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Comparative Efficacy of Choline vs *Clitoria ternatea* Aqueous Root Extract Supplements in Attenuating Maternal Separation Stress Induced Alterations in Postnatal Rat Hippocampus.

Prathibha Maria D Almeida¹, K Dilip Murthy², Shobha U Kamath³, and Kiranmai S Rai^{1*}.

¹Department of Physiology, Melaka Manipal Medical College (Manipal Campus) Manipal University, Maniapl, Karnataka, India, 567104.

²Department of Biomedical Sciences & Therapeutics, Faculty of Medicine & Health Sciences, University Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia.

³Department of Biochemistry, Kasturba Medical College, Manipal University, Manipal, India, 576104.

ABSTRACT

Perinatal maternal separation stress (PMSS) causes brain deficits that last through ageing. Choline is essential for brain development. Alternately, *Clitoria ternatea* aqueous root extract (CTR) is proved to enhance neural plasticity in rat hippocampus. The present study is designed to elucidate the comparative efficacy of choline and CTR in attenuating PMSS induced neural cell deficits in CA1, CA3 and DG sub-regions of hippocampus. Inbred Wistar rat pups were divided into 4 groups - Control, PMSS, PMSS + Choline, PMSS + CTR [n=6 pups/group]. All pups were subjected to PMSS for 6 hours/day from postnatal day (PND) 2 to 21 except the controls. Choline and CTR were supplemented to appropriate groups during the same period. PMSS caused significant neural deficits in all sub-regions of hippocampus compared to the same in normal controls, whereas supplementation of CTR to these rats significantly preserved ($p < 0.001$) these cells in all sub-regions of hippocampus, while choline supplementation preserved ($p < 0.001$) neural cells only in CA3 and DG. Moreover preservation of neural cells in CA1 ($p < 0.05$) and DG ($p < 0.01$) was significantly greater in PMSS + CTR as compared to the same in PMSS+ choline. Thus, supplementation of CTR, compared to choline during developmental periods is more effective in attenuating neural deficits in hippocampus.

Key words: *Clitoria ternatea*, Choline, hippocampus, perinatal maternal separation stress

*Corresponding author

INTRODUCTION

Brain develops rapidly in early postnatal days and is susceptible to adverse environmental influences[1]. Perinatal maternal separation is a stressor to infants during brain growth-spurt period that causes long lasting consequences on brain development[1]. Mother–child interactions are necessary to enhance cognitive, social, emotional and motor development of a child[2].

Chronic perinatal maternal separation stress (PMSS) in rats and monkeys have shown structural disruptions in the brain[3], including decreased neurogenesis in the dentate gyrus[4] (DG) and behavioural alterations[1-5] . Children separated from their mothers for long periods have been observed to have larger amygdala[6] and increase in salivary cortisol levels[7], Children in orphanages are also observed to have alterations in social, behavioural and emotional development[8].

Although these studies have observed the long-term detrimental effects of maternal separation on early brain development and behavioural outcomes in children, very few studies focus on preventive strategies to overcome these alterations. Adequate nutrition and supplementations, even through foster care, for a developing child separated from its mother, may be important to alleviate these long term developmental alterations in the hippocampus[9]. Essential nutrients like choline in both humans and animals derived either from diet or by *de novo* synthesis in liver is important for brain development[10]. Choline a phospholipid precursor is essential for membrane structural integrity, methyl group metabolism and neurotransmitter synthesis and is critical during fetal development[11,12], increases cell proliferation, decreases apoptosis in rodent fetal hippocampus which is important for spatial memory[13].

In India, consumption of dietary supplements during developmental periods, like ‘Chyavanaprash’ that contains many herbal extracts and natural plant foods, is in vogue. One such herbal extract derived from *Clitoria ternatea* (Linn) is used in ‘Shankhapushpi’ a dietary supplement. Studies on *Clitoria ternatea* aqueous root extract (CTR) has proved to enhance acetylcholine content in rat hippocampus [4], increase dendritic arborisation of CA3 pyramidal neurons in the hippocampus and neurons of amygdala correlated with enhanced learning and memory in rats[15,16].

But there are no studies available to show the effect of PMSS on number of surviving cells in CA1, CA3 and dentate gyrus region of hippocampus and the comparative role of Choline or CTR supplementation in attenuating these alterations. We hypothesize that supplementation of either choline or CTR to PMSS rats will attenuate stress-induced alterations in rat hippocampus. Thus, the focus of study was to elucidate the efficacy of the two supplements in attenuating the deficits in the hippocampus.

