Original article

Choline and DHA supplementation ameliorate hippocampal damage in prenatally stressed rats by reducing apoptosis and cortisol levels

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Abstract. *Aim:* Prenatal stress inhibits neurogenesis and increases apoptosis in the hippocampus. Both Choline [C] and docosahexaenoic acid [DHA] are essential nutrients, important for the formation of neural cell membrane phospholipid bilayer. The neuroprotective potential of synergistic supplementation of these nutrients on the outcome of hippocampal neural cell density and neural development in prenatally stressed gestation is seldom evaluated. This study assesses the role of supplemented choline and or DHA in prenatally stressed neonates on their hippocampal neural cell density and serum cortisol levels. *Materials and Methods:* Pregnant rat dams were separated into [NC] - Normal control, [SC] - Saline control, [STR] - Stress, [STR+C] - Stress+Choline, [STR+DHA] - Stress+DHA, and [STR+C+DHA] -Stress +Choline+DHA groups [n= 6/group]. NC dams were undisturbed throughout the gestation. All other experimental groups of dams were supplemented by Saline, C, and DHA throughout the gestation respectively. All STR group dams were exposed with restraint stress from E11 till delivery. On postnatal day 40, pups were sacrificed after blood sample collection to estimate cortisol levels. *Results:* Cresyl violet stained, and caspase-3 labeled hippocampal sections were observed to analysis neural cell density and apoptosis. Significant restoration ($p<0.001$) in the total number of viable neuronal cells in CA1and CA3 subregions of the hippocampus and reduced caspase-3 labeled apoptotic cells were observed in STR+C+DHA rat pups compared to the age-matched NC, SC and stressed pups. In addition, a significant reduction in ($p<0.01$) serum cortisol concentration was found in [STR+C+DHA] pups when compared with age-matched stressed rat pups. *Conclusion:* Oral supplementation of nutrition like choline and DHA during stress among rat dams restores stress induced neonate hippocampal neural cell density and diminishes apoptotic cells in CA1 and CA3 regions with reductions in serum cortisol levels.

Key words: apoptosis, cortisol, choline, DHA, hippocampal neural density, hippocampal development, prenatal stress, neuroprotection, oxidative stress, maternal supplementation

Introduction

Human brain development includes neurulation, neuronal proliferation, neural migration, neural cell differentiation, myelination, and apoptosis that occurs in the third week of intrauterine life as a result of differentiation with the progenitor cells of the neurons and prolongs up to all over the lifetime (1) Hippocampus is a small part of the human and animal brain that lies in the temporal lobe of the cerebrum. It forms the

posterior part of the limbic lobe and is responsible for learning, and memory (2). In both animals and humans, prenatal stress has detrimental effects on the neurobiological and hormonal development of offspring, which can lead to abnormal changes in cognition and behavioral outcomes in adolescent (3,4). Exposure to prenatal stress in monkeys' results in decreased neurogenesis in hippocampus and dentate gyrus (DG), leading to a decrease in the hippocampal volume (5). Exposure to abnormal levels of cortisol in the fetus causes a reduction in the number of neurons in hippocampus that results in reduction of hippocampal size (6). Prenatal stress induces a reduction in hippocampal volume and reduces postnatal neurogenesis in experimental animals. This decrease in hippocampal volumes/weight is due to the reduced number of hippocampal neural cells in prenatally stressed rats compared to normal control rat pups (7). Other animal studies also report that prenatal stress leads to depletion in neuron proliferation in hippocampus(8,9). Prenatal stress causes premature birth, low birth weight, fearfulness, and an increased risk of respiratory and skin illnesses in life (10). Another study reported that maternal stress affects heart rate of the fetus, intrauterine activity of the fetus, sleep cycle, leads to depression, and anxiety in postnatal life (11). Many human studies have expressed that pregnant woman who exposed to stress due to poor economic status, loss of relatives leads to sever consequences to their offspring such as autism and schizophrenia (12). Hormone cortisol plays a crucial role during stress in humans, monkeys, and rodents and affects the hypothalamic-pituitary-adrenal (HPA) axis (13). Chronic prenatal and/or early postnatal stress, resulting in dysregulation of negative HPA feedback via altered glucocorticoid receptor sensitivity to glucocorticoids (14). Stress causes the secretion of high levels of glucocorticoids which decreases glucocorticoid receptors in the hippocampus. The above findings clearly indicate that the negative feedback by HPA axis is impaired. Various human studies show that hyperactivity of the HPA axis is linked with low birth weight (15,16). The higher and prolonged glucocorticoid exposure affects neurotransmitter systems in the brain, especially hippocampus, serotonergic, dopaminergic, GABA-ergic, and noradrenergic systems (17-19). Calcium supplementation in pregnancy also plays a role in reducing maternal and neonatal morbi-mortality by directly reducing the likelihood of developing pre-eclampsia and eclampsia (20). Variations of blood ion concentrations in pregnancy do not only affect maternal physiology but go a long way to impact fetal outcomes (21). Choline is one of the precursors for compounds, like phospholipids, acetylcholine, betaine, and is crucial for the developing fetus and neonate. Oral supplementation of choline for the pregnant mother and thereafter for the infant causes brain development which leads to changes to brain function (22). A high choline diet in pregnancy in animals increases hippocampal stem cell proliferation and inhibits apoptosis (23,24). Thus, in animals, prenatal choline supplementation enhances the brain structure and function of their offspring. Increased brain choline mediates memory function by elevating acetylcholine release (25). DHA enhances memory function by acting on the synaptic membrane fluidity cell signaling and regulating the gene expression (26). The availability of DHA promotes the differentiation of stem cells of central nervous system. In addition, DHA converts these stem cells into mature neural cells, thereby DHA influencing brain development (27). Animals supplemented with DHA during fetal life have high brain DHA which increases hippocampal neurogenesis. In vitro investigation also shows the efficiency of DHA in promoting neurogenesis. DHA not only facilitates the proliferation of embryonic stem cells but also causes neurite outgrowth of differentiated neural cells (28). DHA is vital for neurogenesis and neuritogenesis in prenatal and postnatal development (29). Until recently, no specific therapy has been developed to overcome stress-related neurodevelopmental deficits of the offspring, from dams exposed to stress during gestation. The first three months of pregnancy would be critical since medication would cause malformations in fetus. It is also recommended that certain medications are not to be used by pregnant women, even though no harmful effects have been observed (30). By considering all the above facts, this study was carriedout to analyze the efficacy of individual or combined supplementation of C and DHA during gestational stress in dams on the outcome of hippocampal neural cell density as well as serum cortisol levels in their neonates.

