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RESEARCH ARTICLE

Effect of High cholesterol Diet and Stress on CA1 &CA3 Regions of Hippocampus

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Abstract

Back ground: Dyslipidemia induced cognitive impairment is a matter of controversy. The effects of dyslipidemia coupled with exposure to stress on the hippocampal sub-regions are little known.

Aim: To determine the effect of high fat diet consumption and prolonged exposure to stress on the hippocampal CA1 & CA3 regions.

Materials & methods: Adult male Wistar rats of 2months old were randomly divided into three groups. (n=6). Group-I received normal diet (control), Group-II received high cholesterol diet (HCD) and Group-III received high cholesterol diet and stress (HCD + STS). Body weight, serum total cholesterol (TC), triglycerides (TG) was measured. The spatial memory was assessed by Morris water maze. Rats were sacrificed on 96th day; brains were separated and Coronal sections of CA1 and CA3 areas of hippocampus were processed by Cresyl violet staining and the number of viable neurons was quantified.

Results: Compared to control, (HCD) fed group-II and (HCD + STS) group-III induced showed significant ($p<0.001$, $p<0.01$) increase in body weight, TC and TG levels. Similarly in Morris water maze animals of group-II (HCD) and III (HCD + STS) exhibited significant increase in latency to enter target quadrant ($p < 0.001$, $p < 0.05$) and significant decrease ($p < 0.001$, $p < 0.05$) in the time spent in the target quadrant. Cresyl violet staining of hippocampus also revealed significant ($p < 0.001$, $p < 0.01$) decrease in number of viable neurons in CA1 and CA3 regions among group-II and III respectively compared to control group.

Conclusion: These findings demonstrated that HCD and HCD + STS had effect on memory and was neurotoxic to hippocampus. These findings help us to understand how diet rich in cholesterol and stress affect the hippocampus

INTRODUCTION

Dementia is a syndrome of the brain that results in progressive decline of cognitive function. Alzheimer's disease is the commonest cause for dementia in elderly individuals and has become a major public health challenge. Globally it has been estimated that 10% of individuals aged 70 years or above are suffering from memory loss. It has been stated that by 2050 the estimated prevalence of Alzheimer' disease will multiply by four times. So it is very essential to recognize the probable risk factors to prevent or delay the onset of dementia[1].

Emerging evidence showed that chronic exposure to stress is associated with increased risk of mental illness and psychological disorders. Animal studies have demonstrated the deleterious effects of prolonged stress exposure on cognition in water maze[2].

Research over last five years emphasized the influence of diet on neurological functions such as, mood, memory and learning ability. High fat diet and obesity are known to predispose individuals to type-2diabetes and metabolic syndrome. Evidence from preclinical models and epidemiological studies indicate that high fat diet and hypercholesterolemia can be one of the causes for dementia [3, 4]. Hippocampus lies in the temporal lobe of the cerebrum. It is necessary for the formation of stable declarative memory in humans and spatial memory in rodents[5, 6]. It is divided into two major U shaped interlocking sectors, dentate gyrus and the hippocampus proper (cornu ammonis). Lorento de No divided the hippocampus proper into four regions CA1, CA2, CA3 and CA4 .The effects of stress and diet rich in cholesterol on learning and memory have been well documented but there is scarce information of how these two factors acting alone or in concert influence the hippocampal morphology[7].

AIM

The aim of the present study is to investigate the effects of high fat diet and prolonged stress exposure on CA1 and CA3 regions of hippocampus.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 95 – 120 g (2 months old) were procured from the central animal house, Mamata Medical College, Khammam. Six animals were taken and acclimatized for one week and housed in polypropylene cages (22.5 × 35.5 × 15 cm) at standard temperature 25 ± 2°C, humidity (50-55%) under12 h-light-dark cycles controlled environment with a free access to pelleted chow and tap water *ad libitum.*

The experimental protocol was approved by the institutional animal ethical committee (IAEC/DP-05/C16).All the experimental procedures were carried out during 10 am to 4 pm.

Animals were randomly assigned into three groups of sixeach (n=6).

