**The Pyramid Counteracts Chronic Prenatal Restraint-Stress Effects on the Milestones, Anthropometry, and Body, Brain and Adrenal Gland Weights of Pups in Rats**

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**ABSTRACT**

Prenatal exposure to chronic stress during critical periods of foetal development produces depression, attention and learning deficits, hormonal imbalances and affects the brain. The effect of prenatal restraint-stress on the postnatal developmental milestones, anthropometric measurements, and the body, brain and adrenal gland weights of the pups were examined and compared with the un-restrained control and the restrained group under the pyramid at postnatal day 10 and 21. Pregnant rats were restrained (9h/day) from gestation day 7 until parturition. Results showed significant delay in the milestones by one day in the restraint control (RC) compared to the unrestrained normal control (NC), while pups of the restrained pyramid (RP) group did not show the delay. Significant decreases in the anthropometric measurements, body and brain weights in RC group were observed at both postnatal days, while the RP group results matched with the NC group. Significant increase in the adrenal weights was found in the RC group compared to NC group and not the RP group. Results suggest prenatal restraint-stress definitely hampers the developmental milestones, anthropometric measurements, and body and brain weights of the young offspring. Results suggest, pyramid environment counteracts and protects the deleterious effects of chronic prenatal stress.

**Keywords:**Anthropometry, Milestones, Prenatal stress, Pyramid

**Short title** (running headline): PYRAMID COUNTERACTS PRENATAL STRESS EFFECTS……

**INTRODUCTION**

Any disruption of the maternal environment during gestation leads to behavioural changes in the offspring. Studies have shown that stress during gestation can induce early and long-lasting effects on neurobehavioral development of the pups. Zaneta et al.,1 report high maternal cortisol levels to impact the foetal growth particularly in the male offspring. Earlier reports2-11 show effects of prenatal stress on cognitive, behavioural and psychosocial aspects to be mostly mediated by the effects of maternal stress on the structure and function of the foetal brain. Earlier research10,11 has shown the significant effect of prenatal stress on the CA3 hippocampal pyramidal neurons as well as the beneficial effects of the pyramid environment. However, there is very little information on the effect of gestational stress on the overall postnatal developmental milestones, anthropometric measurements, and body and brain weights in the rats12.This research focused on the effect of prenatal stress outside and under a wooden pyramid on the postnatal development of the offspring at postnatal day 10 and 21.

**MATERIALS AND METHODS**

Sprague Dawley rats weighing 180-250 g were used in the study. Pregnant rats were housed in polypropylene cages (25 x 47.5 x 20 cm) individually. The environmental conditions were controlled at 23±2oC, 50±5% RH on a 12:12 h light/dark cycle. They were allowed to food and water *ad libitum*. All procedures were performed in accordance with the guidelines of National Institute of Health Guide for Care and Use of Laboratory Animals13, and the study was approved by the Animal Experiments Ethics Committee of the Institution. All efforts were made to minimize the suffering and number of animals. Food pellets were purchased from Cargill Farm Animal Food Product which consisted of 16 % protein, 2.5 % crude fat, 18 % crude fibre, 13 % moisture, 0.75 % calcium and 0.45 % phosphorus.

***Prenatal Stress (PS)*** -The pregnant rats were randomly divided into normal control (NC), restrained (stressed) control (RC) and restrained (stressed) under the pyramid (RP) groups. The RC and the RP groups were exposed to restraint stress in a wire-mesh restrainer (L 15cm; W 7cm; H 7cm) for nine hours per day from gestation day 7 until parturition10-11. The NC group of pregnant rats was left un-stressed in their home cages. Pups (n= 10/ group) born to these respective mothers were the subjects of the study. At birth pups were housed with their respective mothers until postnatal day 10 and 21. However, they were observed every day for the development of the milestones. The animals of all the groups were weighed on postnatal days (PND) 10 and 21, euthanized and the brain and adrenal glands were dissected out and weighed (Figure1).