Materials and Methods

In-house bred albino Wistar strain adult male and female rats, obtained from the Central animal research facility, Manipal University, and their neonatal male and female rat pups were used in this study. All pups were maintained at 12:12 hrs. day: night environment, in a well-ventilated room in the Central animal research facility. Rats were fed with water and food *ad libitum*. Experiments were conducted only after the approval from institutional animal ethical committee with the approval number (IAEC/KMC/32/2012) that obey the guidelines enacted by the CPCSEA, New Delhi [India]. Proper care was taken while humanely handling the rats and all precautions were made and, we have used minimum number of animals for generating the necessary data.

Experimental design

E0 day pregnant rat dams were divided into following groups: NC, SC, STR, STR+C, STR+DHA, and STR+C+DHA. Dams from NC group were undisturbed throughout the gestation period. Dams of SC group were supplemented orally with saline for the entire gestation period. STR group of dams were subjected to restraint stress from E11 to delivery. Pregnant dams from the STR+C group were supplemented with Choline from E0 to delivery and subjected to restraint stress from E11 until delivery. Dams from STR+DHA were supplemented with DHA from E0 until delivery and subjected to restraint stress from E11 until delivery. Dams of the STR+C+DHA group were supplemented orally with Choline and DHA from E0 until delivery and subjected to restraint stress from E11 to until delivery. Choline [Extra Pure choline chloride 98% was obtained from Loba Chemical Laboratory Reagents and Fine Chemicals] and dissolved with distilled water to make a dosage of (4.6 mmol/kg/day of choline) (31), and DHA [gelatin capsules consisting of 300 mg DHA were procured from Nouveau Medicament (P) Ltd., located at Chennai] (400 mg/day of DHA) (32). was supplemented orally to the dams using feeding needles. The need for choline and DHA during pregnancy is very high and the demand for these nutrients is increased workload by maternal organs, and to support exponential fetal organ growth (33). Until recently, however, no specific drug therapies have been developed, which is administered during pregnancy, especially when the mother is in the stressful situation, would enhance the development of neurons in CNS especially in the hippocampus of offspring. The first 12 weeks of pregnancy is a critical time when medication can cause malformations of the fetus. So, experts may advise discontinuing medicines over this period unless a woman had multiple episodes of severe stress and depression. It is recommended that certain medications are not used by pregnant women, even though no harmful effects have been observed. The primary dietary factors involved in maintaining homeostasis and energy requirements such as calorie restriction, lipids, vitamins and other special nutritional supplements such as choline and DHA (30). Blood samples from each of the pups were collected for the estimation of cortisol. Then the pups were sacrificed after transcardial perfusion with saline and 10% formalin on postnatal day 40. Their brains were removed without any damage and processed for paraffin sectioning. Altogether, 300 sections of 5 μ thickness were made from the hippocampus. One section of every 30 sections were processed for cresyl violet staining. Randomly selected viable neural cells (cell body of the neurons) from 250 μm area of CA1, CA3, and CA4 regions of the hippocampus, as also from the upper blade portion of the dentate gyrus (DG), were quantified using an ocular micrometer scale by another experimenter, blinded to the study. Non-viable or degenerated neurons were darkly stained, shrunken with fragmented nuclei were excluded from the count. Blinding of the slides from different groups were done prior to the counting to avoid observer bias. Photomicrographs were recorded under the compound Olympus microscope using cellSens Imaging Software at 40x magnification. The apoptosis of hippocampal neurons of CA1 and CA2 regions were observed by labeling caspase-3 expression using the immunofluorescence technique. Immunohistochemistry technique for estimating the apoptosis marker caspase-3 is based on the principle of antigen-antibody interactions. The hippocampal subregions were targeted with rabbit anti-caspase-3 primary antibodies which bind to express caspase-3 enzymes. After appropriate processing, the secondary

sheep antibody tagged with CY-3 immunofluorescence were targeted to bind the primary antibody, and the fluorescence was identified and photomicrographed with a confocal microscope.

