Final Report

Title of the Project: Hormetic effects of dietary restriction on preservation of cytoarchitecture of mouse cortical and hippocampal neurons to youthful conditions during ageing

Name of the Principal Investigator: Dr. A. A. Deshmukh

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Objectives: To study the hormetic effects of food restriction on mouse cortical and hippocampal neurons with respect to

A. Accumulation of lipofuscin granules

B. Deposition of neurofibrillary tangles

C. Deposition of senile plaques

D. Distribution of Nissl substance

E. Lysosomal membrane integrity

Brain is the main part of the central nervous system which controls the functioning of the other body organs. It requires about 20% of total consumed oxygen. 1-2% of oxygen consumed is converted in to Reactive Oxygen Species (ROS) during the oxidative phosphorylation process. In the brain this percentage is increased due to higher oxygen consumption rate. Butterfield *et al.*, (2002) suggested that the biochemical composition of the neurons make them vulnerable to ROS. Neurons contain polyunsaturated fatty acids which are the targets of ROS. Being post mitotic cells, over the period of time the damage worsens that ultimately gets reflected in loss of memory and cognitive functions. Hence in the present investigation brain was selected. Instead of selecting whole brain for the study we selected the specific regions like Cerebral cortex and Hippocampus.

Animal care during studies:-

For the present work the male albino mice were used. The animals of age two months were purchased from the Haffkine Institute for Training, Research and Testing Mumbai. Animals were reared in the departmental animal house. They were under 12 hrs light and 12 hrs dark

cycles. Animals were supplied with fresh water and pelleted feed daily. As the present work is to study the effect of dietary restriction on brain ageing the animals were weighed weekly to ensure that there was no malnutrition of animals while giving the dietary restriction.

Method of Dietary restriction:-

During these studies the animals were grouped in to two groups i.e. control group and the experimental group (i.e. diet restricted group). The control group was fed *ad libitum* i.e. they were supplied with plenty of feed and water. The experimental group was given 40% less feed than the control group. The quantity of *ad libitum* feeding was calculated by substracting the weight of pellets remained in the cage on each day in the control animals from the original weight of pellets added divided by number of animals in the cage.

According to Ikeno *et al.*, (1996) in rat's 40% dietary restriction prolongs the neoplastic and non-neasoplastic diseases. As compared to 40% DR animals the *ad libitum* fed rats were more susceptible to diseases. Also, the studies of Gems and Partridge (2008), Rattan (2008) suggested that restricting the calories up to 20-40% increases the life span in all model organisms. Konno *et al.*, (1991) found that 40% dietary restriction reduces the tumor formation in mice treated with 3-methylcholanthrene.

Through these studies it is seen that 40% dietary restriction prevent the diseased conditions and prolongs the life in many animal models. Hence for the present investigation 40% dietary restriction was selected.

Experimental design:-

The mice were divided in to three groups containing 18 animals in each group.

A. Control group which was fed *ad libitum* (ALF).

B. Experimental group:- The dietary restriction was carried out in two regimens.

i. Dietary restriction after attaining sexual maturity (DRASM):- i. e. after 3 months of age. In mice sexual maturity is attained after 3 months of age. Till attaining the sexual maturity, it is the phase of growth. Therefore, dietary restriction was commenced after three months of age. This age simulates 18 years of age in humans.

ii. Dietary restriction after completion of essential life span (DRAELS):- i.e. after 10 months of age. Essential life span is nothing but the period in life to achieive Darwinian goal i.e. sexual reproduction and perpetuation of species. In mouse, it is 10 months of age, which

