Thai Journal of Physiological Sciences
Vol. 20 No. 2 Sep. 2007–Feb. 2007

Original Article

Neuroprotective Effect of *Centella asiatica* Leaf Extract Treatment on Cognition and Hippocampal Morphology Against Prenatal Stress

Madhyastha S[1], Somayaji SN[2], Bairy KL[3], Prakash[4], Madhyastha P[5]

ABSTRACT **It has been reported that *Centella asiatica* (CeA) has neuroprotective effect on cognition and hippocampal neurons. During early postnatal development and preventing cognitive deficits, the dendritic arborization of hippocampal neurons is promoted. Prenatal stress is known to adversely affect the learning and memory abilities and also damage the neuronal proliferation in hippocampus during pre and postnatal development. In this study, investigated whether leaf extract of CeA can protect against prenatal stress induced cognitive deficit and neuronal loss in hippocampus. Pregnant rats were subjected to restraint stress three times daily for 45 min in day 14-21 of pregnancy. The second group received (200 mg/kg body weight dose) extracts of CeA fresh leaf during entire gestation period. The third group received both stress and CeA treatment. Pups received both prenatal stress and prenatal CeA treatment was allocated in two groups. One group of pups was undisturbed and the other group of pups received CeA extract treatment (20 ml kg^{-1} body weight/oral) from postnatal day 7-60. At postnatal day 45 the learning and memory abilities were tested in the two compartment conditions of avoidance task. After test, hippocampal neuronal population was measured using cresyl violet staining. The results of the present study confirm that prenatal stress has an adverse effect on learning and memory abilities in rats and also on CA3 and CA4 regions of the hippocampal neurons. Administration of CeA fresh leaf extracts during gestation period will not exert any protective effect against loss of hippocampal neurons or at cognition. However postnatal treatment of CeA will protect the hippocampal neurons against prenatal stress and also enhanced learning and memory abilities.**

Key words: ***Centella asiatica*, Hippocampal neurons, Prenatal stress, Learning and memory.**

INTRODUCTION

Prenatal or intrauterine development plays critical role in normal physical, mental and behavioral development of an individual. Prenatal stress in rats results in structural, physiological and behavioral alterations that persist in adulthood. A substantial body of evidence indicates that prenatal stress adversely affects human development, increasing susceptibility to diseases later in life as well as altering behavioral and cognitive development. Prenatal stress is known to impair cognitive function[1,2], increase anxiety and emotionality responses to the open field, decrease tendency to explore[3,4]. There are many reports suggesting the possible mechanisms like altered hypothalamo-pituitary-adrenal axis, neurotransmitters, decreased brain cell proliferations and brain corticosterone level during prenatal development. Furthermore, prenatal stress induced behavioral alteration involves hippocampal neuronal proliferation[5].

[1]*Associate Professor in Anatomy Kasturba Medical College, Mangalore, Karnataka. INDIA*
[2]*Department of Anatomy, Manipal-Melaka Medical College, Manipal*
[3]*Department of Pharmacology, Kasturba Medical College, Manipal*
[4]*Department of Anatomy, Kasturba Medical College, Mangalore*
[5]*Department of Dental Materials, Manipal College of Dental Sciences, Mangalore*
Corresponding address :
Sampath Madhyastha, M.D., Associate Professor in Anatomy Kasturba Medical College, Mangalore, Karnataka. INDIA-575 001; Phone: +91 824 2423452/2423654, 2422271, Fax: +91 824 2428183; E-mail: madhyast@yahoo.com, sampath.m@manipal.edu

CeA is a herb that grows in wet places throughout India. It is used in Ayurvedic preparations either as whole plants or as leaves in the fresh or extract form[6]. CeA has been shown to be very useful in improving learning and memory[7-9]. It is also used as a tonic for promoting brain growth and improving memory[10]. In addition, the plant is used in children with learning difficulties to improve general mental ability and in people suffering from cognitive disorders[8,11-14]. Although the fresh leaf extract of CeA has been claimed to improve learning and memory in different clinical studies[7,8,11-13], there is not any evidence for the effect of this plant extract on the brain regions involved in learning and memory, namely the hippocampus[15-17], amygdala and limbic cortex. The cornu ammonis (CA) region, particularly the CA3 subregion of the hippocampus, is the key structure of the brain involved in learning and memory[18-21].

From these reports it is clear that prenatal stress impairs cognition in offspring which involves proliferative activities of neurons in hippocampus. The neuroprotective efficacy of CeA against prenatal stress were not studied to the best of our knowledge. Since CeA is known to enhance learning and memory abilities in adult rats and also promoting neuronal proliferation in hippocampus, we hypothesize that treatment with fresh leaf extract from CeA would enhance learning and memory abilities and also bring structural changes in the hippocampal neurons in young growing rats against prenatal stress. The major part of neurogenesis occurs during last week of gestation period in rats[22] stress during this period can affect the neurogenisis. Thus, treatment of CeA leaf extract during this period in stressed pregnant rats and also postnatal treatment (when there is active brain cell proliferation during the preweaning period) of CeA to the prenatally stressed pups could affect neurogenisis and neuronal proliferations in hippocampus. There are many studies claiming the adverse effect of prenatal stress on neuronal proliferation during neonatal period, however we would like to evaluate the long lasting effect (in adult rats) on neuronal population in hippocampus and protective effect of CeA leaf extract. We have aimed to conduct the experiment in the way explained in the classic texts of Ayurveda[6], i.e. using fresh leaf extract rather than extraction.