Restraint stress procedure

Restraint stress was performed from embryonic day 11 until delivery. The stress protocol involved placing the pregnant female in a wire mesh restrainer 6 hours per day. Control dams were left undisturbed throughout gestation. The wire mesh restrainer has a wooden base and stainless-steel wire mesh restrainer hinged to the base. A padlock and latch will help to secure the rat in the restrainer. The restrainers of two different dimensions used. The restrainer with 11 cm (length) × 6 cm (breadth) × 6 cm (height) dimensions for restraining the pregnant rats from E1-E17 and restrainer with 11 cm (length) × 8 cm (breadth) × 8 cm (height) dimensions will be used to stress the pregnant rats from E18 till delivery. This type of restrainer claimed to restrict the animal's movement without any pain, discomfort or suffocation (34).

Statistical analysis

Data were analyzed using the method of one-way ANOVA and by Bonferroni's test and was expressed as mean ± SEM. $p < 0.05$ was considered as significant. Entire analysis was done in software Graph pad prism version 5.03.

Results

Serum cortisol levels in prenatally stressed rat pups

Rat pups subject to prenatal stress marked a significant increase (p<0.01) in mean serum cortisol level when compared with age-matched NC and SC group of rat pups. Rat pups subjected to prenatal stress and prenatal supplementation of choline marked a significant decrease (p<0.01) in the serum cortisol concentration when compared with the age-matched rat pup groups which are exposed to prenatal stress (Figure 1).

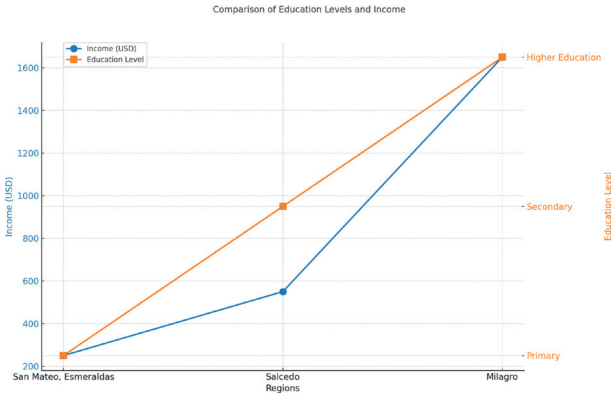

Figure 1. Mean serum cortisol level (ng/mL). n = 6 rats / group. NC & SC vs. STR [**P<0.01], STR vs. STR+C+DHA [^{cc}P<0.01], (One way ANOVA, Bonferroni's test). Normal control (NC), Saline control (SC), Stress (STR), Stress + Choline (STR+C), Sress+DHA (STR+DHA) and Stress+Choline+ Docosahexaenoic acid (STR+C+DHA) group of rat pups.

Prenatal stress and neural cells in hippocampal CA1 region

Rat pups subjected to prenatal stress exhibit a significant decrease (P<0.001) in mean number of viable neurons in CA1 region of the hippocampus when compared to NC & SC group of rat pups. STR+C, STR+DHA groups of rat pups exhibit significant increase (p<0.05) in the mean number of viable neurons, respectively, when compared to STR group of pups. However, STR+C+DHA rat pups exhibit significantly higher mean number of viable neurons (p<0.001) when compared to STR rat pups (Figures 2 and 3).

Prenatal stress and neural cells in hippocampal CA3 region

Rat pups subjected to prenatal stress exhibit significant decrease (P<0.001) in mean number of viable neurons in CA3 region when compared to NC & SC groups. Rat pups are subjected to prenatal stress with the supplementation of choline (p<0.05) or DHA (p<0.05) exhibit higher number of neurons,

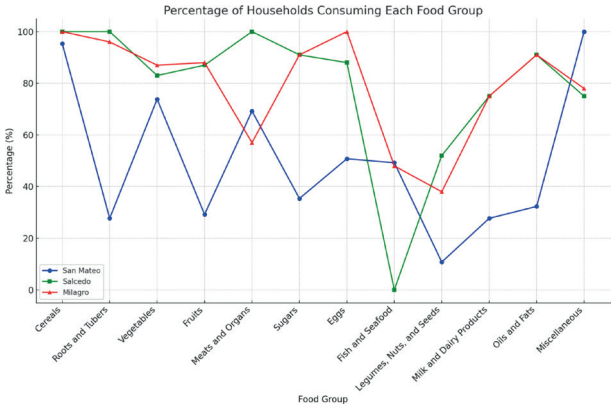

Figure 2. Mean number of viable neural cells across 250µ length in CA1region of hippocampus; Mean ± SD. NC & SC vs. STR [***P<0.001], STR vs. STR+C[aP<0.05], STR vs. STR+DHA[bP<0.05], STR vs. STR+C+DHA [cccP<0.001], (One way ANOVA, Bonferroni's test) Normal control (NC), Saline control (SC), Stress (STR), Stress+Choline (STR+C), Stress+Docosahexaenoic acid (STR+DHA) and Stress+Choline+ Docosahexaenoic acid (STR+C+DHA) group of rat pups.

respectively, when compared to STR groups. However, rat pups subjected to prenatal stress and undergone the supplementation of C+DHA exhibit significantly a greater number of neurons (p<0.001) when compared to STR group (Figures 4 and 5).