Objectives

The present study was designed to observe and analyse PMSS-induced alterations in neural cell counts of CA1, CA3 and DG sub-regions of rat hippocampus and the comparative efficiency of choline or CTR supplementations in attenuating these neural cell deficits.

MATERIALS AND METHODS

Animals

The study was conducted (between Dec 2012- May 2013) in accordance with guidelines laid by Manipal University Institutional Animal Ethics Committee (IAEC/KMC/99/2012). Inbred Wistar rats (females) weighing approximately 200 g were housed in the Manipal University Central Animal Research Facility. Rats were housed in sanitized polypropylene cages containing sterile paddy husk as bedding, maintained under standard controlled conditions of temperature ($23 \pm 2^\circ\text{C}$), humidity ($50 \pm 5\%$) and 12-h light–dark cycle. All animals were allowed free access to water *ad libitum* and fed with standard rat feed (choline content 1mg/kg feed). Female rats in estrus cycle were identified and allowed for mating with male rats at 3:1 ratio. The first day of gestation was determined by the vaginal smear test. Once pregnancy was confirmed pregnant female rats were isolated and provided with nesting material and allowed to litter naturally. Male rat pups aged post natal day (PND) 2-21 were used for the study.

Study design

Study includes four major experimental groups of rat pups(male), n= 6 rat pups / group

Group I: Normal control (NC) + normal saline

Group II: Stressed (PMSS) + normal saline

Group III: Stressed (PMSS) + choline

Group IV: Stressed (PMSS) + CTR

Maternal separation procedure

Rat pups from the PMSS groups were separated from their mothers as per the procedure explained in previous studies[26]. The date of litter birth of the rat pups was recorded as post-natal day 0 (PND 0). Pups from the normal control [NC] group were left undisturbed except during routine cage changes and during normal saline administration. Rat pups from the PMSS groups were separated from their mothers according to the following protocol i.e, 4 hrs separation /day for pups aged PND 2-5, and 6 hrs separation/day for pups aged PND 6-21 as per standard guidelines. During separation procedure the pups from each of the PMSS group were placed in clean cages and housed in a separate room maintained at 31-33°C to prevent hypothermia. This prevents mother-infant ultrasonic communication and also reach of odour of the mother since this may attenuate maternal separation effects on the pups. Handling was kept to a minimum at all times, to prevent buffering of the maternal separation effect. On PND 22, all pups were weaned and housed in groups of two or three pups/cage, to prevent isolation stress.

Supplements and Dosage

Rat pups assigned to Groups I and II received equivolume of normal saline from PND 2 to 21 and pups from Groups III and IV were supplemented orally with Choline (42 mg/kg bw[17]) or *Clitoria ternatea* aqueous root extract (CTR: 100 mg/kg bw[14]) respectively. Choline Chloride obtained from commercial drug supplier and *Clitoria ternatea* roots collected from udupi district were used for the study. Preparation of aqueous root extraction was done as per the method suggested in previous studies[14]. Supplements were mixed with normal saline and administered orally with the help of feeding needle.

Histomorphological analysis

On postnatal day 23 [PND 23] all pups assigned to the afore mentioned groups were decapitated under deep anaesthesia and brains were processed for histological study. Briefly, brains of rat pups from all the experimental groups were collected after killing the animals, preserved in 10% formalin solution and dehydrated. The tissues were impregnated in the paraffin wax and embedded in Leuckhart's brass moulds. Serial 5µm thick sections were sliced using sledge microtome. Sections were processed for Cresyl violet staining which is commonly used to identify surviving and dead neurons. Staining procedure was followed as explained by Jevoor *et al.* 2012). Cleared sections were mounted on glass slides using DPX. The slides were coded prior to observation under light microscope and well stained surviving neurons of hippocampal CA1, CA3, and DG sub-regions were counted using an eye piece grid.

Statistical Analysis

Data are expressed as mean \pm SEM, and analysed using one way ANOVA followed by Bonferroni's post hoc test with significance level at $p < 0.05$. Statistical analysis was done using statistical software Graph Pad Prism version 3.00, Graph Pad Software.

RESULTS

Results of the present study shows that PMSS caused significant decrease ($p < 0.001$) in the number of surviving cells in CA1 sub-region of hippocampus compared to the same age matched normal-control group [Fig 1]. Supplementation of CTR during the PMSS has shown to preserve the CA1 neurons as indicated by the significantly higher number ($p < 0.001$) of CA1 cells when compared to the same in PMSS group. Alternately

supplementation of choline during the PMSS, although did preserve the CA1 neurons to some extent, there was no significant increase in number of surviving CA1 neurons compared to the same in PMSS group.