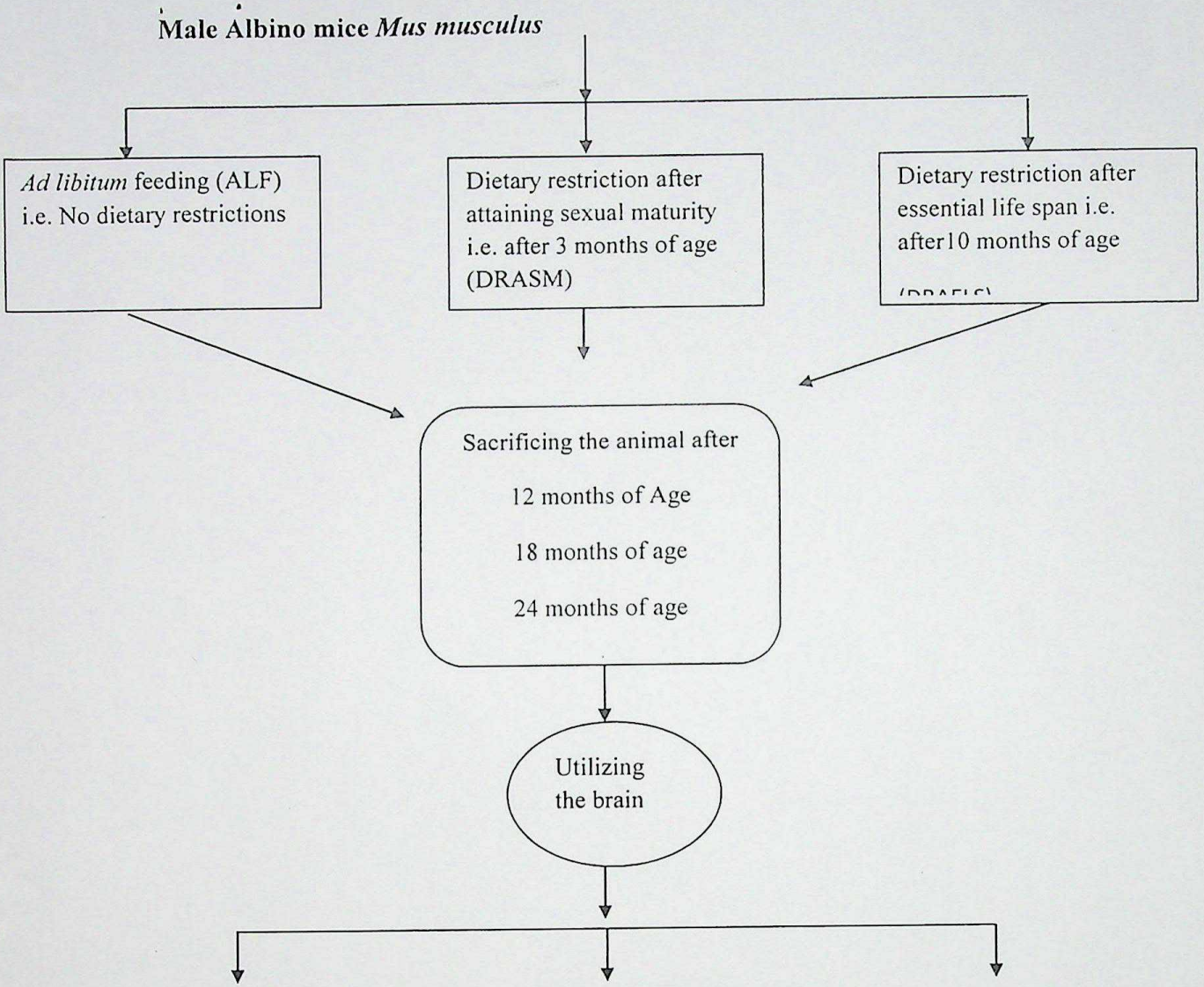
simulates to 40 years of age in humans. Therefore in this group dietary restriction was commenced after 10 months of age.

Table: - Showing the schedule of experimental work

Sr. no	Groups	Dietary restriction after		Age of animals on which animals were sacrificed
		3 months of age	10 months of age	
1	Experimental group	✓	✓	12 months of age 18 months of age 24 months of age
2	Control group	-------	--------	

Six animals from each group were sacrificed by cervical dislocation after 12, 18 and 24 months of age. The brain was excised immediately on prechilled platform at cold temperature. The longitudinal half part of the brain was fixed in 10% formalin for the histochemical studies and remaining half part was dissected to separate the specific regions like Cerebral cortex and Hippocampus for the biochemical studies.

Design of work carried out during the present investigation:-



Histochemical study:-

For the histochemical studies paraffin embedded blocks of brain were prepared.

The longitudinal half of the brain was fixed in 10% Neutral buffered formalin at 4^0C; for 24 hrs. There after it was kept under running tap water for 24 hrs. After washing it was

dehydrated through ascending alcohol grades i.e. 30%, 50%, 70%, 90% and Absolute alcohol for 2hrs each. Then dehydrated brain was transferred in xylene for 10 min and then transferred to melted paraffin for hot impregnation at 60^0c for 90 min. After completion of impregnation blocks were prepared. Each block was trimmed and cut in to sections of 7µm for the histochemical studies.

During present investigation the histochemical studies were carried out to study the deposition of neurofibrillary tangles, accumulation of lipofuscin granules, deposition of β amyloid, and the Nissl substance.

Histochemical demonstration of Neurofibrillary Tangles (Cobb and Bielschowsky's Silver staining method 1925).

The histochemical studies of Neurofibrillary tangles (NFTs) were carried out by Cobb and Bielschowsky's silver staining method. The neufibrillary tangles are generated intraneuronally during ageing. They are formed due to abnormal phosphorylation of microtubule associated protein tau by various kinases. Tau is important for stabilizing the microtubule assembly in the neurons.

Material:- Silver nitrate, Ammonium hydroxide, Formaldehyde, Citric acid, Nitric acid, Sodium thiosulfate , Distilled water etc.

Solutions and Reagents preparation:

10% Silver Nitrate Stock Solution:

Silver nitrate ---------- 5 g

Distilled water ---------- 50 ml

1% Ammonium Hydroxide Solution:

Ammonium hydroxide, concentrated ---------- 1 ml

Distilled water ---------- 100 ml

Developer Stock Solution (make fresh):

37-40% Formaldehyde ---------- 20 ml

Citric acid ---------- ----- ---------- 0.5 g

Nitric acid, concentrated ---------- 2 drops

Distilled water ---------- ---100 ml

Developer Working Solution (prepared freshly immediate before use, and discarded after use):

Developer Stock Solution ---------- 8 drops

Ammonium hydroxide, concentrated --------- 8 drops

Distilled water ---------- 50 ml

5% Sodium Thiosulfate (HYPO):

Sodium Thiosulfate ---------- 5 g

Distilled water ---------- 100 ml

Procedure:

1. Sections were deparaffinized in xylene and hydrated through descending grades of alcohol and finally to distilled water.
2. The slides were placed in pre-warmed (40 °C) 10% silver nitrate solution for 15 minutes sections turned light brown in color.
3. Slides were washed with distilled water for 3 times.
4. To the silver nitrate solution, concentrated ammonium hydroxide solution was added drop by drop until the precipitate was formed and just cleared.
5. Slides were placed back in this ammonium silver solution and incubated at 40 °C for 30 minutes or until sections became dark brown.
6. Later slides were kept directly in developer working solution for one minute.
7. Sections were treated with 1% ammonium hydroxide solution for 1 minute to stop the silver reaction.
8. Further sections were washed in distilled water three times.
9. The sections were placed in 5 % sodium thiosulfate solution for 5 minutes and washed three times with distilled water, and finally dehydrated through ascending grades of alcohol and cleared in xylene and mounted in D.P.X.