Prenatal stress and neural cells in hippocampal CA4 region

Rat pups exposed to prenatal stress exhibit significant (P<0.001) reduction in the mean number of viable neurons in the CA4 region when compared to NC & SC group of pups. Rat pups subjected to prenatal stress and undergone the supplementation of choline (p<0.05); DHA (p<0.05) and C+DHA exhibit a significantly greater (p<0.001) number of neurons when compared to STR group (Figures 6 and 7).

Prenatal stress and neural cells in hippocampal DG region

Rat pups exposed to prenatal stress showed significant reduction in the number of viable neurons (P<0.001) in DG region when compared to NC & SC groups. Rat pups subjected to prenatal stress and undergone the supplementation of choline (p<0.05); DHA (p<0.05) and C+DHA exhibit significantly

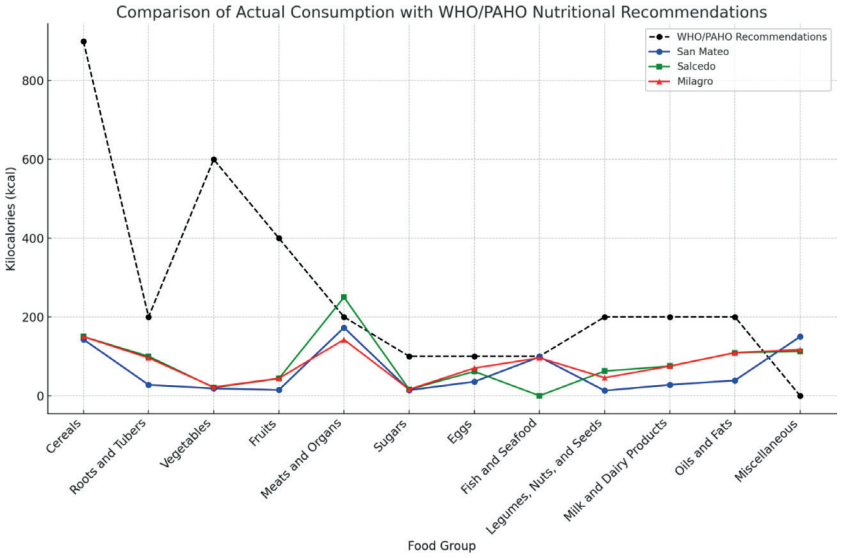

Figure 3. Photomicrographs of CA1 region of hippocampus. Normal control (NC), Saline control (SC), Stress (STR), Stress+Choline (STR+C), Stress+Docosahexaenoic acid (STR+DHA) and Stress+Choline+ Docosahexaenoic acid (STR+C+DHA) group of rat pups. (Cresyl violet stain X 10).

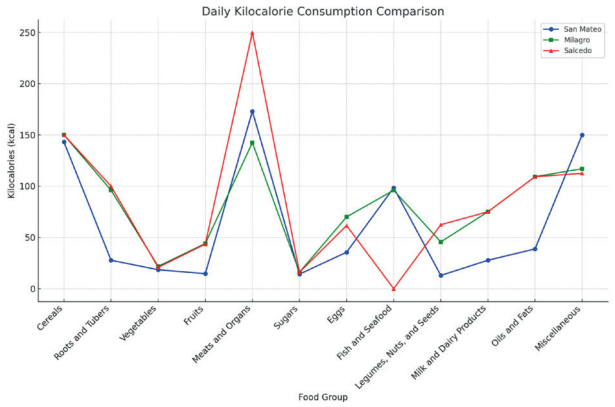

Figure 4. Mean number of viable neurons across 250µ length in CA3 region of hippocampus; Mean ± SD is shown. NC & SC vs. STR [***P<0.001], STR vs. STR+C[^{a}P<0.05], STR vs. STR+DHA[^{b}P<0.05], STR vs. STR+C+DHA [^{ccc}P<0.001], (One way ANOVA, Bonferroni's test) Normal control (NC), Saline control (SC), Stress (STR), Stress+Choline (STR+C), Stress+Docosahexaenoic acid (STR+DHA) and Stress+Choline+ Docosahexaenoic acid (STR+C+DHA) group of rat pups.

greater number of neurons (p<0.001) when compared with STR group of rat pups (Figures 8 and 9).