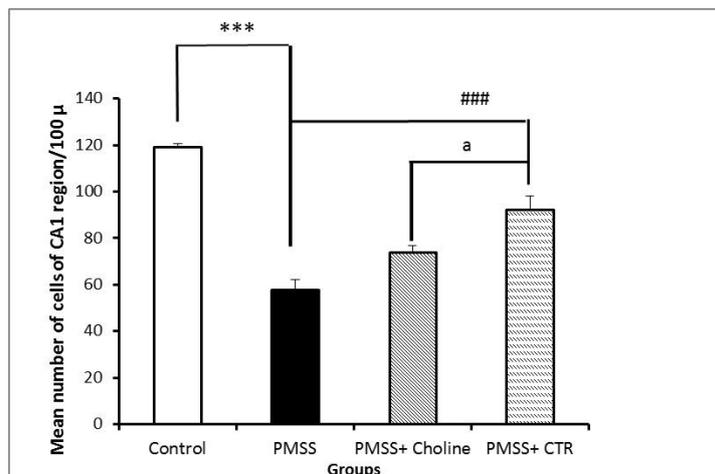


Figure 1: Number of surviving neural cells in CA1sub- region of hippocampus in perinatal maternal separation stress (PMSS) ,PMSS+ supplemented and control groups [n=6/group]..Results are expressed as mean ± SEM surviving CA1 neural cells, further analysed by one way ANOVA followed by Bonferroni’s post hoc test. *** p<0.001 significant deficit in surviving CA1 cells in PMSS Vs control, ### p<0.001 significant greater number of preserved CA1 cells in PMSS+CTR Vs PMSS group and p<0.05 significant greater number (15%) of preserved CA1 cells in PMSS+CTR Vs PMSS + Choline group.

Further, PMSS also caused significant decrease (p<0.001) in the number of surviving neurons in CA3 sub -region of hippocampus compared to the same age matched normal control group [Fig 2]. Supplementation of either Choline or CTR during the PMSS has shown to preserve the CA3 neurons, as indicated by the significantly higher number of surviving cells (p<0.001) when compared to the same in PMSS group, although comparatively there was no difference in surviving CA3 neurons between the two supplemented groups.

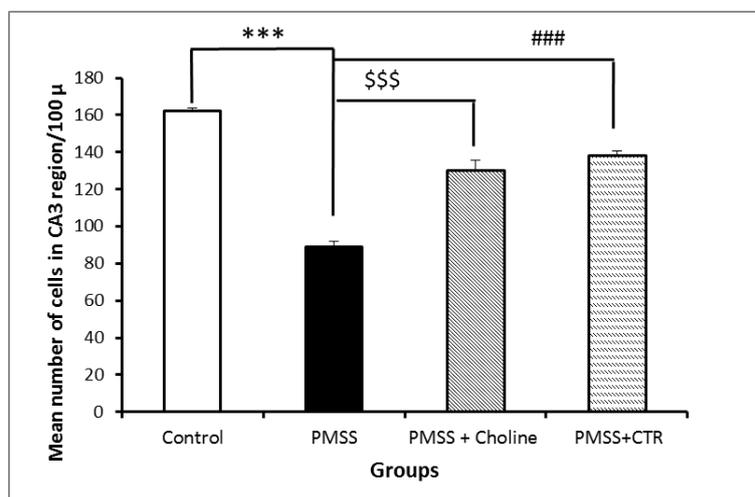


Figure 2: Number of surviving neural cells in CA3 sub- region of hippocampus in perinatal maternal separation stress (PMSS) ,PMSS+ supplemented and control groups [n=6/group]..Results are expressed as mean ± SEM surviving CA3 neural cells, further analysed by one way ANOVA followed by Bonferroni’s post hoc test. *** p<0.001 significant deficit in surviving CA3 cells in PMSS Vs control, \$\$\$ p<0.001 significant greater number of preserved CA3 cells in PMSS+Choline Vs PMSS group and ### p<0.001 significant greater number of preserved CA3 cells in PMSS+CTR Vs PMSS group. No significant difference in surviving CA3 neurons between PMSS+Choline and PMSS+CTR supplemented groups.