Pregnancy

Lactating Period

Post- Weaning

Delivery

Adulthood

Weaning

GD 1

GD 7

PND 0

PND 10

PND 21

PND 40

PND 60

Stress Induction

**GD 7- Parturition:** Stress Induction

**PND 0- 21:** Morphometric Physical Development

Prenatal

Postnatal

**Figure 1** Periods of Prenatal and postnatal development in the rat

***Pyramid model***- This consisted on a wooden pyramid shaped model, locally fabricated having the dimensions of height 30”, base 45” and the sides 41.5”. Holes were drilled on all sides for ventilation and a glass window on one side for observation. The four sides had an angle of 51o to the base and met at the apex of the pyramid as reported earlier8, 10-11, 14-15.

***Pyramid housing***- The pyramid was aligned to face the four cardinal north, south, east and west directions. It was placed to face in the true magnetic north-south axis to provide the maximum beneficial effects as earlier reported8,10-11,15. Pregnant rats were restrained in the wire-mesh restrainers and placed on an elevation at one-third the height (10 inches) from the base of the pyramid to attain the maximum effect of the pyramid environment8,14.

***Statistical analysis*-** Data obtained were analysed using one-way ANOVA and Bonferonni test with SPSS version 17 software. P < 0.005 was considered significant. Results are expressed as the Mean ± SE.

**RESULTS**

The results are expressed as mean ± SEM. There was a delay in the developmental milestones in the restrained control group compared to the unrestrained group; while in those restrained under the pyramid did not show the delay. The body weights and brain weights of pups prenatally exposed to restraint significantly decreased compared to the unrestrained controls at postnatal day 10 and 21 respectively. However, no such change was observed in the pups born to mothers restrained under the pyramid but the results compared well with the unrestrained control group at both the postnatal days. Adrenal glands on the other hand showed a significant increase in weight in the restrained group of pups while the weights of the adrenals of the pups restrained under the pyramid were closer to the unrestrained control group.

*Developmental milestones*

The appearance of fur, opening of the eye and ear as well as detachment of the pinna were observed and compared. There was a delay by one day in all the four milestones observed in the restrained group compared to the unrestrained control group which was significant (p < 0.003), while there was no difference in the pyramid restrained group but compared well with the unrestrained control group (Table 1).

**Table 1-** Effect of chronic restraint-stress on the day of appearance of developmental milestones (in days); [Values are Mean ± SE from 10 rats/group; \*\*\*ANOVA p<0.003]

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Milestone** | **NC**  **(n=10)** | **RC**  **(n=10)** | **RP**  **(n=10)** | **ANOVA** | |
| **F** | **p** |
| Appearance of fur (day) | 5.10 ± 0.18 | 6.00 ± 0.21 | 5.00 ± 0.21 | **7.51** | **0.003**  **\*\*\*** |
| Detachment of pinna (day) | 5.30 ± 0.21 | 6.20 ± 0.20 | 5.10 ± 0.18 | **8.75** | **0.001**  **\*\*\*** |
| Opening of the ear (day) | 15.20 ± 0.20 | 16.20 ± 0.20 | 15.10 ± 0.23 | **8.26** | **0.002**  **\*\*\*** |
| Opening of the eyes (day) | 16.00 ± 0.21 | 17.00 ± 0.21 | 15.90 ± 0.18 | **0.17** | **0.001**  **\*\*\*** |

*Anthropometry*

Anthropometric measurements of the lengths of the head, body and tail were also compared at postnatal day 10 and 21among the groups. Once again there were significant (p < 0.003) decreases in the head, body and tail lengths in the RC groups compared to the unrestrained NC groups, while the results of the groups restrained under the pyramid (RP) compared well with the unrestrained NC controls (Figures2 and 3).