MATERIALS AND METHODS

Animals

Inbred albino rats of Wistar strain of either sex of 90-120 days old, weighing 150-200g were selected. The rats were maintained in 12 hours light and dark cycle in temperature and humidity controlled environment, and were fed with standard food pellet and water ad libitum. Breeding and maintenance of the animals were done as per the guidelines of Government of India for use of Laboratory animals (Government of India notifies the rules for breeding and conducting animal experiments, proposed in the gazette of India Dec 15, 1998: which was reproduced in Ind. Journal of Pharmacol 1999; 31:92-5).

Mating of rats and animal groups

Female rats (n=40) were divided into five groups. Rats from each group were allowed to mate with one fertile sexually active male for 4 hours per day (separate male rats for each group). At the end of 4 hours, females were separated and vaginal smears taken to detect the presence of sperm for the confirmation of pregnancy and the rats were designated as day 0 of pregnancy. The pregnant rats were housed individually in separate cages and were randomly allocated into 4 groups of six each (n=6).

Stressing procedure

The pregnant rats were stressed (restraint stress) using a wire mesh restrainer[23] for three times daily for 45 min from gestation day 14 to 21. The wire mesh restrainer will have a wooden base and stainless steel wire mesh restrainer hinged to the base. A pad lock and latch will help to secure the rat in the restrainer. The restrainer with dimensions 11 cm (L) x 6cm (B) x 6 cm (H) was used for G 3-14 groups. Restrainer of 11cm (L) x 8 cm (B) x 8 cm (H) was used for G 14-21 group. This type of restrainer will only restrict the animal movement with out any pain, discomfort or suffocation.

Animal groups: (Diagram 1)

Group-1. (Control) Pregnant rats received saline throughout pregnancy instead of CeA.

Group-2. Pregnant rats received restrain stress from gestation day 14 to 21.

Group-3. Pregnant rats received leaf extracts of CeA (200mg/kg body weight) throughout the gestation period.

Six pups (3 male+3 female) from group 1, 2 & 3 were selected randomly for histological studies on postnatal day 60.

Group-4. Pregnant rats received restrain stress from gestation day 14 to 21 and CeA treatment during entire gestation period.

Group-4a. Pups from Group 4 rats received saline instead of CeA (equal volume) were undisturbed and processed for histological study on postnatal day 60 (n=6, 3 male + 3 female)

Group-4b. Pups from Group 4 rats received leaf extract of CeA (20 ml kg-1 body weight/oral) from postnatal day 7-60, then processed for histological study on postnatal day 60 (n=6, 3 male + 3 female).

Extraction and Administration of CeA Leaf Juice:

The fresh leaves of CeA were obtained locally and were authenticated by Dr.Gopalakrishna Bhat, Professor of Botany, Poorna Prajna College, Udupi, India. Fresh, 15-20-day-old leaves of CeA were collected in the morning. Fresh leaf juice was extracted from these leaves after washing, air-drying and homogenizing in a glass vessel and finally filtering through a sterile gauge cloth. Leaves were maximally extracted, so that from a given weight of leaves (5.0 g), a known volume was extracted (1.63 ± 0.15 g, n = 6). Since soil water was kept uniform we could extract the same from a given weight of leaves on different days. Furthermore, we have established that the dry weight of a given volume (1 ml) of juice prepared on different days was the same (0.079 ± 0.01 g, n = 6). The fresh leaf juice so obtained was fed to the rat pups through a gastric capillary tube attached to a 1 ml hypodermic syringe. Since the volume of juice to be fed to each individual rat pup was very little, the dose (20 ml kg-1 body weight/oral) was blended with an appropriate volume of saline for convenient feeding. Control rats were fed with a volume of saline equivalent to the volume of extract that the age-matched experimental rats received on each day. The dose selected for pups were according to the earlier study by Mohandas Rao et al[24]. Pregnant rats of group 3 and 4 received oral CeA leaf extract (200 mg/kg body weight) every morning through out the gestation period. The dose for pregnant rats was selected as per the previous study by Gnanapragasam et al[25].

Since standard extraction procedures (which involve boiling in water, ethyl alcohol or other organic solvents) may alter the structure of bioactive principles, we avoided them. Although there may have been minor variation in our daily preparations, it would have been minimal as leaves of equal maturation were collected from the same place on all days. This minor daily variation would have been compensated for by the long period (2, 4 or 6 weeks) of treatment. It has been shown in a recent study has shown that a CeA plant extract obtained from ethanol extraction was different from one obtained from water extraction in terms of its biological activity[26].

Conditioned avoidance test (shuttle box test):

The shuttle box consisted of a closed wooden box with shutters in front. The floor area was made up of grids, which were separated into two parts by a median grid. Each part was connected to separate electric circuits and a buzzer was fixed inside the box. Individual rats were allowed to explore the test box for 5 minutes. After 10 seconds, a discriminative stimulus was given through a buzzer. During that period, the rat could avoid the shock by crossing to the other compartment. If the rat failed to respond during the discriminative stimulus period, it received a shock of 2.5 mA for a maximum period of 10 seconds, during which time it could escape by crossing to the other side. The contingency for avoiding the shock was a single crossing over the median grid from one side of the shuttle box to the other. The test consisted of 30 trials daily for 5 consecutive days. The number of shock avoidances increases from day 1 to 5 of the test under normal circumstances. The average shock avoidance numbers on all 5 days were termed as the mean score during 5 days of testing. Any decrease in this score is an indication of learning impairment. Each rat was retested one week after the last trial to assess retention of memory. A comparison of rat's performance with its previous performance gives the assessment of memory and is presented as the retention score (RTS), which was calculated as in Jensh et al[27].

$$RTS = \frac{\text{Mean of retest score} \times \text{Mean scoring during 5 days of test}}{\text{Mean score during day 5 of the test}}$$

Any decrease in the retest score and retention score is an indication of memory impairment. Each rat was trained for 4 days before starting the test.