Caspase – 3 immunofluorescence expression in neurons of hippocampal CA1 region

NC & SC group of rat pups showed very few and random signals of caspase - 3 in CA1 sub-region of hippocampus whereas the caspase - 3 expression of CA1 neurons of STR group were observed to be more while comparing to NC and SC groups. However caspase - 3 expressions of apoptotic CA1 neurons in hippocampal sections of all supplemented groups of rat pups [(STR+C), (Stress+DHA) and (STR+C+DHA)] were markedly reduced compared to STR group of rat pups (Figure 10).

Caspase – 3 immunofluorescence expression in neural cells of hippocampal CA3 region

NC & SC group of rat pups showed very few and random signals of caspase - 3 in CA3 sub-region of hippocampus whereas the caspase - 3 expression of CA3 neurons of STR group were observed to be more while comparing to NC and SC groups. However

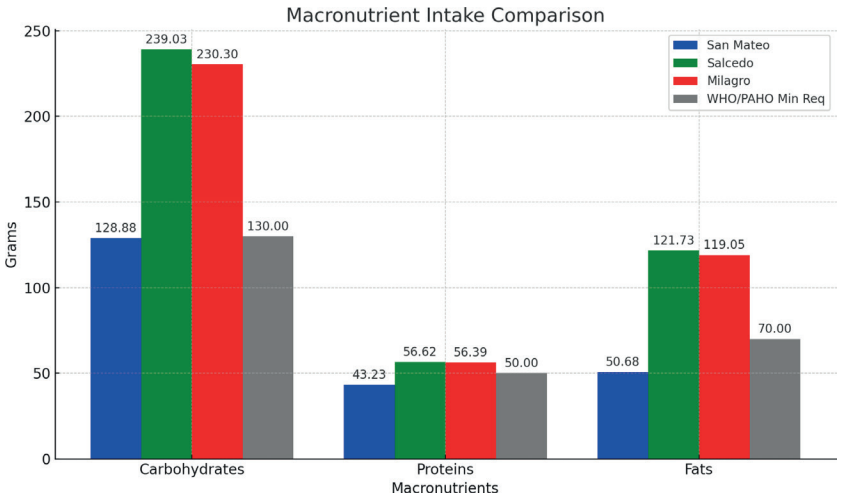

Figure 5. Photomicrographs of CA3 region of hippocampus. Normal control (NC), Saline control (SC), Stress (STR), Stress+Choline (STR+C), Stress+Docosahexaenoic acid (STR+DHA) and Stress+Choline+ Docosahexaenoic acid (STR+C+DHA) group of rat pups. (Cresyl violet stain X 10).

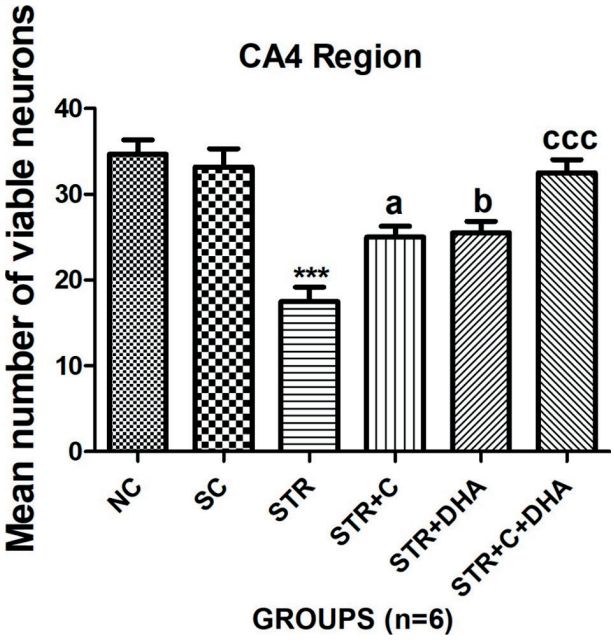

Figure 6. Mean number of viable neurons across 250µ length in CA4 region of hippocampus; Mean ± SD is shown. NC & SC vs. STR [***P<0.001], STR vs. STR+C[aP<0.05], STR vs. STR+DHA[bP<0.05], STR vs. STR+C+DHA [cccP<0.001], (One way ANOVA, Bonferroni's test) Normal control (NC), Saline control (SC), Stress (STR), Stress+Choline (STR+C), Stress+Docosahexaenoic acid (STR+DHA) and Stress+Choline+ Docosahexaenoic acid (STR+C+DHA) group of rat pups.

caspase - 3 expressions of apoptotic CA3 neurons in hippocampal sections of all supplemented groups of rat pups [(STR+C), (Stress+DHA) and (STR+C+DHA)] were markedly reduced compared to STR group of rat pups (Figure 11)

Discussion

Results from the present study shows that postnatal rat pups from gestationally stressed dams have significantly higher serum cortisol concentration/levels when compared with the rat pups from non-stressed age-matched NC and SC dams. High cortisol levels during pregnancy is associated with results in abortion of the fetus, delay in fetal growth, premature delivery, and very low birth weight of offspring, respectively (35,36). Constriction of placental arteries are one of the complications of the maternal stress, which leads to reduced blood flow to the fetus thereby decreasing the availability of essential nutrients and oxygen to the offspring (37). The amount of cortisol produces during stress will have an adverse effect on HPA axis of the developing fetus which suppresses fetal growth and cause premature delivery (38). Rat pups from gestationally