Histochemical demonstration of Lipofuscin granules (Zeihl-Neelson corbol fuscin method, Troyer 1980)

The lipofuscin is an autoflurescent yellowish brown to reddish brown pigment. It is present in all types of cells. It is undigested waste within the secondary lysosomes containing lipids and lipoproteins. The oxidation of these molecules occurs slowly so the pigment exhibit variation in staining reaction and its shape and size.

Material: - Brain tissue, alcohol grades, Basic fuscin, Phenol crystals etc.

Preparation of carbol fuchsin stain:

Basic fuchsin ---------- 1 gm

Phenol ---------- 5 gm

Ethanol ----------10 ml

Distilled water ---------- 100 ml

Basic fuscin, phenol and ethanol were added in distilled water stirred well and filtered through the filter paper to remove the dust particles if any.

Staining Procedure:

1. The sections were dewaxed in xylene for 30 min.
2. The dewaxed sections were hydrated through descending grades of alcohol (Absolute alcohol, 90%, 70%, 50%, 30%) and finally brought to distilled water.
3. Sections were placed in carbol fuchsin stain for 3 hrs. at 600c maintained oven and were washed under running tap water
4. Later sections were differentiated in acid alcohol (1% HCl in 70% ethanol), until excess of stain was washed off and counter stained lightly with 0.5% toluidine blue for 10 min.
6. Further slides were washed under running tap water and dehydrated through ascending grades of alcohol (30%, 50%, 70%, 90% and Absolute alcohol) cleared in xylene and mounted in D.P.X.

Histochemical demonstration of Senile plaques (Amyloid β) by (Highmans congo red method described Puchtler *et al.*, 1962):-

The deposition of senile plaque was studied histochemically by modified Highmans Congo red method described by Puchtler *et al.*, (1962).

Amyloid is an extracellular deposition of protein and mucopolysacharide complexes mostly occuring in ageing tissues of animals. It is formed mainly due to abnormal cleavage of amyloid precursor protein present in the cell membrane.

Material:- Congo red, Alcohol, Haematoxylin, Sodium hydroxide, Distilled water. etc.

Preparation of Congo red stain:-

Sodium chloride stock solution saturated:-

Sodium chloride	300 gm
Distilled water	200 ml

Mixed well, and then 800 ml ethanol was added.

Working solution of sodium chloride

Stock sodium chloride	50 ml
1% sodium hydroxide	0.5 ml

Congo red stock solution saturated:-

Congo red stain	1.0 gm
Stock sodium chloride	500 ml

Working solution for Congo red stain:-

Working solution was prepared freshly by adding 0.5 ml of 1% sodium hydroxide in to 50 ml of stock Congo red solution.

Procedure:

1. Sections were deparaffinized for 30 minutes in xylene
2. Hydrated with descending grades of alcohol i.e. Absolute alcohol, 90%, 70%, 50%, 30% alcohol and lastly in distilled water for 5 min each.
3. Sections were stained with haematoxylin for 10 min.
4. Rinsed in distilled water for 5 min.
5. Later sections were treated with solution containing 0.5 ml of 1% aq. Sodium hydroxide in 50 ml of 80% ethanol saturated with NaCl for 20 min.
6. Sections were stained in solution containing 50 ml of 80% ethanol saturated with Congo red and NaCl containing 0.5 ml of 1% aq.sodium hydroxide for 20 min.
7. Dehydrated rapidly in 90% and Ab. Alcohol.
8. Cleared in xylene and mounted in D.P.X

Histochemical demonstration of Nissl substance by Nissl staining method (Powers and Clark 1955):-

Principle:-

Neurons contain Nissl substance, which is primarily composed of rough endoplasmic reticulum. Its content and distribution varies in different types of neurons. Nissl substance is basophilic in nature because of the RNA content, and gets very sharply stained with basic aniline dyes. By varying the pH and the degree of differentiation, both Nissl substance and nuclei or only Nissl substance may be demonstrated.

Preparation of Solution:

Cresyl violet:

Cresyl violet-acetate ---------- 0.2 g

Distilled water --- ----------150.0 ml.

Buffer solution (0.1M, pH 3.5):

0.1M acetic acid --- 94.0ml

0.1M Sodium acetate ---- 6.0 ml

Working solution:

Buffer solution ---------- 100.0 ml

Cresyl violet solution---------- 6-12 ml

Procedure:

1. Sections were deparaffinized and hydrated to distilled water through descending grades of alcohol.
2. Sections were stained for 20 minutes with working solution and then rinsed quickly in 70% ethyl alcohol and 95% ethyl alcohol, dehydrated in isopropyl alcohol, 2 changes of 3-4 minutes each.
3. Finally cleared in xylene and mounted in D.P.X.