Histological study of Hippocampus:

Perfusion: Each animal was deeply anesthetized with ether and secured on a dissection board, and its chest cavity was opened to expose the heart. About 15 mL of 0.9% saline was perfused through the left ventricle at the rate of 1mL/min. This was followed by perfusion of 10% formalin at the same flow rate. The animal was decapitated and the brain was removed and kept in 10% formalin for 48h (post fixation). Paraffin blocks were made in an embedding bath. Coronal sections of 3-5-μm thickness were cut in the dorsal hippocampus using a rotary microtome (Jung Biocutt 2035, Leica, Germany). Twenty five sections from each animal were mounted serially on air dried gelatinized slides.

Staining: The sections were stained with cresyl violet stain. One hundred milligrams of cresyl violet was dissolved in 100 ml of distilled water. To this 0.5 ml of 10% acetic acid was added to give a pH of 3.5-3.8. The stain was filtered before use[28].

Scoring: In each hippocampal section, cornua amonis (CA1, CA2, CA3 and CA4; 200 μm length) of hippocampus were selected (using an oculomicometer) and the number of viable neurons were counted. The slides were screened using a light microscope (100X). Ten sections from each rat were considered. Slides from different groups of rats were decoded to avoid manual bias while counting the cells. The cell counts were expressed as the number of cells per unit length of the cell field (cells/200 μm).

Statistical analysis:

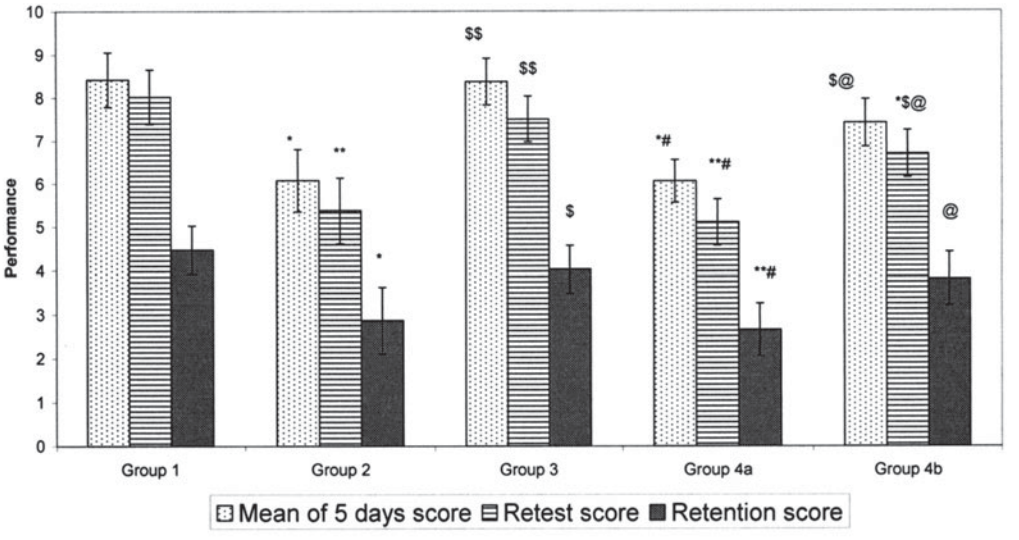
The data were expressed as mean ± SE. The significance of differences among the groups were assessed using one way analysis of Variance (ANOVA) test followed by Bonferroni multiple comparison test using Graph Pad in Stat (GPIS) software, version 1.13. P values < 0.05 were considered as significant.

RESULTS

Gestational period stress as well as CeA extract treatment did not had significant effect on gestational length ($p=0.62$, $F=0.6557$) and litter size ($p=0.124$, $F=1.946$). The mean gestational length for group 1 was 22.6±0.2, for group 2, 22.4±0.2, for group 3, 22.4±0.3, for group 4a, 22.5±0.4 and for group 4b, 22.6±0.4. The mean litter size of group 1 was 6.86±0.4, for group 2, 5.9±0.9, for group 3, 6.3±0.8, for group 4a, 6.4±0.6 and for group 4b, 6.7±0.8. There was no sexually dimorphic effect was observed in assessed parameters, hence mean values for both male and female were considered.