Group 1: (control) fed with standard chow diet for three months

Group 2: fed with high fat diet for three months

Group 3: fed with high fat diet for three months and received stress for 21 days (69^{th} day-90^{th} day)

Deoxycholic acid (5g): Nice chemicals, Cochin kerala

Pure cholesterol powder (5g): Nice chemicals, Cochin kerala

Total cholesterol and triglycerides kits: Eumic laboratory, Trichy,kerala

Cresylviolet powder: Lobachemie

Induction of hyperlipidemia protocol

Hyperlipidemia has been induced by feeding with cholesterol-rich diet for 3 months. Deoxycholic acid (5g) was mixed thoroughly with 700g of powdered rat chow diet. Simultaneously cholesterol (5g) was dissolved in 300g warm coconut oil. This oily solution was added slowly into the powdered mixture to obtain a soft homogenous cake. This cholesterol-rich diet was made into pellets of about 3g each and given to the animals[8].

Stress Induction protocol

Male Wistar rats were placed in a wire mesh restrainer for 21 days, daily 6 hours, from 69^{th} to 90^{th} day. The restrainer was made up of a wooden base to which stainless steel wire mesh was hinged. A pad lock and latch were used to secure the rat. The dimensions were 8 cm (Length) x 4cm (Breadth) x 4 cm (Height). This type of wire mesh restrainer can only restrict the animal movement without any uneasiness, pain or suffocation[9].

Weight gain

Weight of the animals was measured at the beginning of the study and at the end (after 3 months) ***Lipid profile***

Blood samples were obtained by retro-orbital puncture, serum cholesterol and triglycerides were estimated at the beginning of the study and at the end (after 3 months).[10]

Morris water maze test

Spatial memory of the rats was tested on 90^{th} day by using Morris water maze. The water maze consists of a circular tank of 1.80 m diameter and 75 cm depth. The pool was filled with water and maintained at a temperature of 24-26 º C to a depth of about 50 cm. It was divided into four quadrants and an escape platform of size 4"×4" was hidden approximately 2cm below the water surface in the target quadrant. Water in the pool was made opaque by adding milk just before the experiment. Permanently positioned distinctive objects were placed for facilitating spatial

orientation of the animal. Positions of the cues were kept unchanged throughout the period of experiment. The rats were trained in the water maze in 10 sessions on 5 consecutive days, two sessions on each day. Each session consists of four trails. In each trial time taken to reach hidden platform was recorded. If the rat was unable to find the platform within two minutes, the training session was terminated and a maximum score of two minutes was assigned.

Twenty four hours after the last session, rats were subjected to a probe trial. This session was for 30 seconds in which the hidden platform was removed. Here time taken to reach the target quadrant and time spent by the rats in search of the platform was measured. Greater latency to reach the target quadrant and less time to spend in the target quadrant suggests memory impairment[11].

Cresyl violet staining procedure

The animals were profoundly anesthetized with diethyl ether and fixed to dissection board. The chest cavity was opened and heart was exposed. About 15ml of 0.9% heparinized saline was perfused with help of perfusuion pump through left ventricle at the rate of 1ml/min followed by 250ml of 10% formalin until the liver was blanched. The animal was decapitated and the brain was isolated. 5-6mm thick coronal sections of brain tissue were made and kept in 10% formalin for 48 hours (post fixation).Tissues were processed through different grades of alcohol (50%, 70% for 24 h, 90%, and 100% for 12 h) and were immersed in xylene for 1-2 h. Paraffin blocks were made and sections of 5 microns thickness were cut from the mid dorsal hippocampal level, using a rotary microtome. Sections were selected and mounted serially on air dried gelatinized slides.

Preparation of Cresyl violet stain (0.1%)

100mg of Cresyl violet was dissolved in 100ml of distilled water. To this 0.5 ml of 10% Acetic acid was added to give a pH of 3.5 to 3.8.The stain was filtered before use.