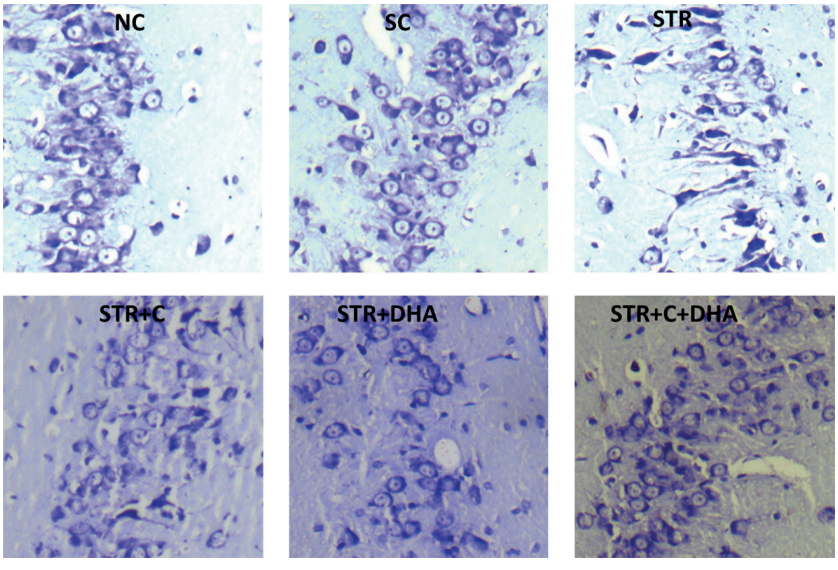

Figure 7. Photomicrographs of CA4 region of hippocampus. Normal control (NC), Saline control (SC), Stress (STR), Stress+Choline (STR+C), Stress+Docosahexaenoic acid (STR+DHA) and Stress+Choline+ Docosahexaenoic acid (STR+C+DHA) group of rat pups. (Cresyl violet stain X 10).

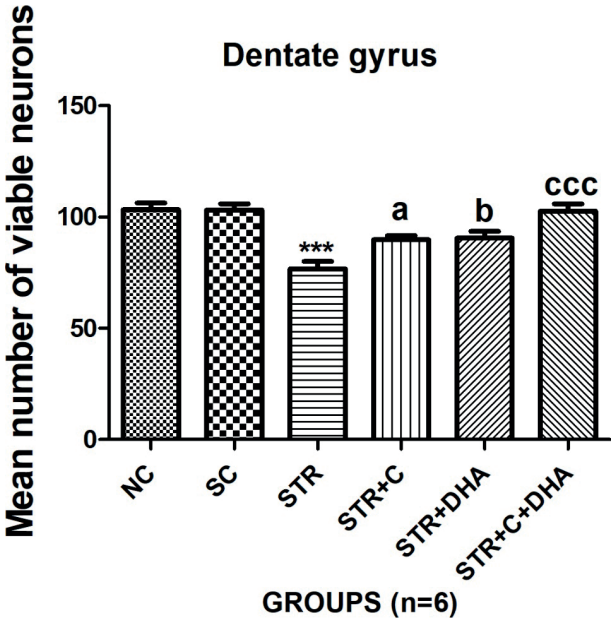

Figure 8. Mean number of viable neurons across 250µ length in DG region of hippocampus; Mean ± SD is shown. NC & SC vs. STR [***P<0.001], STR vs. STR+C[^{a}P<0.05], STR vs. STR+DHA[^{b}P<0.05], STR vs. STR+C+DHA [^{ccc}P<0.001] (One way ANOVA, Bonferroni's test) Normal control (NC), Saline control (SC), Stress (STR), Stress+Choline (STR+C), Stress+Docosahexaenoic acid (STR+DHA) and Stress+Choline+ Docosahexaenoic acid (STR+C+DHA) group of rat pups.

stressed dams when supplemented prenatally with choline and DHA showed a significant decrease in serum cortisol concentrations when compared to rat pups from age-matched non-supplemented stressed dams. These findings could be better explained by understanding various mechanisms through which choline produces higher methylation of CRH (corticotropin releasing hormone) genes of the placenta, this results in lower CRH transcription in the placenta, which leads to low concentrations of cortisol in the cord blood of the fetus. Studies have also reported that prenatal choline supplementation regulates the expression of genes that alter the fetal HPA axis sensitivity via epigenetic mechanisms (39,40). PUFAs, especially DHA and arachidonic acid, are fundamental elements of membrane phospholipids of the brain which are needed for optimal cerebral function (41). The hippocampus is the major constituent of the limbic system, which regulates the stress response. This part of the brain is also vulnerable to Glucocorticoids since mineralocorticoid receptors and glucocorticoid receptors are expressed in this limbic region (42). Feeding dams with DHA is proved to prevent stress-induced neural cell damage, apoptosis, and mitochondrial damages in hippocampus of their fetuses as we observed