**Figure 2** Effect of chronic restraint-stress on anthropometric measurements

at postnatal day (PND) 10

[NC, normal un-restrained control; RC, restrained control outside; RP, restrained under the pyramid. Values are Mean ± SE from 10 rats/group; \*\*\*ANOVA, p< 0.001]

**Figure 3** Effect of chronic restraint-stress on anthropometric measurements

at postnatal day (PND) 21

[NC, normal un-restrained control; RC, restrained control outside; RP, restrained under the pyramid. Values are Mean ± SE from 10 rats/group; \*\*\*ANOVA, p< 0.001]

*Body and Brain Weights*

The RC groups of animals showed a significant reduction in their body weights at PND 10 (11.94 ± 0.29 ; p < 0.001) and PND 21 (22.47 ± 0.47) compared to NC groups (16.01 ± 0.22 and 31.77 ± 0.26; p < 0.001) respectively. On the other hand the RP groups of animals’ body weights (15.72 ± 0.23 and 27.16 ± 0.13) were similar to those of the unrestrained NC groups at both PND 10 and 21 respectively (Figures4 and 5).

**Figure 4** Effect of chronic restraint-stress on the body weight on postnatal day (PND) 10

NC, normal un-restrained control; RC, restrained control outside; RP, restrained under the pyramid. Values are Mean ± SE from 10 rats/group; \*\*\*ANOVA, p< 0.001]

**Figure 5** Effect of chronic restraint-stress on the brain weight on postnatal day (PND) 21

[NC, normal un-restrained control; RC, restrained control outside; RP, restrained under the pyramid. Values are Mean ± SE from 10 rats/group; \*\*\*ANOVA, p< 0.001]

*Adrenal gland weights*

Weights of the adrenal glands were used as an indicator of the stress. The restrained RC groups adrenal glands weighed significantly (p < 0.001) more (27.01 ± 1.42) compared to the unrestrained NC groups (15.81 ± 0.70), while the restrained-pyramid (RP) groups adrenals (16.80 ± 0.56) were closer to those of the unrestrained NC groups at PND 10. Similar trend was also seen at PND 21 (Figure6).

**Figure 6** Effect of chronic restraint-stress on the adrenal gland weight on postnatal day (PND) 10 and postnatal day (PND) 21

[NC, normal un-restrained control; RC, restrained control outside; RP, restrained under the pyramid. Values are Mean ± SE from 10 rats/group; \*\*\*ANOVA, p< 0.001]

**DISCUSSION**

Though stress is essential and triggers homeostatic mechanisms to combat, yet chronic stress is found to be deleterious to the wellbeing. Gestation period is vulnerable to various external stimuli, such as stress. It affects the postnatal development of the central nervous system inducing neurological deficits as reported earlier16-20 report prenatal continuous light exposure, a form of stress to have adverse behavioural effects leading to increased chronic oxidative stress and altered gene expression. On the other hand, other researchers21-23 have shown environmental enrichment during gestation effectively prevents behavioural deficits and abnormal structure of synapses in prenatal stressed offspring.

Results of the offspring were compared the between the unrestrained control, restrained control and those restrained under a wooden pyramid. The offspring of the stressed group of rats showed a significant delay in the opening of the eyes and ears growth of fur, and detachment of the pinna compared to the control group. However, these changes of delay were not seen in the stressed groups under the wooden pyramid. In fact the results were similar to the unstressed control group. In other words, the animals did not show evidence of stress when kept under the pyramid. These results are in agreement with the results on the corticosterone levels and the dendritic branching of the CA3 hippocampal neurons under similar conditions reported earlier10-11.

Dancause et al.,24 havereported prenatal stress results in shorter long bones in adulthood, independently of effects on overall body size. The present study adds to a growing body of evidence suggesting prenatal stress is a risk factor for not only poor linear growth of bones as earlier reported23,25, but the overall postnatal physical development of the pups. Maternal stress retards foetal development in rats with delay in the milestones reached postnatal. Present results also suggest that the geometric shape of the pyramid and the energy within reduces or ameliorates the effects of restraint stress. Results of the present study compare well with previously reported results on the plasma levels of corticosterone and dendritic arborisation of CA3 neurons in the hippocampus10,11 and the effect seen on the oxidative stress parameters26. Seckl et al.,27 state that the excess maternal cortisol (corticosterone in rodents), is typically changed by the foetal-placenta into the inactive form (cortisone), which reaches the foetus in high concentrations and is responsible for the alterations in the foetal development and growth.