Quantification of cells

In each hippocampal section, cornuaamonis(CA1 and CA3; 250 µm length) was selected using an ocular micrometer. The number of viable neurons were counted and averaged under (40X) magnification (Magnus, Olympus Pvt. Ltd. New Delhi, India). Darkly stained, shrunken and cells with fragmented nuclei were excluded from the count. To avoid manual bias ten sections from each rat were considered. The cell counts were expressed as the number of cells per unit length of the cell field (cells/250 µm) [12].

Statistical analysis

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

RESULTS

Weight, cholesterol & triglycerides

Weight of the animals was measured during the study period (after three months). There was significant increase in the body weight of the animals fed with only high cholesterol diet (HCD: 264± 6.90grams, ($p<0.001$) and high cholesterol diet + stress (HCD+STS: 172 ± 9.61 grams ($p<0.01$), when compared to control group animals (C: 151±8.16 grams). (Fig: 1) After three months serum cholesterol and triglyceride levels were elevated in both groups namely high cholesterol diet (HCD) group (166±4.64mg/dl, 149 ±6.31mg/dl), ($p<0.001$) and HCD + stress group (106±9.70mg/dl, 108 ± 8.52mg/dl), ($p<0.01$) as compared to the control group (90.7±4.18mg/dl, 93± 5.96mg/dl). (Fig: 2, 3)

Behavioural test (Morris water maze): Water maze performance during the training sessions*:* Analysis of the spatial memory test on the water maze has shown that during the first session all the rats failed to reach the escape platform. During the second and third day the control group animals were able to reach the platform in less time when compared to the other two groups suggesting of memory impairment in the groups fed with high cholesterol diet and induced to stress. In the fourth and fifth day rats fed with high cholesterol diet and stress induced group took longer time to escape on to the platform compared to the group fed with high cholesterol diet alone. This can be attributed to the deleterious effects of stress and high cholesterol diet together on spatial memory.

Latency to enter the target quadrant

This was done on 96th day, twenty four hours after the 10th session. Compared to control (C: 3.32 ± 0 .76seconds), rats exposed to stress along with high fat diet (HCD+STS: 7.67 ± 0.77seconds) took significantly longer time ($p<0.001$) to reach the target quadrant (probe) than rats fed with high cholesterol diet alone (HCD: 4.66 ±0 .84

seconds, ($p<0.05$)**.** The above results imply that the two factors, stress and high cholesterol diet, given in combination causes substantial memory impairment. (Fig: 4)

Time spent in the target quadrant

Compared to control group (C: 24± 3.01) high cholesterol diet plus stress induced rats spent (HCD+STS: 10 ± 1.37seconds) significantly ($p<0.001$) lesser amount of time in the target quadrant (probe) in search of the missing platform. Similarly rats fed with only high cholesterol diet (HCD: 19 ±2.72seconds) showed significant ($p<0.05$) results but the time spent in the target quadrant (probe) is more than that of High cholesterol diet +stress induced group. This shows that both stress and high cholesterol diet when given in combination produces more memory deficits. (Fig: 5)

Cresyl violet staining: viable neurons in CA1 and CA3 of hippocampus

Compared to control group (C: 34± 1.47) the respective hippocampal CA1 and CA3 regions of High colesterol diet +stress group (HCD+STS: 28±1.33 & 18± 1.47) showed significant ($p < 0.001$) decrease in the number of viable neurons than only high cholesterol diet group (HCD: 31± 1.76, $p < 0.01$). This again reemphasizes the concept of both stress and high cholesterol diet together damage the sub regions of hippocampus and affects the learning behaviour and memory in rats. (Fig, 6, 7, 8, 9)

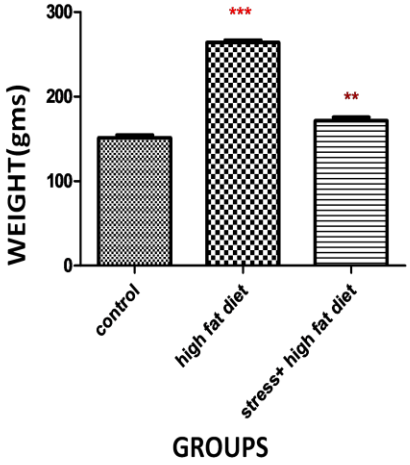

Figure 1: Bar graph showing the weight (gms)after three months. Bars represent mean ± SEM (control vs. HFD, ***$p < 0.001$; control vs. HFD+STS, **$p < 0.01$.