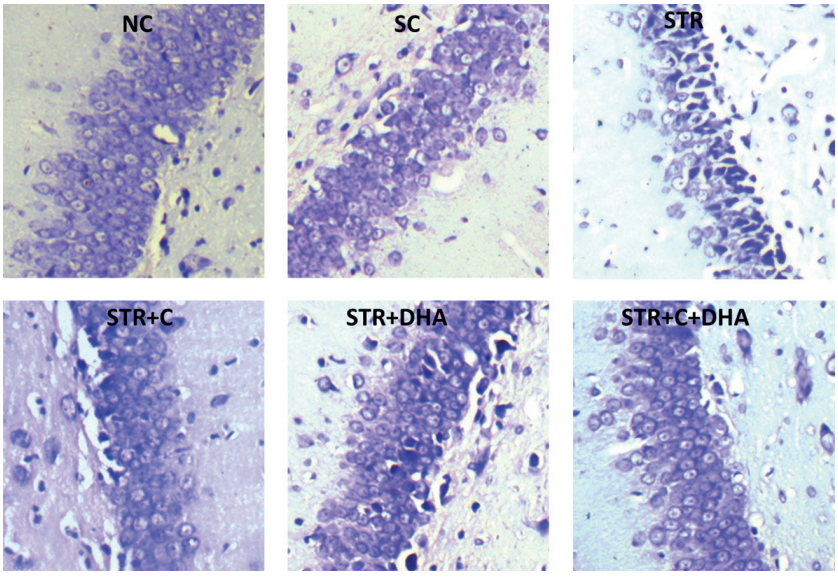

Figure 9. Photomicrographs of DG region of hippocampus. Normal control (NC), Saline control (SC), Stress (STR), Stress+Choline (STR+C), Stress+Docosahexaenoic acid (STR+DHA) and Stress+Choline+ Docosahexaenoic acid (STR+C+DHA) group of rat pups. (Cresyl violet stain X 10).

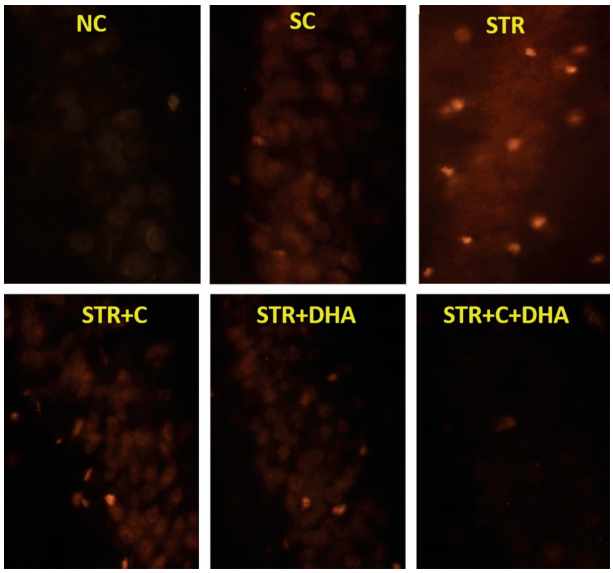

Figure 10. Photomicrographs of CA1 neurons showing the signals of caspase -3 expression in different groups. Normal control (NC), Saline control (SC), Stress (STR), Stress + Choline (STR+C), Sress+DHA (STR+DHA) and Stress+Choline+ Docosahexaenoic acid (STR+C+DHA) group of rat pups.

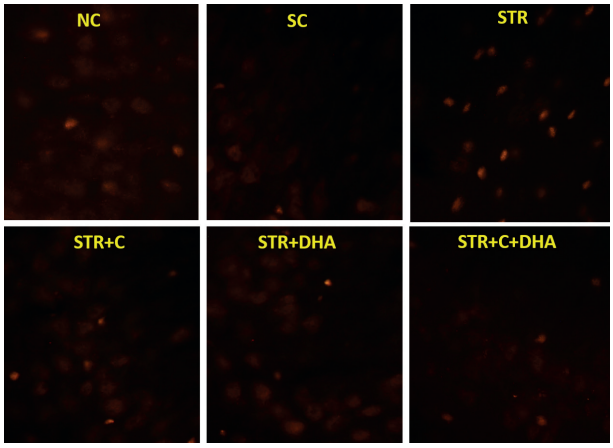

Figure 11. Photomicrographs of CA3 neurons showing the signals of caspase -3 expression in different groups. Normal control (NC), Saline control (SC), Stress (STR), Stress + Choline (STR+C), Sress+DHA (STR+DHA) and Stress+Choline+ Docosahexaenoic acid (STR+C+DHA) group of rat pups.