**CONCLUSION**

Stress and the environment in which it is experienced have deleterious effects on the overall physical development, particularly being most vulnerable during gestation. Effects are seen on the development of the offspring born to the mothers stressed during gestation. The current results also suggest that the pyramid’s geometric shape helps reduce stress and its deleterious effects. However, it will be interesting to explore whether the pyramid environment can reverse the effects of chronic stress. Further studies on analysis of the quality and quantity of energy developed within the pyramid structure should help us better understand how such environment acts as an anti-stressor.

**CONFLICT OF INTEREST STATEMENT**

The authors have no conflict of interest to declare regarding the study described in this article and in the preparation of the article.

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**REFERENCES**

1. Zaneta M, Alan BF & Christopher WK. 2012. Maternal cortisol disproportionately impacts fetal growth in male offspring: Evidence from the Philippines *Am J Hum Biol,* 24: 1.
2. Rao, B.S.S, Madhavi R, Sunanda & Raju TR. 2001. Complete reversal of dendritic atrophy in CA3 neurons of the hippocampus by rehabilitation in restraint stressed rats. *Current Science,* 80: 653.
3. Fujioka A, Fujioka T, Ishida Y, Maekawa T & Nakamura S. 2006. Differential effects of prenatal stress on the morphological maturation of hippocampus neurons. *Neuroscience,* 141:907.
4. Kawamura T, Chen J, Takahashi T, Ichitani Y & Nakahara D. 2006. Prenatal stress suppresses cell proliferation in the early developing brain. *NeuroReport*, 17: 1515.
5. Kraszpulski M, Dickerson PA & Salm AK. 2006. Prenatal stress affects the developmental trajectory of the rat amygdala. *Stress*, 9: 85.
6. Lemaire V, Lamarque S, Le Moal M, Piazza PV & Abrous DN. 2006. Postnatal manipulation of the pups counteracts prenatal stress-induced deficits in hippocampal neurogenesis. *Biol Psychiatry,* 59: 786.
7. Michelsen KA, van den Hove DL, Schmitz C, Segers O, Prickaerts J & Steinbusch HW. 2007. Prenatal stress and subsequent exposure to chronic mild stress influence dendritic spine density and morphology in the rat medial prefrontal cortex. *BMC Neurosci*, 8: 107.
8. Bhat S, Rao G, Murthy KD & Bhat PG. 2007. Influence of alignment of the pyramid on its beneficial effect. *Indian J Exptl Biol,* 45: 455.
9. Arnaud C, Laplante DP, Vaillancourt C & King S. 2010. Prenatal stress and brain development. *Brain Res Rev,* 65: 56.
10. George MC, *Effects of Prenatal stress on the rat offspring’s hippocampal CA3 neurons and the influence of pyramid environment: A morphological and biochemical study*, Master’s thesis, Universiti Malaysia Sabah, Kota Kinabalu, Sabah, Malaysia, 2013.
11. Murthy KD, George CM, Ramasamy P & Mustapha AZ. 2013. Housing under the pyramid reduces susceptibility of hippocampal CA3 pyramidal neurons to prenatal stress in the developing rat offspring. *Indian J Exptl Biol,* 51: 1070.
12. Dom`enec JS, Montserrat, Maria LA, Mercedes G, Victoria L & Jos´e LD. 2006. Exposure of pregnant rats to uranium and restraint stress: Effects on postnatal development and behavior of the offspring. *Toxicology,* 228: 323.