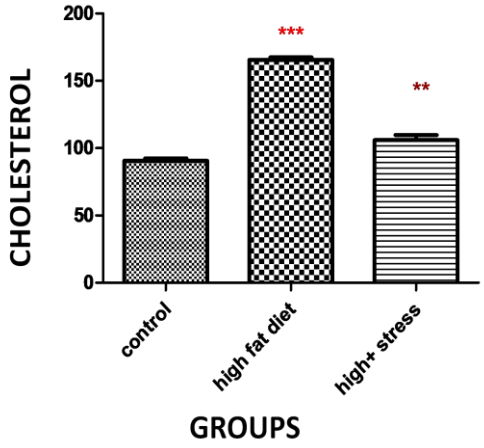

Figure 2: Bar graph showing the cholesterol (mg/dl). Bars represent mean ± SEM (control vs. HFD, ***$p < 0.001$; control vs. HFD+STS, **$p < 0.01$.

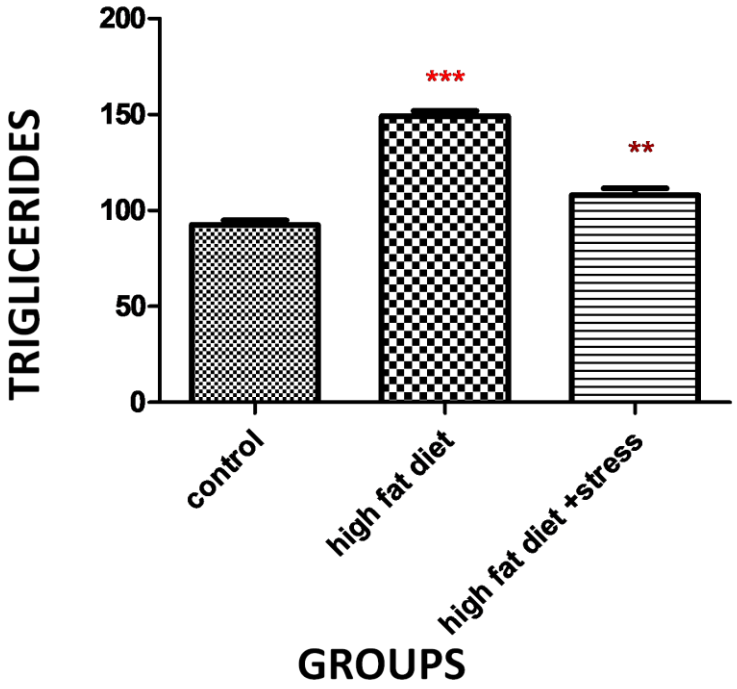

Figure 3: Bar graph showing the triglycerides(mg/dl). Bars represent mean ± SEM (control vs. HFD, ***$p < 0.001$; control vs. HFD+STS, **$p < 0.01$.

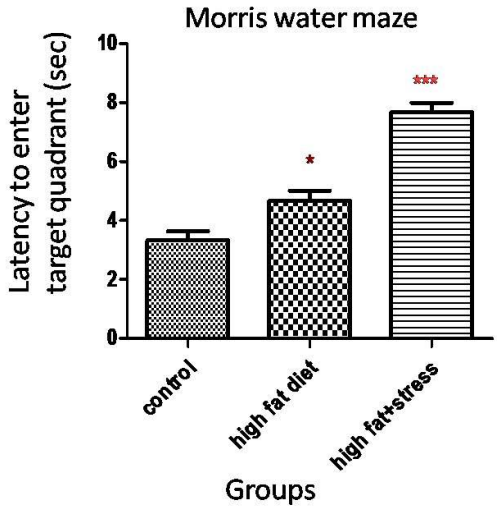

Figure 4: Bar graph showing the latency to enter target quadrant (sec). Bars represent mean ± SEM (control vs. HFD, *$p < 0.05$; control vs. HFD+STS, ***$p < 0.001$.