Biochemical studies:-

After fixing the longitudinal half part of the brain for histochemical studies in 10% neutral formalin the remaining half part of the brain was used for the biochemical studies. The brain regions like Cerebral cortex and Hippocampus were separated on prechilled glass plate and used for the biochemical assays.

Homogenate preparation:-

After separating the brain regions they were weighed separately and kept in the deep freezer for hardening. After hardening the regions were crushed with the help of chilled pestle and mortar and homogenates were prepared by using the respective homogenizing medium as per the enzyme assay.

Table: showing the homogenizing medium used for the enzyme assay.

Sr.no.	Parameter	Homogenizing medium
1	Lipid peroxidation	Reaction mixture i.e. 75mM potassium phosphate buffer (pH 7.04) containing 1mM ascorbic acid, 1mM $FeCl_3$
2.	Acid phosphatase	10 mM Tris-acetate containing 1mM EDTA (tris EDTA buffer pH 7.2)

The homogenates required for the assay of acid phosphatase activity was subjected to cell fractionation. The enzyme activity was assayed in the post lysosomal and lysosomal fraction to study the lysosomal membrane integrity.

During cell fractionation the homogenates were subjected to cold centrifugation at 1500 rpm for 10 min. at 10^0C. At this speed the nuclei and unbroken cells settled in the pellet. Pellet was discarded and supernatant (the post nuclear fraction) was centrifuged 12,000 rpm for 20 min. at 10^0C. The supernatant was used for the estimation of microsomal i.e. post lysosomal acid phosphatase activity and pellet was resuspended in 0.8ml distilled water and 0.2 ml Triton X 100 (10%) and kept at 10^0C for 20 min to rupture the lysosomes. Triton X 100 being an anionic detergent, the lysosomes get burst in it and lysosomal enzymes were released in the solution. Mixture is again centrifuged for 20 min. at 10,000 rpm. The supernatant was the lysosomal fraction which was used for the assay of lysosomal activity.

Measurement of Lipid peroxidation (Wills 1966):-

Principle: - Malondialdehyde (MDA) is the end product of lipid peroxidation in the tissue. One molecule of MDA reacts with two molecules of thiobarbituric acid (TBA) to produce red colored product malondialdehyde.

Reagents:-

Ascorbic acid (1mM)

Potassium phosphate buffer pH 7.04 (75mM)

$FeCl_3$ (1mM)

Trichloroacetic acid 20%

Thiobarbituric acid (TCA) 0.67% (W/V)

Preparation of reaction mixture (prepared freshly)

Potassium phosphate buffer pH 7.04 (75mM) + Ascorbic acid (1mM) + $FeCl_3$ (1mM).

Procedure:-

1. The tissue homogenate was prepared in reaction mixture.
2. Aliquots of 0.2 ml of homogenate were added to 1.8 ml of distilled water.
3. 1 ml of 20% TCA and 2 ml of 0.67% TBA were added and tubes were placed in boliling water bath for 10 min.
4. All tubes were cooled.
5. The absorbance was recorded at 532 nm. Blank was prepared by adding 2 ml D.W. 1 ml TCA (20%), 2 ml TBA (0.67%).
   Zero absorbance was set by control.

Formula:-

$$\text{Lipid peroxidation/ mg tissue} = \frac{\text{O.D. of sample}}{0.156 \times \text{amount of tissue in mg in 0.2 ml sample}}$$

Where, 0.156 = the absorbance for 1 nM solution of malondialdehyde in a 1cm thick cell at 532nm.

0.2 ml of sample contains 0.4 mg tissue

Estimation of Acid Phosphatase (Linhardt and Walter 1965)

Acid phosphatases (APs) family of enzymes that are widespread in nature. This enzyme is present in many animal and plant species. The biochemical assay of acid phosphatase was carried out by the method described by Linhardt and Walter (1965).

Principle:

Acid phosphatase is an enzyme, which cleaves phosphate group from substrate (*P*- nitro phenyl phosphate) at acidic pH 4.8 at 37^0C.This results in yellow colored product (*p*-nitrophenol).

Reagents:

1. Sodium citrate buffer 0.05 M (pH 4.8) containing 5.5×10^{-3} M P- nitrophenyl phosphate
2. 0.1 N NaOH

Procedure:

Aliquots of 0.2 ml sample were added in 0.8 ml of 0.05 M citrate buffer pH 4.8 containing 5.5×10^{-3} .M *P*- nitrophenylphosphate, incubated at 37^0C for 30 min. Reaction was inhibited by addition of 4 ml 0.1 N NaOH. Absorbance of the mixture was measured at 405 nm by setting the zero with blank tube which was devoid of sample.

Calculations:-

$$\text{Acid phosphatase activity in terms of µmols of p-nitrophenol/mg protein} = \frac{\text{Optical density} \times 2.76 \times 1000}{\text{Amount of protein/ mg tissue}}$$

Statistical analysis:- The results were the mean values of 6 animals from each group. The data was analyzed by statistically using unpaired student t test.

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}}$$

Where,

X1 = Mean of control group

X2 = Mean of experimental group

S1 = Standard deviation of control group

S2 = Standard deviation of experimental group

n1 = Total number of animals from control group

n2 = Total number of animals from experimental group

The level of significance was calculated by knowing the p values.