The mean score during 5 days of testing, the retest score and the retention score decreased significantly ($p<0.001$) in parentally stressed pups (group 2) when compared with control offspring (group 1). Similar results were obtained when prenatally CeA treated pups (group 3) were compared with parentally stressed offspring (group 2). This clearly indicates that prenatal stress impairs learning and memory abilities in offspring. Offspring which received both prenatal stress and CeA treatment (group 4a) did not differ ($p>0.05$) from offspring received only prenatal stress (group 2) in all the 3 parameters assessed in this test. This result clearly indicates that prenatal CeA treatment has not affected learning and memory abilities in offspring. Offspring of group 4b (received prenatal stress, prenatal CeA treatment and postnatal CeA treatment) showed a significant increase in mean of 5 days score ($p<0.01$), mean of retest score ($p<0.01$) and retention score ($p<0.05$) when compared to group 4a. This result demonstrates that postnatal CeA treatment not only minimizes the prenatal stress effects, but also enhances the learning and memory abilities in offspring. The memory retention and mean of 5 days score did not differ between group 4b and control offspring (group 1). These results indicate that postnatal CeA treatment has maintained learning and memory abilities in prenatally stressed offspring almost equivalent to control offspring (Figure 1).

Prenatal stress has produced regional specific effect on hippocampal neurons. The CA1 and CA2 region of the hippocampus did not show any significant structural or numerical changes in response to prenatal stress. Prenatal stress (group 2) has significantly ($p<0.001$) affected in CA3 and CA4 hippocampal neurons when be compared to control group (group 1) as well as prenatal CeA treatment (group 3). Pups received prenatal stress and prenatal CeA treatment showed significant ($p<0.00$) decrease in number of neurons when be compared to control (group 1) and group 3. From this result, it is clear



Footnote:

ANOVA significance for mean of 5 days score, F=23.17, Inter group comparison - Group 1 Vs 2 * p<0.001, Group 1 Vs 4a * p<0.001, Group 2 Vs 3 $^{\$\$}$ p<0.001, Group 2 Vs 4b $^{\$}$ p<0.01, Group 3 Vs 4a $^{\#}$ p<0.001, Group 4a Vs 4b $^{@}$ p<0.01

ANOVA significance for mean of retest score, F=26.82, Inter group comparison - Group 1 Vs 2 ** p<0.001, Group 1 Vs 4a ** p<0.001, Group 1 Vs 4b * p<0.01, Group 2 Vs 3 $^{\$\$}$ p<0.001, Group 2 Vs 4b $^{\$}$ p<0.01, Group 3 Vs 4a $^{\#}$ p<0.001, Group 4a Vs 4b $^{@}$ p<0.01

ANOVA significance for retention score, F=9.48, Inter group comparison - Group 1 Vs 2 * p<0.01, Group 1 Vs 4a ** p<0.001, Group 2 Vs 3 $^{\$}$ p<0.05, Group 3 Vs 4a $^{\#}$ p<0.01 Group 4a Vs 4b $^{@}$ p<0.05

Fig. 1 Observation of offspring performance in conditioned avoidance test. Values are expressed as mean ± SD (n=6 animal in each group). Error bar indicates ± SD.

Table 1 Number of hippocampal neurons (in 200 μm length area) in male Wistar rats. Values are expressed as means ± SD (n=60 slides). Group 1 -control, Group 2- pups received prenatal stress, Group 3- pups received prenatal CeA, Group 4a- pups received both prenatal stress and prenatal CeA treatment and Group 4b- pups received prenatal stress, prenatal and postnatal CeA treatment.

Hippocampal region	Group 1	Group 2	Group 3	Group 4a	Group 4b
CA1	17.03±0.541	16.96 ± 0.919	17.05±0.528	16.78±0.691	16.93±0.606
CA2	14.23±0.582	14.0 ± 0.920	14.13±0.669	14.23±0.697	14.28±0.64
CA3	15.63±0.66	15.08 ± 0.65*	15.72±0.536 $^{\$\$}$	15.01±0.655 * $^{\#}$	15.51±0.567 $^{\$@}$
CA4	15.41±0.84	14.1 ± 1.02 *	15.53±0.56 $^{\$\$}$	14.02±0.75 * $^{\#}$	15.23±0.57 $^{\$\$@}$

ANOVA significance for CA1 neurons

P<0.0001, F=1.519, No significant difference between the groups

ANOVA significance for CA2 neurons

P=0.0045, F=1.475, No significant difference between the groups

ANOVA significance for CA3 neurons

P=0.3758, F=16.67
Group 1 Vs Group 2 * p<0.001, Group 1 Vs Group 4a * p<0.001
Group 2 Vs Group 3 $^{\$\$}$ p<0.001, Group 2 Vs Group 4b $ p<0.01
Group 3 Vs Group 4a $^{\#}$ p<0.001
Group 4a Vs Group 4b $^{@}$ p<0.001

ANOVA significance for CA4 neurons

P<0.0001, F=55.08
Group 1 Vs Group 2 * p<0.001, Group 1 Vs Group 4a * p<0.001
Group 2 Vs Group 3 $^{\$\$}$ p<0.001, Group 2 Vs Group 4b $$ p<0.01
Group 3 Vs Group 4a $^{\#}$ p<0.001,
Group 4a Vs Group 4b $^{@}$ p<0.001