13. Garber JC, Barbee RW, Clayton LA, Donovan JC, Kohn DF, Lipman NS, Locke P, Melcher J, Quimby FW, Turner PV, Wood GA & Wurbel H, *Guide for the care and use of laboratory animals*, 8th edition. (The National Academic Press, U.S.A.), 2011.
14. Schul B & Pettit E, *The Secret Power of Pyramids.* (CBS Publications, New York) 1975.
15. Bhat S, Rao G, Murthy KD & Bhat PG. 2010. Alterations in stress parameters in rats housed in a pyramid model: seasonal variations. *Int J Pharma Bio Sci,* 1: *http://www.ijpbs.net/issue-2/168.pdf*
16. Solano ME, Jago C, Pincus MK & Arck PC. 2011. Highway to health; or How prenatal factors determine disease risks in the later life of the offspring. *J Reprod Immunol,* 90: 3.
17. Bale TL. 2011. Sex differences in prenatal epigenetic programming of stress pathways. *Stress,* 14: 348.
18. Gerecke KM, Kishore R, Jasnow A, Quadros-Menella P, Parker S, Kozub FJ, Lambert KG & Kinsley CH. 2012. Alteration of sex-typical microanatomy: Prenatal stress modifies the structure of medial preoptic area neurons in rats. *Dev Psychobiol,* 54: 16.
19. Mychasiuk R, Gibb R & Kolb B. 2012. Prenatal stress alters dendritic morphology and synaptic connectivity in the prefrontal cortex and hippocampus of developing offspring. *Synapse*, 66: 308.
20. [Voiculescu SE](http://www.ncbi.nlm.nih.gov/pubmed/?term=Voiculescu%20SE%5BAuthor%5D&cauthor=true&cauthor_uid=27566064), [Duc DL](http://www.ncbi.nlm.nih.gov/pubmed/?term=Duc%20DL%5BAuthor%5D&cauthor=true&cauthor_uid=27566064), [Roșca AE](http://www.ncbi.nlm.nih.gov/pubmed/?term=Ro%C8%99ca%20AE%5BAuthor%5D&cauthor=true&cauthor_uid=27566064), [Zeca V](http://www.ncbi.nlm.nih.gov/pubmed/?term=Zeca%20V%5BAuthor%5D&cauthor=true&cauthor_uid=27566064), [Chiţimuș DM](http://www.ncbi.nlm.nih.gov/pubmed/?term=Chi%C5%A3imu%C8%99%20DM%5BAuthor%5D&cauthor=true&cauthor_uid=27566064), [Arsene AL](http://www.ncbi.nlm.nih.gov/pubmed/?term=Arsene%20AL%5BAuthor%5D&cauthor=true&cauthor_uid=27566064), [Drăgoi CM](http://www.ncbi.nlm.nih.gov/pubmed/?term=Dr%C4%83goi%20CM%5BAuthor%5D&cauthor=true&cauthor_uid=27566064), [Nicolae AC](http://www.ncbi.nlm.nih.gov/pubmed/?term=Nicolae%20AC%5BAuthor%5D&cauthor=true&cauthor_uid=27566064), [Zăgrean L](http://www.ncbi.nlm.nih.gov/pubmed/?term=Z%C4%83grean%20L%5BAuthor%5D&cauthor=true&cauthor_uid=27566064), [Schöneberg T](http://www.ncbi.nlm.nih.gov/pubmed/?term=Sch%C3%B6neberg%20T%5BAuthor%5D&cauthor=true&cauthor_uid=27566064) & [Zăgrean AM](http://www.ncbi.nlm.nih.gov/pubmed/?term=Z%C4%83grean%20AM%5BAuthor%5D&cauthor=true&cauthor_uid=27566064). 2016. Behavioral and molecular effects of prenatal continuous light exposure in the adult rat. *Brain Res,* 1650: 51.
21. Morley-Fletcher S, Rea M, Maccari S & Laviola G. 2003. Environmental enrichment during adolescence reverses the effects of prenatal stress on play behaviour and HPA axis reactivity in rats. *Eur. J Neurosci,* 18: 3367.
22. Veena J, Srikumar BN, Raju TR & Shankaranarayana Rao BS. 2009. Exposure to enriched environment restores the survival and differentiation of new born cells in the hippocampus and ameliorates depressive symptoms in chronically stressed rats. *Neurosci Lett,* 455: 178.