$p > 0.05$ the difference is not significant

$p < 0.05$ the difference is said to be statistically significant

$p < 0.01$ the difference is said to be moderately significant

$p < 0.001$ the difference is said to be highly significant

LIST OF CHEMICALS

Chemical	Company	Batch No.
Basic fuchsin	Sd fine chemicals	K06/1006/0805/71
Phenol crystals	Molychem	MCR-6058
Hydrochloric acid	Thomas baker	PK/12/3416/1270-12/13/16
Toludine blue	Himedia	92-31-9
xylene	Sd fine chemicals	I11A/0811/1708/25
Formaldehyde	Merck	AJof 600883
Sodium diphosphate orthophosphate	Molychem	MCR-4780
Sodium phosphate dibasic	Sd fine chemicals	I12A/1411/1709/31
Silver nitrate	Sd fine chemicals	L12A/0812/2211/21
Ammonium hydroxide	Sd fine chemicals	K12A/0912/0111/13
Citric acid	Sd fine chemicals	K02Y/0802/1410/21
Nitric acid	Thomas baker	PG/12/296/375-12/13116
Sodium tiosulphate	Sd fine chemicals	I12A/1812/0109/13
Congo red	Sd fine chemicals	E112/1410/3010/74
Haematoxylin	Himedia	517-28-2
Sodium hydroxide	Merck	MCR-4861
Sodium chloride	Merck	MB 1B602595
Cresyl violet acetate	Sigma Aldrich	10510-540
Acetic acid glacial	Sd fine chemicals	K12A/0912/2910/21
Sodium acetate	Sd fine chemicals	0698/1298/3105/21
Isopropyl alcohol	Sd fine chemicals	K11A/0711/2107/31
Tris amino methane	Sd fine chemicals	C03X/1003/2602/31
Ethylene diamine tetra acetic acid	Thomas baker	PL/12/3732/1398-12/13/01
Potassiumdihydrogen orthophosphate	Sd fine chemicals	AO72/3006/2812/08
Potassium phosphate dibasic	Merck	MCR-1152
Tri-sodium citrate	Qualigens	NL 3151 6109 S
p-nitrophenylphospate	Himedia	2-1299
Sodium carbonate	Merck	MLOM603798
Copper sulphate	Sd fine chemicals	J12A/3912/1509/21
Potassium sodium L(+) tartrate	Sd fine chemicals	D12A/2112/2703/21
Phenol reagent	Qualigens	48/20/21/22, 68
Bovine serum albumin	Himedia	9048-46-8
L-Ascorbic acid	Sd fine chemicals	L12A/1812/0711/62
Ferric chloride	Sd fine chemicals	A13A/1013/0301/13
Trichloroacetic acid	Sd fine chemicals	D12A/0112/1403/13
Thiobarbituric acid	Sigma Aldrich	504-17-6
Triton X 100	Sd fine chemicals	G06A/0406/1907/13

Results:

A. Study of lipofuscin granules

Effect of dietary restriction on the accumulation of lipofuscin granules in the neurons of Cerebral cortex and hippocampus of mice brain.

Lipofuscin is a yellowish brown, autoflurescent age pigment. Main components of lipofuscin are undigested proteins and lipids accumulated within the lysosomes. Lipofuscin granules get accumulated progressively in the post mitotic cells such as neurons, cardiac myocytes and retinal pigment epithelial cells. This affects the autophagoctotic capacity and alters the cellular recycling mechanism. The lipofuscin loaded lysosomes become susceptible to oxidative damage which in turn affects the lysosomal membrane integrity. The damage to lysosomal membrane results in to release of lysosomal content into cytosol causing cell death resulting in to degeneration of cells during ageing process (Sulzer *et al.*, 2008).

Lipofuscinogenesis in Cerebral cortex and Hippocampus of 12 month old mice:- The observations are presented in the Table No.1 (Graph No. 1, 2)

In the neurons of cerebral cortex of 12 month old *ad libitum* fed mice 4.14 ± 0.023 lipofuscin granules per neuronal cell were observed. In the mice subjected to 40% dietary restriction after 3 months of age 1.54 ± 0.023 lipofuscin granules were observed per neuron. As compared to values of *ad libitum* fed mice 2.68 fold lower accumulation of lipofuscin granules was observed. This difference was highly significant ($p<0.001$). In the mice subjected to 40% dietary restriction after 10 months of age 2.37 ± 0.061 lipofuscin granules per neuron were observed. Comparison of these results with *ad libitum* fed mice suggests 1.74 fold less accumulation of lipofuscin granules. This difference was highly significant ($p<0.001$).