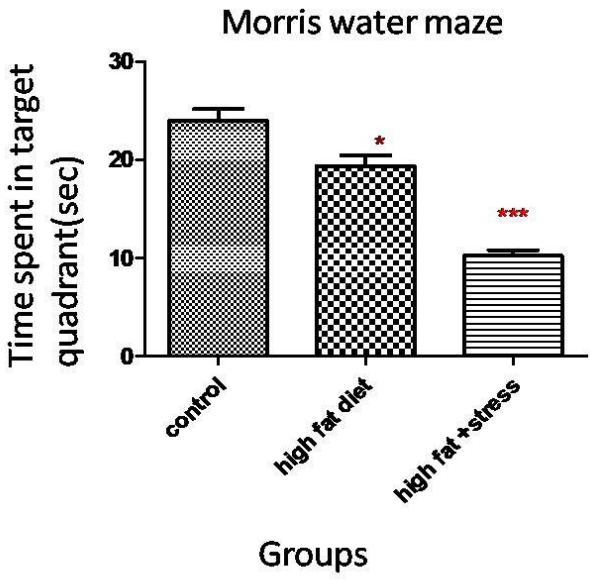

Figure: 5 Bar graph showing the time spent in enter target quadrant (sec). Bars represent mean ± SEM (control vs. HFD, *$p < 0.05$; control vs. HFD+STS, ***$p < 0.001$

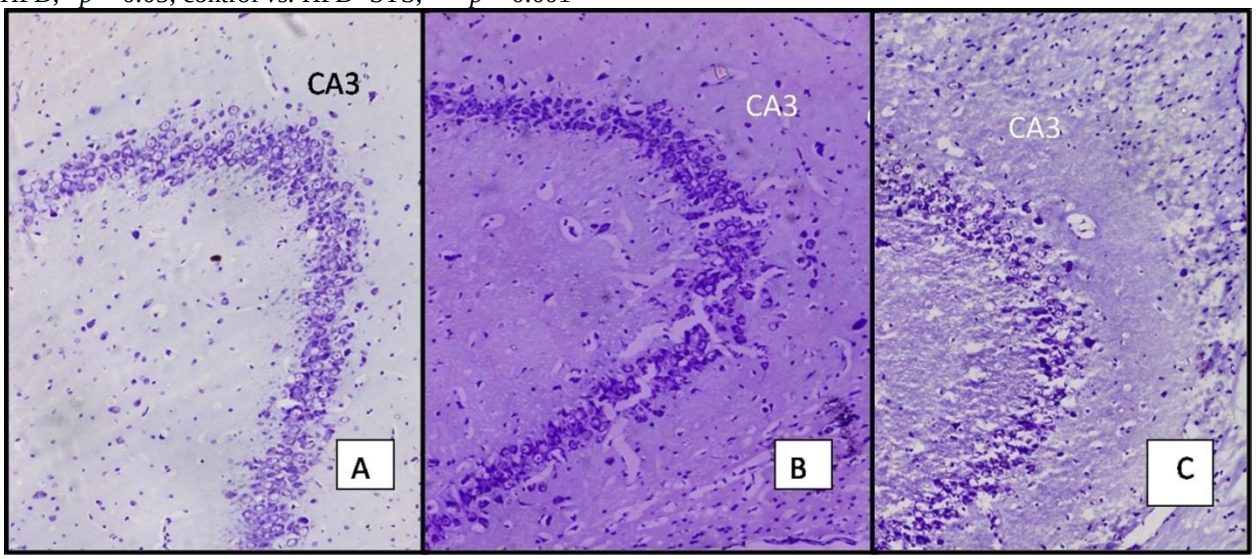

FIG 6 (Cresyl violet staining under 10X magnification. Coronal section of **CA3** region of the hippocampus A: control group, B: High Fat Diet, C: High Fat Diet + Stress.

