, 9, and 11.

		General parameters											
		Weight (g)		Crown-rump length (CRL)		Thorax circumference		Thorax width		Naso-anal length		Ano-genital distance	
Age	Case numbers (CMS; Control)	CMS	Control	CMS	Control	CMS	Control	CMS	Control	CMS	Control	CMS	Control
7th week	Male (4; 15)	88.8 ± 7.1	98.2 ± 15.3	125.0 ± 0.0	128.3 ± 4.4	101.7 ± 6.9*	113.2 ± 3.1	44.2 ± 1.5	45.8 ± 3.3	144.7 ± 16.0	154.0 ± 10.5†	29.0 ± 1.1*†	25.9 ± 4.3†
	Female (20; 18)	85.7 ± 4.0*	96.9 ± 11.0	122.5 ± 6.1*	128.6 ± 7.2	103.9 ± 5.3*	110.6 ± 4.4	42.3 ± 2.1*	44.4 ± 2.4	139.7 ± 10.9	148.0 ± 5.9	14.5 ± 2.5	15.6 ± 2.0
9th week	Male (4; 15)	137.6 ± 9.8*†	156.7 ± 6.6	138.7 ± 7.5†	143.3 ± 5.5	108.5 ± 4.3*	127.6 ± 6.1	49.0 ± 1.1†	51.1 ± 3.8	157.5 ± 5.0†	158.4 ± 7.7	23.7 ± 2.5†	22.2 ± 3.1†
	Female (20; 18)	113.9 ± 7.3*	155.7 ± 6.3	130.4 ± 4.8*	140.3 ± 5.5	110.0 ± 5.7*	126.1 ± 9.6	45.1 ± 2.1*	49.0 ± 3.7	143.2 ± 6.9*	152.0 ± 8.7	15.0 ± 2.0	15.8 ± 1.4
11th week	Male (4; 15)	147.5 ± 8.6	152.6 ± 2.5†	147.5 ± 8.6	153.3 ± 2.4	115.0 ± 0.0	119.7 ± 8.0	49.2 ± 1.5†	49.8 ± 2.8	166.2 ± 4.7†	154.0 ± 5.7	29.0 ± 1.1*†	25.3 ± 3.1†
	Female (20; 18)	127.9 ± 5.8*	148.2 ± 18.2	140.2 ± 3.7*	150.5 ± 5.6	112.5 ± 3.4*	120.0 ± 8.6	44.8 ± 1.8*	48.5 ± 5.0	155.5 ± 6.4*	165.5 ± 13.6	14.6 ± 2.4	13.9 ± 2.1

$^{*}p < 0.05$. Difference between rats of same gender in CMS and control groups (male–male, female–female).
$^{\dagger}p < 0.05$. Difference between males and females within CMS and control groups.

Table VI. Arithmetic means (mm) and standard deviations of morphometric parameters pertaining to the cranium and thorax of male and female pups in the chronic mild stress (CMS) and control groups measured at weeks 7, 9, and 11.

		Cranium and thorax parameters									
		Skull length		Face length		Bi-orbital diameter		Head circumference		Bi-parietal diameter	
Age	Case numbers (CMS; control)	CMS	Control	CMS	Control	CMS	Control	CMS	Control	CMS	Control
7th week	Male (4; 15)	43.7 ± 9.1*	52.6 ± 2.0	24.0 ± 0.8	24.1 ± 1.5	18.0 ± 3.5	21.0 ± 1.0	66.2 ± 4.7	67.8 ± 3.7	22.0 ± 2.8	24.3 ± 0.8
	Female (20; 18)	41.5 ± 6.8*	51.1 ± 2.9	23.4 ± 1.3*	24.6 ± 1.4	17.8 ± 2.7*	20.9 ± 1.6	65.5 ± 4.4*	69.8 ± 2.5	22.5 ± 1.6*	24.2 ± 1.1
9th week	Male (4; 15)	42.5 ± 2.8†	44.4 ± 1.8	26.5 ± 1.7	27.2 ± 2.7	21.0 ± 1.1†	21.2 ± 1.3	78.7 ± 4.7†	82.0 ± 3.6	30.2 ± 2.0†	29.6 ± 3.1
	Female (20; 18)	37.4 ± 2.8*	44.8 ± 3.2	25.4 ± 1.9*	27.8 ± 2.5	19.3 ± 0.9*	21.5 ± 1.6	72.7 ± 2.8*	80.0 ± 5.0	27.2 ± 2.2*	29.6 ± 3.3
11th week	Male (4; 15)	39.2 ± 2.9†	40.9 ± 1.0	27.0 ± 0.0*†	32.2 ± 2.8	20.0 ± 0.0*†	21.4 ± 1.5	74.0 ± 1.1.8†	76.5 ± 3.1†	30.0 ± 1.6†	30.1 ± 1.4
	Female (20; 18)	35.9 ± 3.2*	42.0 ± 6.1	25.3 ± 0.9*	30.9 ± 3.8	17.9 ± 0.3*	20.7 ± 1.9	71.2 ± 2.2*	73.8 ± 3.2	28.2 ± 0.6*	29.3 ± 0.9