Similarly, comparison between both the experimental groups (i.e. animals subjected to dietary restriction after 3 months of age and 10 months of age) the animals subjected to dietary restriction after 3 months of age showed 1.53 fold lower accumulation of lipofuscin granules than the animals subjected to dietary restriction after 10 months of age. **(Plate No. 1. Fig. 1, 2, 3).**

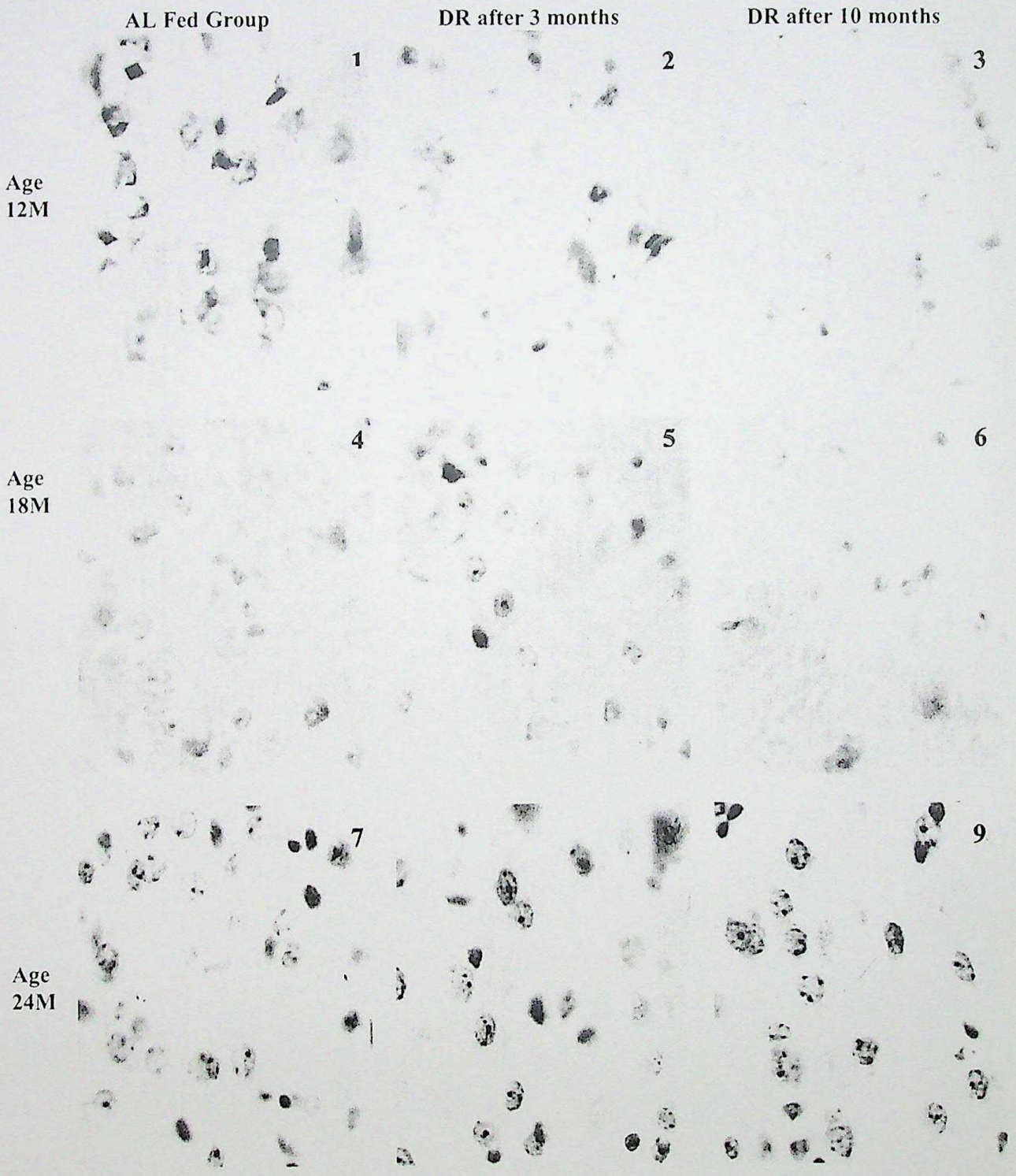
In case of hippocampus of *ad libitum* fed mice, 3.98 ± 0.023 lipofuscin granules per neuronal cell were observed. Whereas in mice subjected to dietary restriction after three months of age there was three fold lower accumulation of lipofuscin granules i.e.

Table No. 1:- Effect of dietary restriction on accumulation of Lipofuscin granules in Cerebral cortex and Hippocampus of mice.

Organ	Age of animals In months	Control group (*ad libitum* fed animals)	Experimental group (Dietaryrestriction after 3 months of age)	Experimental group (Dietaryrestriction after 10 months of age)
Cerebral cortex	12 months	4.14 ± 0.023	1.54 ± 0.023***	2.37 ± 0.061***
	18 months	4.57 ± 0.082	3.14 ± 0.052***	3.84 ± 0.065***
	24 months	6.34 ± 0.16	3.56 ± 0.17 ***	5.67 ± 0.02 ***
Hippocampus	12 months	3.98 ± 0.023	1.31 ± 0.023***	2.39 ± 0.023***
	18 months	4.6 ± 0.086	3.06 ± 0.044 ***	3.47 ± 0.038***
	24 months	6.31 ± 0.011	3.66 ± 0.09 ***	5.78 ± 0.01 ***