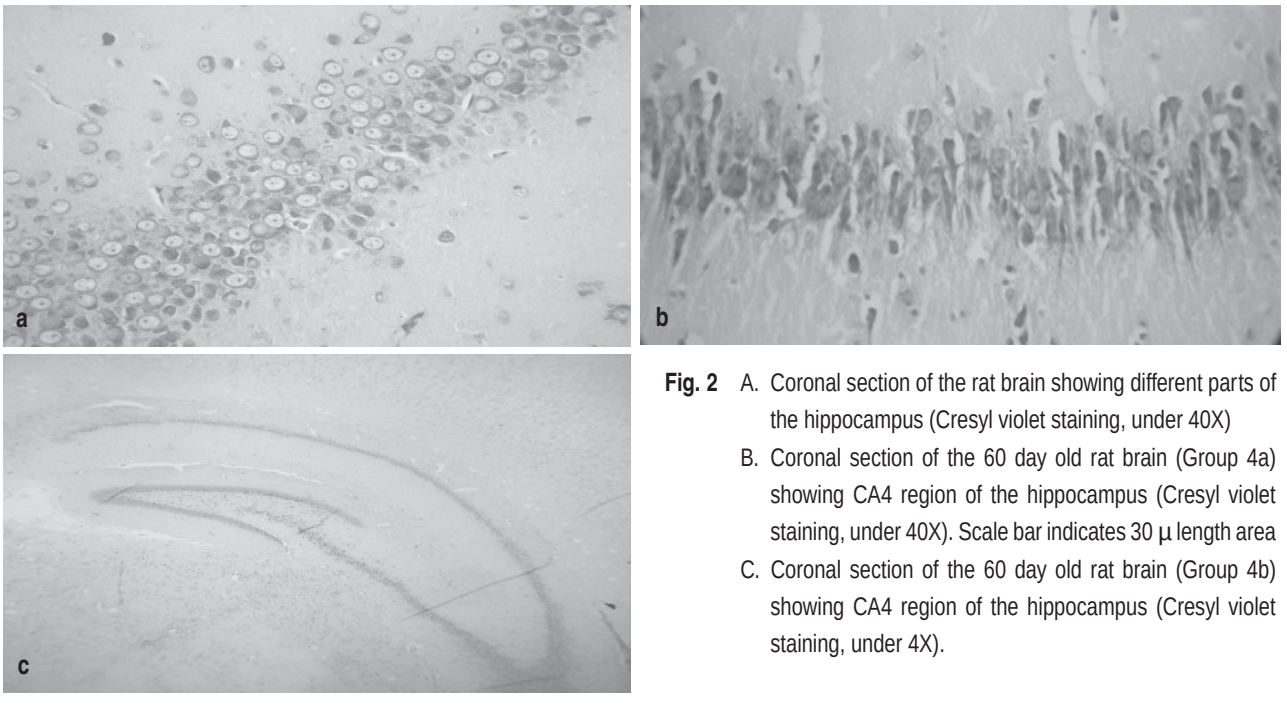


Fig. 2 A. Coronal section of the rat brain showing different parts of the hippocampus (Cresyl violet staining, under 40X)
B. Coronal section of the 60 day old rat brain (Group 4a) showing CA4 region of the hippocampus (Cresyl violet staining, under 40X). Scale bar indicates 30 µ length area
C. Coronal section of the 60 day old rat brain (Group 4b) showing CA4 region of the hippocampus (Cresyl violet staining, under 4X).

that prenatal CeA treatment did not have any protective effect against prenatal stress. Pups which received both prenatal and postnatal CeA treatment showed a significant ($p<0.001$) protective effect against prenatal stress. The protective effect is induction neuronal loss when group 4a and 4b was compared. Histopathological changes characterized by densely stained, distorted cells with pyknotic appearance were observed in CA3 and CA4 region of the hippocampus (Table-1, Figure 2a, 2b & 2c).

DISCUSSION

Results of the present study confirm that prenatal stress impairs learning and memory abilities in offspring with considerable damage to CA3 and CA4 region of the hippocampus. This study demonstrates that prenatal CeA treatment has no protective effect against prenatal stress in learning and memory abilities of offspring, and also on neuronal population of hippocampus. The novel finding of the present study was postnatal CeA treatment not only minimizes the prenatal stress effects, but also enhances the learning and memory abilities in offspring. Postnatal CeA treatment has maintained learning and memory abilities in prenatally stressed offspring almost equivalent to control offspring. Postnatal CeA treatment has enhanced the neuronal proliferative activities in CA3 and CA4 region of the hippocampus against prenatal stress which induced neuronal loss.

Prenatal stress has impaired learning and memory abilities in offspring. Prenatal stress is known to increase anxiety behavior and alter cognitive functions, in postnatal life[1,2]. The mechanism accounting for these neurotoxic effects are not fully understood. However, involvement of stress induced corticosterone secretion, brain cell proliferation, brain derived neurotrophic factor and brain amines are known. The impact of prenatal stress on neuronal proliferation may be the major cause of neurotoxicity, such as learning and memory dysfunction which is often associated with hippocampal neuronal loss. In the present study prenatal stress has considerably damaged the CA3 and CA4 region of the hippocampus. Our findings were supported by findings of many reports[29,5,30]. Short-lasting, mild prenatal stress enhanced neonatal neurogenesis and differentiation of processes of hippocampal neurons, whereas long-lasting, severe stress impaired their morphology[5]. Similar effect was also observed in hypothalamic paraventricular neurons[31]. Prenatal stress resulted in an approximately 50% decrease in brain cell proliferation just after birth in both genders within the hippocampus at postnatal day 1 and at post-