Additionally, PMSS also caused a significant decrease (p<0.001) in the number of surviving neurons in DG sub -region of hippocampus compared to the age matched normal control group [Fig 3]. Supplementation of either Choline or CTR during the PMSS preserved the DG neurons, as indicated by the significantly higher

number of surviving cells ($p < 0.001$) when compared to the same in PMSS group, although comparatively there was a trend towards higher preservation of surviving DG neurons in the CTR supplemented group ($p < 0.001$) compared to the same in choline supplemented group

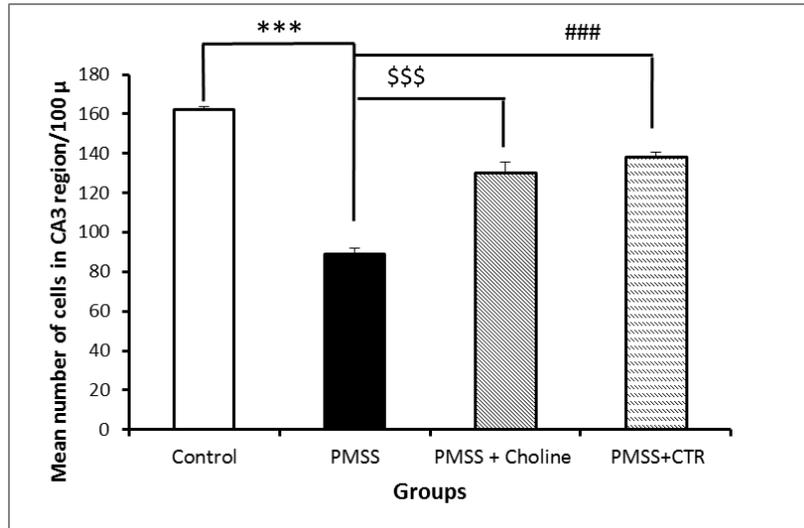


Figure 3 : Number of surviving neural cells in dentate gyrus [DG] sub- region of hippocampus in perinatal maternal separation stress (PMSS), PMSS+ supplemented and control groups [n=6/group]. Results are expressed as mean \pm SEM surviving DG neural cells, further analysed by one way ANOVA followed by Bonferroni's post hoc test. *** $p < 0.001$ significant deficit in surviving DG cells in PMSS Vs control, \$\$\$ $p < 0.001$ significant greater number of preserved DG cells in PMSS+Choline Vs PMSS group, ### $p < 0.001$ significant greater number of preserved DG cells in PMSS+CTR Vs PMSS group and aa $p < 0.01$ significantly greater number (50%) of preserved DG cells in PMSS+CTR Vs PMSS+Choline group.