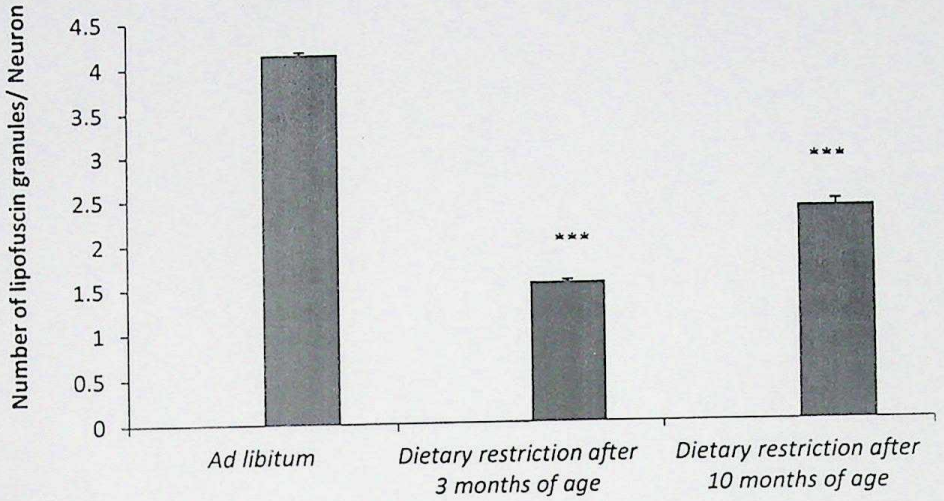
Results expressed as mean ± SD of 6 animals
***p<0.001= highly significant

PLATE NO: 1 Results of histochemical studies on Lipofuscin granules in Cerebral cortex of mouse brain (1000X)



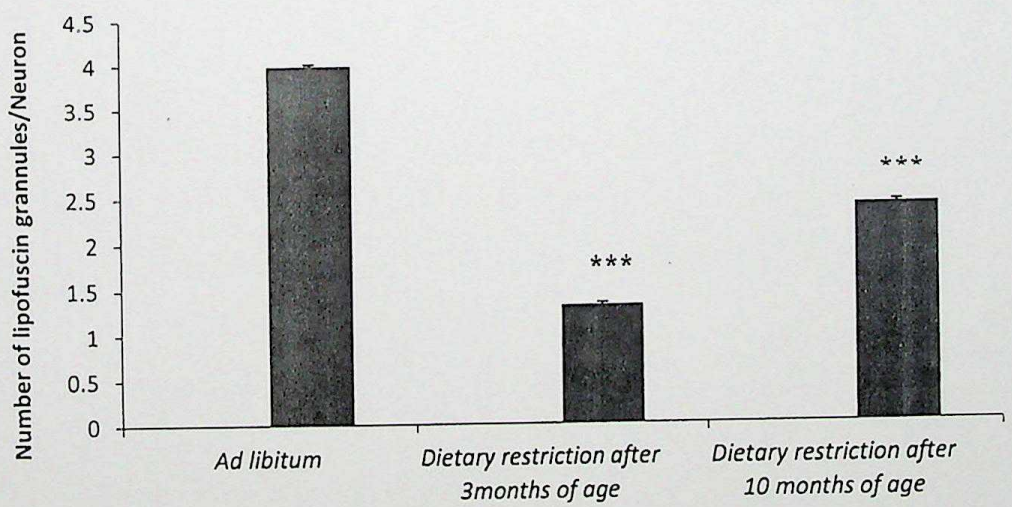
White arrow indicates Lipofuscin granules

Graph No. 1. Effect of dietary restriction on accumulation of lipofuscin granules in cortical neurons of 12 months old mice. (Average ± SD)



*** indicates p<0.001 i.e. highly significant

Graph No. 2. Effect of dietary restriction on accuumulation of lipofuscin granules in hippocampal neurons of 12 months old mice. (Average ± SD)



*** indicates p<0.001 i.e. highly significant

1.31± 0.023 per neuron. This depletion was highly significant ($p<0.001$). Mice subjected to dietary restriction after 10 months of age exhibited 2.39 ± 0.023 lipofuscin granules per neuron. This difference was highly significant ($p<0.001$). There was 1.67 fold lower accumulation of Lipofuscin granules as compared to *ad libitum* fed group.

Whereas, comparison between both the experimental groups showed 1.8 fold lower accumulation of lipofuscin granules in dietary restriction after 3 months of age than dietary restriction after 10 months of age. This difference was highly significant ($p<0.001$). **(Plate No. 2. Fig. 1, 2, 3)**

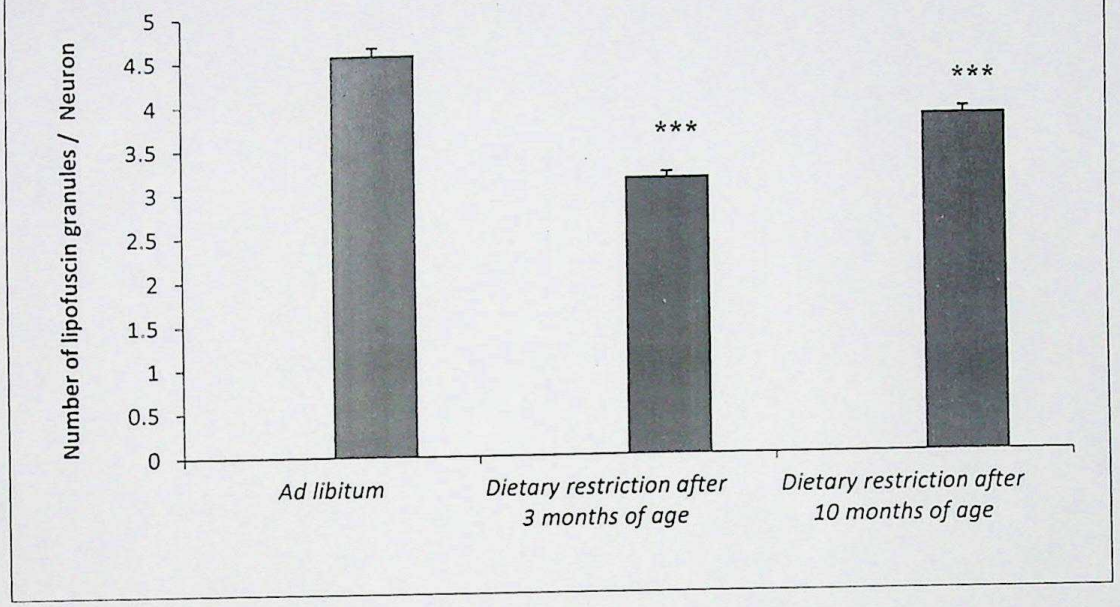
Lipofuscinogenesis in Cerebral cortex and Hippocampus of 18 month old mice:- The observations are presented in the Table No. 1. (Graph No. 3, 4)