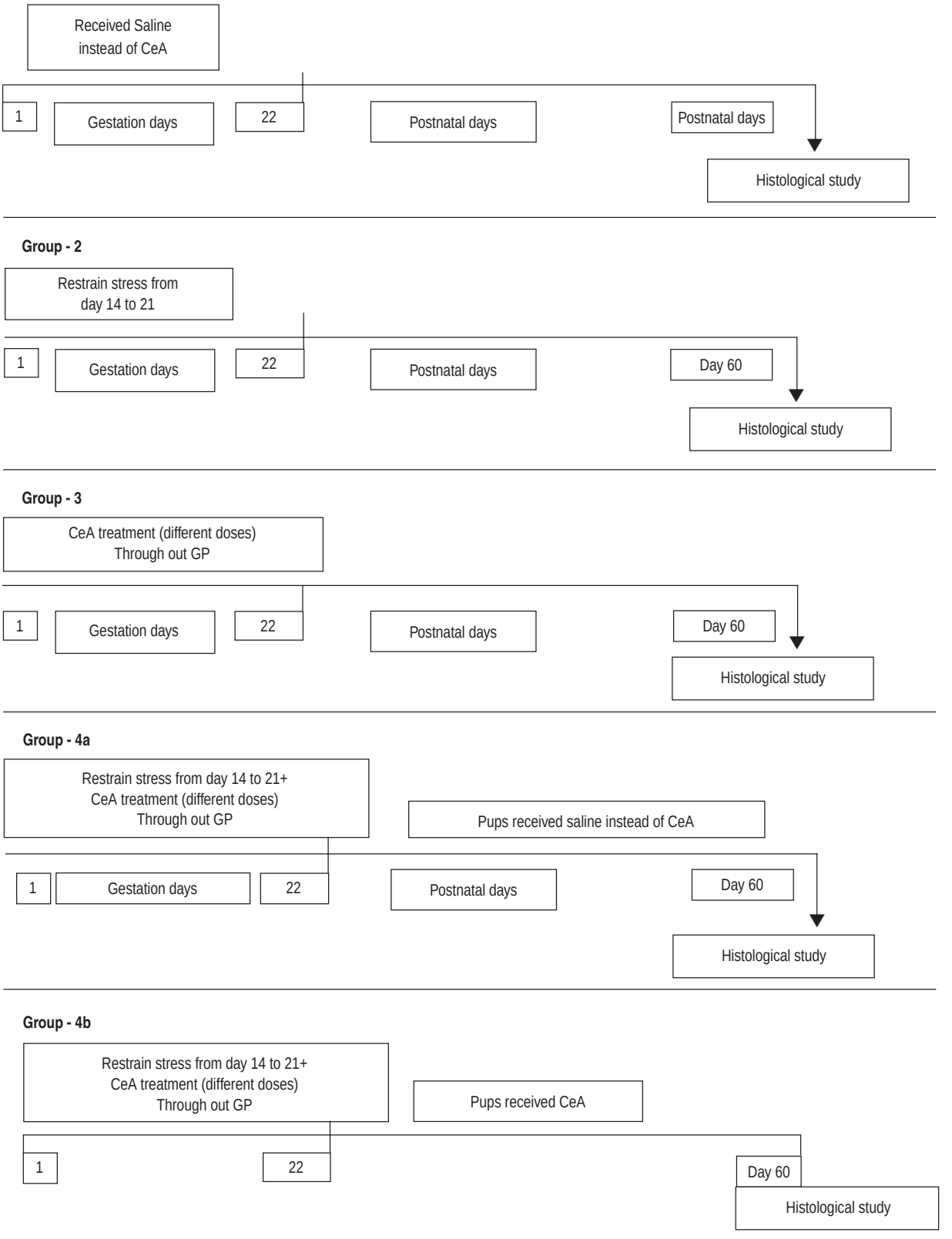


Diagram 1 Group of study

natal day 5[32]. Both middle and late gestational stress caused a significant decrease in the number of hippocampal neurons in the female, but not in the male offspring[29]. Prenatal stress is known to decrease 5-HT receptor binding site in ventral hippocampus[30] of 4-week old rats.

It has been reported that CeA has specific neuroprotective effect on hippocampal neurons. Thus, we investigated whether CeA can protect against prenatal stress induced neuronal loss in hippocampus. In the present study gestational treatment of CeA had not any protective effect against neuronal loss in hippocampus. It can be concluded that the protective effect of CeA extract is not carried to the offspring. Although a preliminary study, the estimation of active constituents of CeA in both maternal and offspring could give more information in this regard. It is interesting to know that postnatal treatment of leaf extract of CeA from day 7 to 60 in prenatally stressed rats showed a significant protective effect against prenatal stress induced neuronal loss in hippocampus. There are many reports supporting our findings in which postnatal treatment of CeA had a significant effect on neurons (increased neuronal proliferation) and enhancement of memory. The memory-enhanced property of fresh leaf extracts of CeA in neonatal rats has been reported before Rao Mohandas et al[33]. Nalini et al[34] reported the memory-enhanced effect of aqueous extract of CeA in adult rats. In addition to its effect on behavior, asiatic acid, asiaticoside 6 and SM2, which are the active components of the plant, have a neuroprotective property. A strong inhibition of beta amyloid and free radical which induced cell death in B103 cell cultures and hippocampal slices was shown[35]. The changes taking place in the neuronal population of hippocampus may be due to these neuroprotective active components and the key neuronal basis for improved learning and memory in these rats.