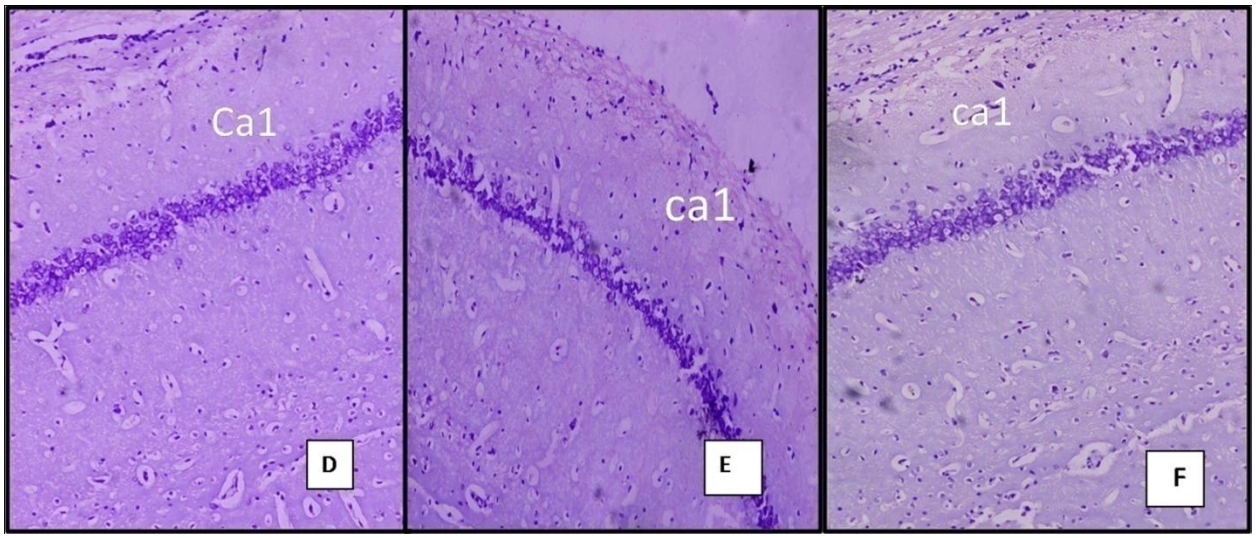

FIG 7 (Cresyl violet staining under 10X magnification). Coronal section of **CA1** region of the hippocampus D: control group, E: High Fat Diet group, F: High Fat Diet + Stress group.

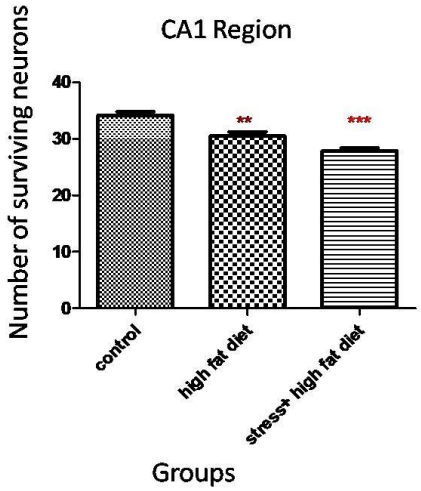

Figure 8: Bar graph showing the number of viable neurons in the CA1 region of hippocampus of rat brain. Bars represent mean ± SEM (control vs. HFD, $^{**}p < 0.01$; control vs. HFD + STS, $^{***}p < 0.001$.

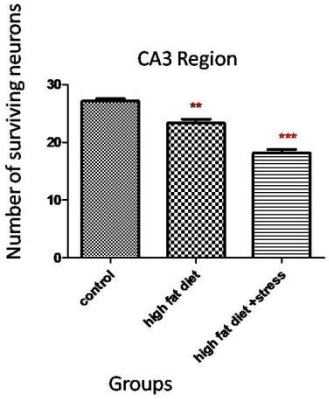

Figure 9: Bar graph showing the number of viable neurons in the CA1 region of hippocampus of rat brain. Bars represent mean ± SEM (control vs. HFD, $^{**}p < 0.01$; control vs. HFD + STS, $^{***}p < 0.001$.