in our histochemical evaluations (43). Gestational stress affecting the fetus prenatally caused significantly lower numbers of surviving neural cells in postnatal rat hippocampus specifically in CA1, CA3, CA4, and DG regions when compared with NC and SC group of rat pups. The number of surviving neurons in these sub-regions of the hippocampus was observed to be significantly increased by prenatal supplementation of choline and DHA alone or together during gestational stress. Additionally, surviving neural cells were observed to be significantly greater when both choline and DHA were supplemented in combination during the prenatal stress period. Chronic stress is associated with structural changes in the sub-regions of the hippocampus and can impair neurogenesis in the DG region (44). Maternal stress during gestation reduces hippocampal neurogenesis in primates (5). Maternal restraint stress even during the third trimester of gestation causes a significant decrease in neuronal proliferation in dentate gyrus (45). Maternal stress in mice reduces the number of dendritic spines and neuronal synapses in CA3 pyramidal cells (46). Neuronal proliferation of the hippocampus was decreased in PND 10 male rat pups in addition to the apoptosis in the sub-regions of the hippocampus caused by the chronic stress (47). Another study reported that stress causes elevation of cortisol and reductions in body weight and apoptosis in sub-regions of the hippocampus and cortex (48). Prenatal supplementation of choline, which is an important methyl donor, influences neurogenesis and apoptosis in fetal hippocampus (49). Apoptosis in the nervous system is modulated by neurotrophins and sex hormones (50). It has been proved that choline-regulated intermediate signals that mediate apoptosis are induced by deficiency of choline in the cortex and hippocampus of the fetus (51,52). Choline deficiency was associated with a decrease in phosphatidyl choline concentrations in some subcellular compartments, leading to the induction of apoptosis (53). Thus, choline deficiency causes apoptosis in neurons by altering the cell cycle, as these neurons normally do not progress past G0/G1 (52). Representative photomicrographs of CA1 and CA3 sub-regions of the hippocampus from NC and SC rat pups were observed to have very few and arbitrary signals of caspase - 3 expression. Whereas caspase - 3 expressions were observed to be more in CA1 and CA3 neurons from prenatally stressed rat pups when compared to non-stressed NC and SC rat pups. Moreover, caspase - 3 expression in CA1 and CA3 neurons of hippocampal regions were found to be markedly reduced in

prenatally stressed rats supplemented with choline and DHA separately or both together prenatally when compared to non-supplemented prenatally stressed rat pups. Prenatal supplementation of choline causes neurogenesis by preventing apoptosis in the develpoing hippocampus (49). Apoptosis is programmed cell suicide, appearing even during normal development (54). It has been proved that apoptosis in the developing hippocampus as well as other part of the brain is facilitated by choline deficiency (51). Choline deficiency associated with a decrease in PtdCho concentrations, and some subcellular compartments leads to the induction of apoptosis (55). Thus, choline deficiency causes apoptosis in neurons by the disturbance in the cell cycle, as a result, cell division is impaired (52). Maternal choline supplementation during E11 and 17 results in significant changes of the developing hippocampus and other parts of the brain by altering apoptosis (51). Availability of choline during development stimulates neuronal cell division of the brain whereas choline deficiency inhibits proliferation of neuronal precursor cells and stimulates apoptosis (23,55). Supplementation of choline in gestation boosts up neurogenesis in the hippocampus and dentate gyrus (56). This effect on adult neurogenesis is believed to be brought by increased hippocampal concentrations of trophic factors like BDNF, insulin like growth factor 2 (IGF2), as well as vascular endothelial growth factor (VEGF) which help to increase the size of cholinergic neurons of the brain, facilitate acetylcholine synthesis and better cognitive function (57). Recent studies explore the coordinated roles of metabolism of choline and DHA during fetal development (58). The metabolic coordination of DHA and C has been well explained in vivo, in Pemt−/− mouse model. The PEMT enzyme increases the synthesis of phosphatidylcholine by using phosphatidylethanolamine as a precursor, that increases the requirement of choline in the tissues. Maternal DHA supplementation increases the concentration of brain PEMT enzyme thereby it increases the production of choline (55). Additionally, PEMT prefers phosphatidylethanolamine that contains DHA which is the long-chain PUFA and increases the development of DHA-enriched phosphatidylcholine in cell membranes of the neurons mainly in the brain (59). This metabolic coordination between the choline and DHA through the PEMT enzyme restores neural cell density in the hippocampus by reduced apoptosis and low serum cortisol concentration.

Conclusion

In conclusion, our findings documenting for the first time that supplementation of choline-DHA during prenatal stress in rats restores stress-induced neonate hippocampal neural cell density in CA1, CA3, and CA4 subregions of the hippocampus, and the dentate gyrus and diminishes apoptotic cells in CA1 and CA3 regions with reductions in serum cortisol levels**.** The result of this study suggests for further studies to revel the mechanism and the implication of the findings in humans.

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Conflict of Interest: Each author declares that he or she has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article.

Author Contribution: KSR, KMRB and RHT were involved in the conception and design of the study. Data collection and analysis were performed by RHT and. The first draft of the manuscript was written by RHT, and it was critically reviewed by KSR. The final draft of the manuscript was approved by all the authors.

Limitation: Study was limited to early adolescent period. The study showed only the neural cell density and serum cortisol of particular age group of rat pops. The mechanisms for alterations in neural cell density and serum cortisol need to be evaluated.

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