$^{*}p < 0.05$. Difference between rats of same gender in the CMS and control groups.
$^{\dagger}p < 0.05$. Difference between rats of different gender in the CMS and control groups.

Table VII. Arithmetic means (mm) and standard deviations of morphometric parameters pertaining to the limbs of male and female pups in the CMS and control groups measured at weeks 7, 9, and 11.

		Limb parameters					
		Forearm length		Leg length		Bi-acetabular distance	
Age	Case numbers (CMS; control)	CMS	Control	CMS	Control	CMS	Control
7th week	Male (4; 15)	36.0 ± 3.5	36.7 ± 1.7	33.5 ± 1.9	34.8 ± 1.6	40.0 ± 0.0★	46.8 ± 4.5†
	Female (20; 18)	34.2 ± 2.8★	36.9 ± 1.8	32.2 ± 2.4★	34.7 ± 1.2	41.5 ± 3.5	42.3 ± 2.5
9th week	Male (4; 15)	42.5 ± 2.8†	42.8 ± 2.0	37.0 ± 6.2	40.0 ± 2.2	55.2 ± 2.0★	59.4 ± 2.6†
	Female (20; 18)	40.0 ± 3.8★	41.7 ± 3.3	35.6 ± 2.3★	39.0 ± 2.8	53.1 ± 2.3	53.6 ± 5.9
11th week	Male (4; 15)	38.7 ± 4.7	43.0 ± 2.1	35.0 ± 5.7	39.8 ± 0.9	40.0 ± 0.0†	59.2 ± 5.3†
	Female (20; 18)	41.7 ± 3.8★	44.1 ± 1.5	36.3 ± 3.7★	39.6 ± 3.6	53.2 ± 2.9	55.5 ± 3.1

*$p < 0.05$. Difference between rats of same gender in the CMS and control groups.

†$p < 0.05$. Difference between rats of different gender in the CMS and control groups.

prenatal and postnatal growth retardation [4,28,29,34,35]. Intrauterine growth retardation is a pathology of various etiology and recent studies implicated stress as the most probable cause of intrauterine growth retardation [14]. In these studies, premature birth, low birthweight, small stature, structural malformations, growth retardation, and alterations in male/female ratio have been reported [2,3,14,28,34,35]. It also causes delay in newborn reflexes and delayed motor development [3,14]. However, in the present study, premature birth, structural malformations, and marked growth retardation were not found in any of the pups in the CMS or control groups but birthweight and other morphometric growth parameters (general, cranial, and limbs) were significantly smaller than the controls ($p < 0.05$, Tables II–IV).

The effects of prenatal stress on birthweight and body weight in later stages have been examined in previous studies [14,15,28,32,36–38]. The relation between maternal stress and fetal weight between 16 and 29 weeks of gestation was explored and a negative correlation between these parameters was found [32]. It was found in some studies that, in comparison to the controls, pups of rats exposed to prenatal stress during gestation had low birthweight and their body weights later in life were also less than the controls [14,28,33,37] while other studies reported no such difference between groups and no effect on birthweight and body weight [15,36,38]. In our study, birthweights and body weights in later stages of pups of rats exposed to CMS prior to the fertilization were significantly lower than the controls.

We were unable to find any publications that thoroughly examined the postnatal development using general growth parameters and growth parameters pertaining to the cranium, thorax, and limbs in rats exposed to prenatal stress. Significant differences in all parameters were determined between the CMS and control groups in periods when the gender of the pups could not be differentiated, with CMS group having smaller ($p < 0.05$, Tables II–IV).