DISCUSSION

The present study underlines the collective influence of consumption of cholesterol rich diet and stress on the CA1 and CA3 regions of hippocampus and spatial memory in male wistar rats.

Cholesterol is an essential component of the cell membranes' which plays an important role in the maintenance of the homeostasis and trans- membrane communication within and between cellular compartments. Cholesterol with in the brain is mainly synthesized by the neurons and astrocytes. Central nervous system contains 23% of the total cholesterol pool found in the whole body. Therefore brain is the cholesterol rich organ[13]. The role of high fat diet rich in cholesterol as susceptible factor for neurodegenerative disorders is still controversial, since cholesterol cannot pass through blood brain barrier so the exact mechanism of cholesterol rich diet affecting the brain is still elusive[14].

Few mechanisms were put forward to explain how plasma cholesterol causes neurodegenerative diseases. Sterols (27-hydroxy cholesterol or 24s-hydroxy cholesterol) derivatives of cholesterol pass through the blood brain barrier and cause increase in generation of free radicals and super oxide production, rats that were fed with high fat diet induced memory impairment and reduced hippocampal neurogenesis[15, 16].

Molteni, *etal*[17] reported that high fat diet along with refined sugar intake reduces brain derived neurotrophic factor (BDNF) in hippocampus. According to Lindqvist, *etal*[18] high fat diet impairs hippocampal neurogenesis through increased serum corticosterone levels in the brain. Ceineullrich, *etal*[19] stated that high fat diet impairs the cholinergic system in rats which in turn leads to memory deficit. Thirumangalakudi L, *etal*[20]observed the hypercholesterolemia associated increase in inflammatory markers in the brain milieu.

Few epidemiological studies state that increased serum cholesterol is a risk factor for development of dementias (Alzheimer's disease old age)[21,22] but the study done on Japanese-American population by Kalmijin, et al[23] did not find any relationship between serum cholesterol and dementia in old age population. Pre-clinical studies have shown that dietary cholesterol can be the cause for neurodegenerative diseases and dementia. In animal studies, rats that were fed with high fat diet performed poorly on various cognitive Tests[24, 25] but these findings were different from those reported by Anastasia, et al[26] in which high fat diet did not cause any cognitive deficits in the rats. This change in result could be due to the period for which the animals were fed with high fat diet (three months) or type of diet given i.e. pure cholesterol powder was was mixed in the coconut oil and given in our experiment.

· Present study, shows that diet rich in cholesterol resulted in memory impairment and affected CA1 and CA3 sub regions of hippocampus.

Stress is one of the major causes in many neurological disorders. Stress activates the hypothalamo-pituitary adrenal (HPA) axis, which results in the release of CRH (corticotroph releasing hormone), ACTH from pituitary and glucocorticoids from adrenal cortex. [27.] Mason JW, etal[28]has demonstrated that hippocampus has a major role to play on HPA axis modulation. Lesions of hippocampus tend to increase the HPA activity especially as a consequence of stress.

Adrenal steroids, in particular, Glucocorticoids (corticosterone) play an important role in decreasing the hippocampal plasticity .Sapolsky *etal*[29] reported that hippocampus is damaged with prolonged exposure to glucocorticoids. In contrast IritAkirav, etal and McLaughlin,*etal* observed Corticosterone and chronic stress enhanced the spatial memory and did not affect the sub regions of hippocampus[30, 31.]The difference in these findings might be due to the variations in the duration of stress and type of stress given, since shorter duration of stress period may serve as an adaptive function while longer duration can cause disruption of neurons in the hippocampal region.

CONCLUSION

The results of the present study are in line with the previous studies that support the hypothesis that stress and high fat diet are one of the causes for neurodegenerative diseases. This study can be useful in understanding the complications of stress and diet on hippocampal sub regions.

Further investigations are required to delve deeper into the causality between cholesterol alteration and brain disorders. A better comprehension of the basic molecular mechanisms by which imbalance in cholesterol homeostasis is linked to neurological disorders could provide useful information for designing novel and effective therapeutic approaches.

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