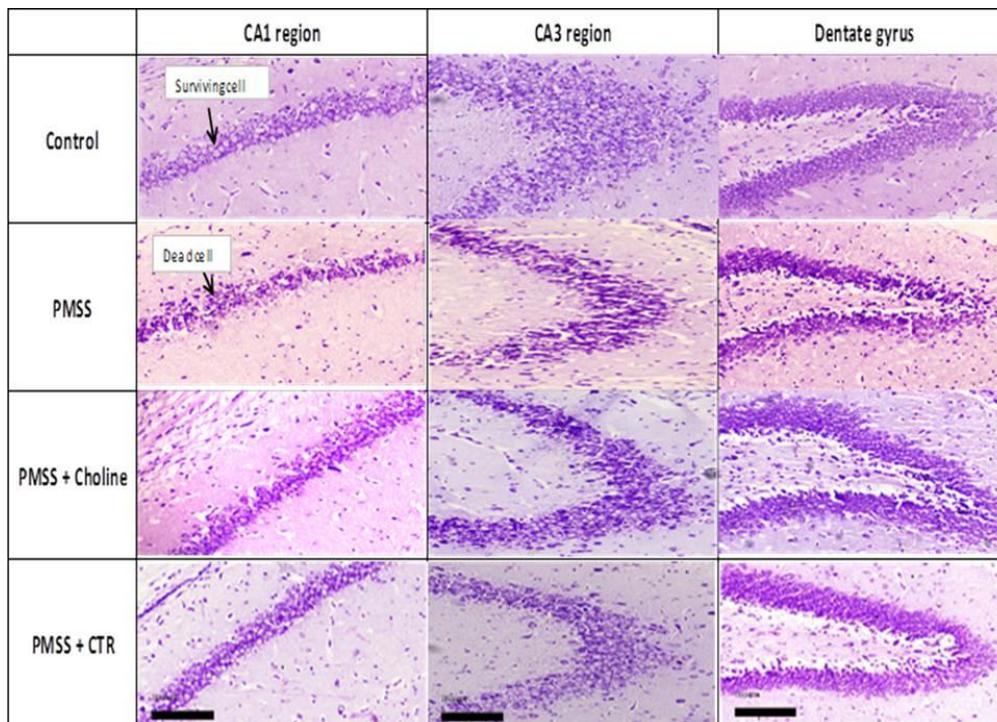


Figure 4: Photo-micrographs showing number of surviving and dead neural cells in CA1 ,CA3 and dentate gyrus [DG] sub-regions of hippocampus in control group, perinatal maternal separation stress (PMSS) group, PMSS+ Choline supplemented group and PMSS + CTR supplemented groups. Scale bar =100 microns

DISCUSSION

The results of this study showed that perinatal maternal separation stress [PMSS] of 6 hours/day for 21 days in rat pups causes decrease in neural cell counts in all sub-regions of the hippocampus. Studies show that early postnatal period in rat pups is a stress-hypo-responsive period, with very little response by the HPA axis to any form of stress[18]. Alternately, other studies similar to the present study has shown that chronic prolonged separation of the pups from the mother, increases the response of HPA axis to stressful situations[18]. Alterations in HPA axis increases the levels of circulating ACTH that further stimulates increased release of corticosterones [19]. Studies show that increased levels of corticosterone in rat pups during the first postnatal week causes down regulation of glucocorticoid receptor expression and mineralocorticoid receptor expression in CA1 region of hippocampus[20]. Additionally increased corticosterone decreases proliferation of granule cells and increases pyknotic cell numbers in the dentate gyrus [21]. Studies also have documented that PMSS causes overexpression of corticotropin releasing factor that impairs spatial learning and memory and decreases dendritic spines of CA3 neurons [22].

Further in the present study, administration of choline throughout the 21day-period of PMSS in rat pups significantly preserved CA3 neurons and neural cells in dentate gyrus although in the CA1 sub-region of hippocampus there was only increasing trend towards preserving the cells compared to the same in PMSS rat pups. Choline is a precursor of phosphatidylcholine, a component of neural cell membranes[23] and is also required for the synthesis of acetylcholine a neurotransmitter that improves cognition[23]. Supplementing choline thus helps to maintain enough free choline levels in the ECF for cholinergic neural functions, thereby preventing neural cell membrane 'auto-cannibalism'[24] and preserving the membrane integrity of hippocampal neural cells. Studies show that PMSS causes increase in lipid peroxidation[25,26] that damages neural cell membrane leading to neural cell death[25]. Thus, choline supplementation may also preserve neural cells by decreasing oxidative stress[27]. Additionally supplementing choline also activates cholinergic systems in the brain that enhances neurogenesis in the DG of the hippocampus[28].

Alternately, supplementation of *Clitoria ternatea* aqueous root extract [CTR] to PMSS rat pups was observed to significantly preserve neural cells to a greater extent [23%] than the choline supplemented groups in all sub-regions of the hippocampus when compared to the same in PMSS-only group. Previous studies have also shown that supplementation of CTR to neonatal and young adult rat enhances ACh levels in the hippocampus[15]. Moreover when aSVZ neural precursor cells (aSVZ NPC-s) at 7 days *in vitro* was treated with 200ng/ml of CTR extract, significant increase in proliferation and growth of neurospheres with increase in the yield of differentiated neurons was observed[29]. Studies show that the roots of CTR have antioxidant activity[30] and this could be one of the mechanisms in preserving neural cells of all the sub-regions of hippocampus in PMSS rats. Preliminary phytochemical screening of the aqueous root extract of *Clitoria ternatea* was observed to have presence of amino acids, proteins, carbohydrates and absence of alkaloids, saponins, flavonoids, coumarins. Further fractionation and analysis had shown proteins as the most active sub-fraction[16]. Additional study is required to isolate the active principles in CTR. Further investigations are required to elucidate the mechanism by which CTR supplementation during PMSS helps in improved preservation of neural cells in different sub-regions of the hippocampus and DG neurogenesis as compared to the same in Choline supplemented rats.

CONCLUSION

Supplementation during perinatal maternal separation stress in rats with CTR is comparatively better than Choline in attenuating the detrimental effects of stress on neural cells in the hippocampus and helps in better preservation of its cellular integrity.

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