The neurons of hippocampal CA3 region receive extrinsic and intrinsic inputs from the different parts of the limbic system, which is concerned with learning and memory. The extrinsic afferents are mainly from the entorhinal cortex, septal area, mamillary body and nuclei of the brain stem[36,37]. Moreover, the CA3 neurons also receive a significant number of intrinsic afferents from the axons of dentate granule cells and the contralateral CA3 region[38,39]. Alterations such as increases or decreases in neuronal cell population may result in alterations in learning and memory behavior.

In the present study neuronal population of CA3 and CA4 region of the hippocampus in pups treated with CeA was increased during postnatal development. This result suggests that such doses of plant extract were adequate to retain the number of neurons against prenatal stress induced neuronal loss. Such a protection against neuronal loss after prenatal stress may be effective in enhancement of learning and memory[40]. Rao et al[41] reported that CeA treatment during postnatal developmental stage influenced the neuronal morphology and promoted the higher brain function of juvenile and young adult mice, which support our findings. Centella asiatica is also known to be a potent antioxidant and exerts significant neuroprotective effect and proved efficacious in protecting rat brain against age related by oxidative damage[42]. Aqueous extract of C. asiatica has cognitive enhancing effect involving an antioxidant mechanism[43]. CeA treatment has reduced corticosterone level in serum and increase of the contents of serotonin, norepinephrine, dopamine and their metabolites in rat brain[44]. These factors could also involve the protective mechanism of CeA against prenatal stress, as prenatal stress is known to alter the brain monoamine level.

Thus, from the present study it is evident that administration of fresh leaf extracts of CeA during gestation period will not exert any protective effect against loss of hippocampal neurons. However, postnatal treatment of CeA will protect the hippocampal neurons against prenatal stress which induced less loss. Children with congenital cerebral or hippocampal atrophy with delayed milestones due prenatal insults like stress can be treated with leaf extract of CeA. The results of this study will have impact in delivery of better health care which is less cumbersome, more functional and would be within the reach of common man from the economy aspect as well.In view of developing cost effective remedy to behavioral disorders in children with congenital cerebral atrophy in poor countries, the utility of the plant product as therapeutic agent is in vogue. The plant in the present study was in use in this part of the world since decades without much research. This study is very relevant in delivering the health care at the doorstep of the common man.