Not many studies exist regarding the morphometric effects of prenatal stress on male and female pups. Bowman and colleagues' study [6] reported higher anxiety in females exposed to prenatal stress. Moreover, Kofman et al. [5] showed that prenatal stress induced changes in dopamine distribution and that female rats were more affected than males. It has been argued that prenatal stress delays neonatal development of sensorimotor reflexes in male rats and spontaneous alternation behavior in female rats [5]. Neuromotor activity was found higher in male offspring of rats exposed to prenatal stress [39]. Demasculinizing effects of prenatal stress in male offspring have also been reported [5]. When we compared the ano-genital distance between stressed males and control males and between stressed females and control females (male–male, female–female comparisons), we found no significant difference between females while the difference in males was significant. Our finding is in agreement with those of Gerardin et al. [15] and Pereira et al. [33] who also reported decreased ano-genital distance in male pups following prenatal stress. Overall, our results suggest that exposure to prefertilization stress affects the postnatal morphometric development of females more than that of males. However, the small number of male pups in the present study was a constraint of this study that prevented us to draw more reliable biological conclusions. Therefore, more studies are required to elucidate the differential effects of prenatal stress on postnatal morphometric development with respect to gender.

There are contradicting arguments on which mechanisms prenatal stress exerts its effects on fetuses and offspring in the prenatal and postnatal periods. Diego and coworkers [32] attributed the adverse effects on fetal development to maternal neuro-endocrine functions. Drago et al. [14], on the other hand, argued that prenatal stress not only increases the secretions of corticotropin releasing hormone and adrenocorticotropic hormone but also decreases growth hormone synthesis. Gerardin and colleagues [15] suggested that decrease in ano-genital distance resulted from insufficient activity or secretion of testosterone secondary to exposure to stress in prenatal period [33]. Stress increases the synthesis of catecholamines [40]. Studies on rats exposed prenatally to substances that increase catecholamines such as dexamethasone, rather than prenatal stress, showed that morphometric parameters of development in rats exposed to dexamethasone were smaller than controls [41]. Prenatal exposure to dexamethasone precipitates low birthweight and low birthweight is associated with less bone and muscle mass in adulthood [41]. We believe that prenatal stress, acting via similar mechanisms, exerted adverse effects on morphometric development of fetuses and pups in pre- and postnatal periods which resulted in pups in the CMS group having lower birthweight and subsequent body weight than the controls. Moreover, dams in the CMS group gained less weight than the controls and this can be held accountable for the prenatal and postnatal growth retardation and its consequences.

With regard to the differential effects of prenatal stress on gender: Huizink et al. [2] reported that responses elicited by prenatal stress acting on the hypothalamo-pituitary axis

(HPA) are different for genders. While prenatal stress decreases glucocorticoid density in male cerebral cortex, it increases the density in female brain [5]. Female rats are more susceptible to the effects of prenatal stress on HPA [2]. Swolin-Eide et al. [40] stated that female offspring of dams exposed prenatally to dexamethasone had smaller CRLs than the controls in the first 5 weeks of postnatal period. Adverse affects observed in female rats in the present study are believed to be associated with the mechanisms presented above.

Diversity of prenatal stressors used in previous studies, timing of the exposure to stress and differences between subjects exposed to stress will contribute to the variable results observed pre- and postnatally [2,3,5]. In short, it is safe to say that the effects of prenatal stress on offspring are multifactorial. The present study is the first to utilize stress before fertilization. Our results show that exposure to prefertilization stress adversely affects prenatal development and postnatal growth in males and females alike though these effects on females were more pronounced.

According to retrospective studies, offspring of mothers disturbed emotionally or those with high level of anxiety during gestation are restless, more irritable, and less responsive and they sleep less [6]. It has also been shown that maternal anxiety at 32 weeks of gestation caused hyperactivity and attention deficit in males, behavioral problems in females and emotional problems in both males and females during childhood. Furthermore, it has been argued that children born as a result of unwanted pregnancy are at greater risk to develop schizophrenia during adulthood [6].

In conclusion, prenatal stress is a very important maternal factor that affects the fetal development. Mothers have to lead a psycho-socially comfortable and serene pregnancy for the fetus to live a morphologically, physiologically, and psycho-socially healthy life. With new studies aimed to look into the subject from other points of view, it would be possible to gain insight into and understand the effects of prenatal stress on offspring.

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