23. Li M, Wang M, Ding S, Li C & Luo X. 2012. Environmental enrichment during gestation improves behaviour consequences and synaptic plasticity in hippocampus of prenatal-stressed offspring rats. *Acta Histochem Cytochem*, 45: 157.
24. [Dancause KN](http://www.ncbi.nlm.nih.gov/pubmed?term=Dancause%20KN%5BAuthor%5D&cauthor=true&cauthor_uid=22826037), [Cao XJ](http://www.ncbi.nlm.nih.gov/pubmed?term=Cao%20XJ%5BAuthor%5D&cauthor=true&cauthor_uid=22826037), [Veru F](http://www.ncbi.nlm.nih.gov/pubmed?term=Veru%20F%5BAuthor%5D&cauthor=true&cauthor_uid=22826037), [Xu S](http://www.ncbi.nlm.nih.gov/pubmed?term=Xu%20S%5BAuthor%5D&cauthor=true&cauthor_uid=22826037), [Long H](http://www.ncbi.nlm.nih.gov/pubmed?term=Long%20H%5BAuthor%5D&cauthor=true&cauthor_uid=22826037), [Yu C](http://www.ncbi.nlm.nih.gov/pubmed?term=Yu%20C%5BAuthor%5D&cauthor=true&cauthor_uid=22826037), [Laplante DP](http://www.ncbi.nlm.nih.gov/pubmed?term=Laplante%20DP%5BAuthor%5D&cauthor=true&cauthor_uid=22826037), [Walker CD](http://www.ncbi.nlm.nih.gov/pubmed?term=Walker%20CD%5BAuthor%5D&cauthor=true&cauthor_uid=22826037) & [King S](http://www.ncbi.nlm.nih.gov/pubmed?term=King%20S%5BAuthor%5D&cauthor=true&cauthor_uid=22826037). 2012. Brief communication: prenatal and early postnatal stress exposure influences long bone length in adult rat offspring. [*Am J Phys Anthropol*.](http://www.ncbi.nlm.nih.gov/pubmed/22826037) 149: 307.
25. [Fontanetti PA](http://www.ncbi.nlm.nih.gov/pubmed/?term=Fontanetti%20PA%5BAuthor%5D&cauthor=true&cauthor_uid=26278318), [Nervegna MT](http://www.ncbi.nlm.nih.gov/pubmed/?term=Nervegna%20MT%5BAuthor%5D&cauthor=true&cauthor_uid=26278318), [Vermouth NT](http://www.ncbi.nlm.nih.gov/pubmed/?term=Vermouth%20NT%5BAuthor%5D&cauthor=true&cauthor_uid=26278318) & [Mandalunis PM](http://www.ncbi.nlm.nih.gov/pubmed/?term=Mandalunis%20PM%5BAuthor%5D&cauthor=true&cauthor_uid=26278318). 2014. Prenatal Exposure to Continuous Constant Light Alters Endochondral Ossification of the Tibiae of Rat Pups. [*Cells Tissues Organs*](http://www.ncbi.nlm.nih.gov/pubmed/26278318)*,* 200: 278.
26. Bhat S, Rao G, Murthy KD & Bhat PG. 2006. Housing in pyramid counteracts neuroendocrine and oxidative stress caused by chronic restraint in rats. *eCAM*. 4: 35.
27. Seckl JR & Holmes MC. 2007. Mechanism of disease: glucocorticoids, their placental metabolism and foetal “programming” of adult pathophysiology. ***Nat Clin Pract Endocrinol Metab****,* 3: 479.

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**Statement of the contributions made to the study by each of the listed authors:**

**Mitchel Constance George**: Postgraduate: Acquisition, analysis and interpretation of data as part of Master’s thesis research

**Krishna Dilip Murthy:** Principal Investigator: Study conception and design as well as drafting of the manuscript

**Zainal Arifin Mustapha:** Co-investigator of the research and critical revision of the manuscript.

**ABBREVIATIONS**

**PND** = Postnatal day

**NC** = Normal Control (Born to Unstressed Mothers)

**RC** = Restrained Control (Born to stressed Mothers outside)

**RP** = Restrained in the Pyramid (Born to stressed Mothers inside a pyramid)