REFERENCES

1. Barker DJ. Fetal programming of coronary heart disease. Trends Endocrinol Metab 2002; 13:364-8.
2. Charmandari E, Kino T, Sourvatzoglou E, et al. Pediatric stress: hormonal mediators and human development. Horm Res 2003; 59:161-79.
3. Weinstock M, Matlina E, Maor GI, et al. Prenatal stress selectively alters the reactivity of the hypothalamic-pituitary adrenal system in the female rat. Brain Res 1992; 595:195-200
4. Poltyrev T, Keshet GI, Kay G, et al. Role of experimental conditions in determining differences in exploratory behavior of prenatally stressed rats. Dev Psychobiol 1996; 29:453-62.
5. Fujioka A, Fujioka T, Ishida Y, et al. Differential effects of prenatal stress on the morphological maturation of hippocampal neurons. Neuroscience 2006; 141(2):907-15.
6. Sharma PV.; 13th edition New Delhi, India Chaukhamba Publications Vishwa Bharati Academy Dravyaguna Vignana 1992; pp.3-5.
7. Sivarajan VV. Indira Balachandran;New Delhi, India Oxford and IBH Publishing Company Ayurvedic Drugs and Their Plant Sources 1994; 97:pp.289-90.
8. Dash PK, Mistry IU, Rao AR, et al. Role of medhya rasayana in school children. Ayu 1996; 12:15.
9. Satyavati GV, Gupta AK, Tandon N.; 1st edition New Delhi Indian Council of Medical Research. Medicinal Plants of India 1976; pp. 216-20.
10. Anbuganapathi GA. Synergetic effect of vallarai and brahmi on learning ability of albino mice and school children Paper presented at the International Seminar on Recent Trends in Pharmaceutical Sciences Ootacamund February, 1995; pp. 18-20
11. Rajagopalan V. Effect of Ayushman-8 in manasa mandata (mental retardation) Paper presented at the Seminar on Research in Ayurveda and Siddha New Delhi March, 1995; CCRAS pp.20-2.
12. Shah LP. An open clinical trial of mentat in hyperkinetic children. Probe 1992; 31:125-9.
13. Appa Rao MVR, Srinivasan K, Rao KT. The effect of mandookaparni (Centella asiatica) on the general mental ability (medhya) of mentally retarded children. J Res Indian Med 1973; 8:9-16.
14. Joshi H and Parle M. Brahmi rasayana improves learning and memory in mice. Evid Based Complement Alternat Med 2006; 3:79-85.
15. Galani R, Weiss I, Cassel JC, et al. Spatial memory habituation and reactions to spatial and nonspatial changes in rats with selective lesions of the hippocampus, the entorhinal cortex or the subiculum. Behav Brain Res 1998; 96:1-12.
16. Batch ME, Hawkins RQ, Osman M, et al. Impairment of spatial but not contextual memory in Ca MKII mutant mice with a selective loss of hippocampal LTP in the range of theta sequency. Cell 1995; 81:905-15.
17. Kesner RP and Hopkins RD. Short-term memory for duration and distance in humans: role of the hippocampus. Neuropsychology 2001; 15:58-68.
18. Jensen O and Lisman JE. Hippocampal CA3 region predicts memory sequences accounting for the phase precession of place cells. Learn Mem 1996; 3:279-87.
19. Lassale JM, Bataille T, Halley H. Reversible inactivation of the hippocampal mossy fiber synapses in mice impairs spatial learning, but neither consolidation nor memory retrieval, in the Morris navigation task. Neurobiol Learn Mem 2000; 73:243-57.
20. Christian EP and Deadwyler SA. Behavioral functions and hippocampal cell types: evidence for 2 non-over lapping populations in the rat. J Neurophysiol 1986; 55:331-48.
21. Stubley Weatherly L, Harding JW, Wright JW. Effect of discrete kainic acid induced hippocampal lesions on spatial and contextual learning in rats. Brain Res 1996; 716:29-38.
22. Dobbing J and Sands J. Comparative aspects of the brain growth spurt. Early Human Dev 1979; 3:79-83.
23. Sunanda, M.S.Rao, T.R.Raju. Effect of chronic restraint stress on dendritic spines and excrescences of hippocampal CA3 pyramidal neurons- a quantitative study. BrainRres 1995; 694:312-7.
24. Mohandas Rao KG, Muddanna Rao S, Gurumadhva Rao S. Centella asiatica (L.) Leaf Extract Treatment during the Growth Spurt Period Enhances Hippocampal CA3 Neuronal Dendritic Arborization in Rats. Evid Based Complement Alternat Med 2006; 3(3):349-57.
25. Gnanapragasam A, Ebenezar KK, Sathish V, et al. Protective effect of Centella asiatica on antioxidant tissue defense system against adriamycin induced cardiomyopathy in rats. Life Sci 2004; 76(5):585-97.
26. Soumyanath A, Zhong YP, Gold SA, et al. Centella asiatica accelerates nerve regeneration upon oral administration and contains multiple active fractions increasing neurite elongation in-vitro. J Pharm Pharmacol 2005; 57:1221-9.
27. Jensh RP, Brent RL and Vogel WH. Studies of effect of 0.4Gy AND 0.6Gy prenatal X-irradiation on postnatal adult behaviour in the wistar rats. Teratology 1987; 35:53-61.
28. Sampath Madhyastha, Somayaji SN, Rao MS, et al. Hippocampal brain amines in methotrexate-induced learning and memory deficit. Can.J.Physiol.Pharmacol. 2002; 80:1076-84.
29. Zhu Z, Li X, Chen W, et al. Prenatal stress causes gender-dependent neuronal loss and oxidative stress in rat hippocampus. J Neurosci Res 2004; 78(6):837-44.
30. Van den Hove DL, Steinbusch HW, Scheepens A, et al. Prenatal stress and neonatal rat brain development. Neuroscience 2006; 137(1):145-55.
31. Fujioka T, Sakata Y, Yamaguchi K, et al. The effects of prenatal stress on the development of hypothalamic paraventricular neurons in fetal rats. Neuroscience 1999; 92(3):1079-88.
32. Van den Hove DLA, Lauder JM, Scheepens A, et al. Prenatal stress in the rat alters 5-HT1A receptor binding in the ventral hippocampus. Brain Research 2006; 1090:29-34.
33. Rao Mohandas KG, Rao Muddanna S, Rao Gurumadhva S.

Centella asiatica (linn) induced behavioural changes during growth spurt period in neonatal rats. Neuroanatomy 2005; 4: 18-23.
34. Nalini K, Aroor AR, Karanth KS, et al. Effect of Centella aciatica fresh leaf extract on learning and memory and biogenic amine turnover in albino rats. Fitoterapia 1992; 63:232-8.
35. Mook Jung I, Shin JE, Yun SH, et al. Protective effects of asiaticoside derivatives against beta amyloid neurotoxicity. J Neurosci Res 1999; 58:417-25.
36. Van Hoesen GW and Pandya DN. Some connections of the entorhinal cortex (area 28) and perirhinal (area 35) cortices of rhesus monkey, III Efferent connections. Brain Res 1975; 95:39-59.
37. Raisman G, Cowan WM, Powell TPS. The extrinsic afferent commissural and association fibers of the hippocampus. Brain 1965; 88:963-96.
38. Hamlyn LH. The fine structure of the mossy fiber endings in hippocampus of the rabbit. J Anat 1962; 96:112-20.
39. Amaral DG and Witter MP. The three dimensional organization of the hippocampal formation. A review of anatomical data. Neuroscience 1989; 31:571-91.
40. Tang AC. Neonatal exposure to novel environment enhances hippocampal dependent memory function during infancy and adulthood. Learn Mem 2001; 8:257-64.
41. Rao SB, Chetana M, Uma Devi P. Centella asiatica treatment during postnatal period enhances learning and memory in mice. Physiol Behav 2005; 86(4):449-57.
42. Subathra M, Shila S, Devi MA, et al. Emerging role of Centella asiatica in improving age-related neurological antioxidant status. Exp Gerontol 2005; 40(8-9):707-15.
43. Veerendra Kumar MH, Gupta YK. Effect of different extracts of Centella asiatica on cognition and markers of oxidative stress in rats. J Ethnopharmacol 2002; 79(2):253-60.
44. Chen Y, Han T, Rui Y, et al. Effects of total triterpenes of Centella asiatica on the corticosterone levels in serum and contents of monoamine in depression rat brain. Zhong Yao Cai. 2005; 28(6):492-6.