In the cortical neurons of *ad libitum* fed mice of 18 months 4.57± 0.082 lipofuscin granules per neuron were observed. Whereas the mice subjected to 40% dietary restriction after 3 months of age showed 3.14 ± 0.052 lipofuscin granules per neuron. As compared to *ad libitum* fed mice 1.45 fold decrease in number of lipofuscin granules was observed. This decrease was highly significant ($p<0.001$). The mice subjected to 40% dietary restriction after 10 months of age showed 3.84 ± 0.065 lipofuscin granules. As compared to *ad libitum* fed mice, the accumulation of lipofuscin granules was lowered to 1.19 fold in this group. This decrease was highly significant ($p<0.001$). Similarly, comparison between both the experimental groups, suggest that dietary restriction after 3 months of age showed 1.22 fold decrease in the lipofuscin granules than to dietary restriction after 10 months of age. **(Plate No. 1. Fig. 4, 5, 6).**

In hippocampal neurons of *ad libitum* fed mice 4.6± 0.086 lipofuscin granules per neuron were observed. While the mice subjected to dietary restriction after three months of age showed 3.06 ± 0.044 lipofuscin granules per neuron. The comparison between these two groups indicated 1.5 fold lower number of lipofuscin granules and it was highly significant ($p<0.001$). The mice subjected to dietary restriction after 10 month of age showed 3.47 ± 0.038 lipofuscin granules per hippocampal neurons. This decrease was 1.32 fold as compared to *ad libitum* fed values. This decrease was highly significant ($p<0.001$).

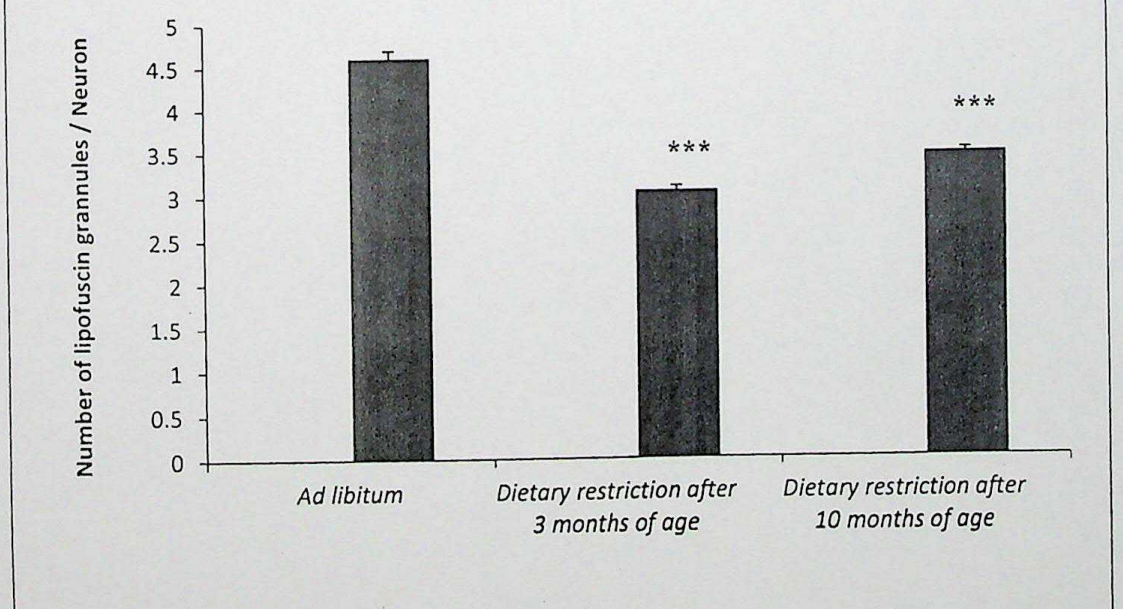
Whereas, the comparison between both the experimental groups shwos 1.13 fold less accumulation of lipofuscin granules in mice subjected to dietary restriction after 3 months of age than the mice which were subjected to dietary restriction after 10 months of age. This difference was highly significant ($p<0.001$). **(Plate No. 2. Fig. 4, 5, 6).**

Graph No. 3. Effect of dietary restriction on accumulation of lipofuscin grannules in cortical neurons of 18 months old mice . (Average ± SD)



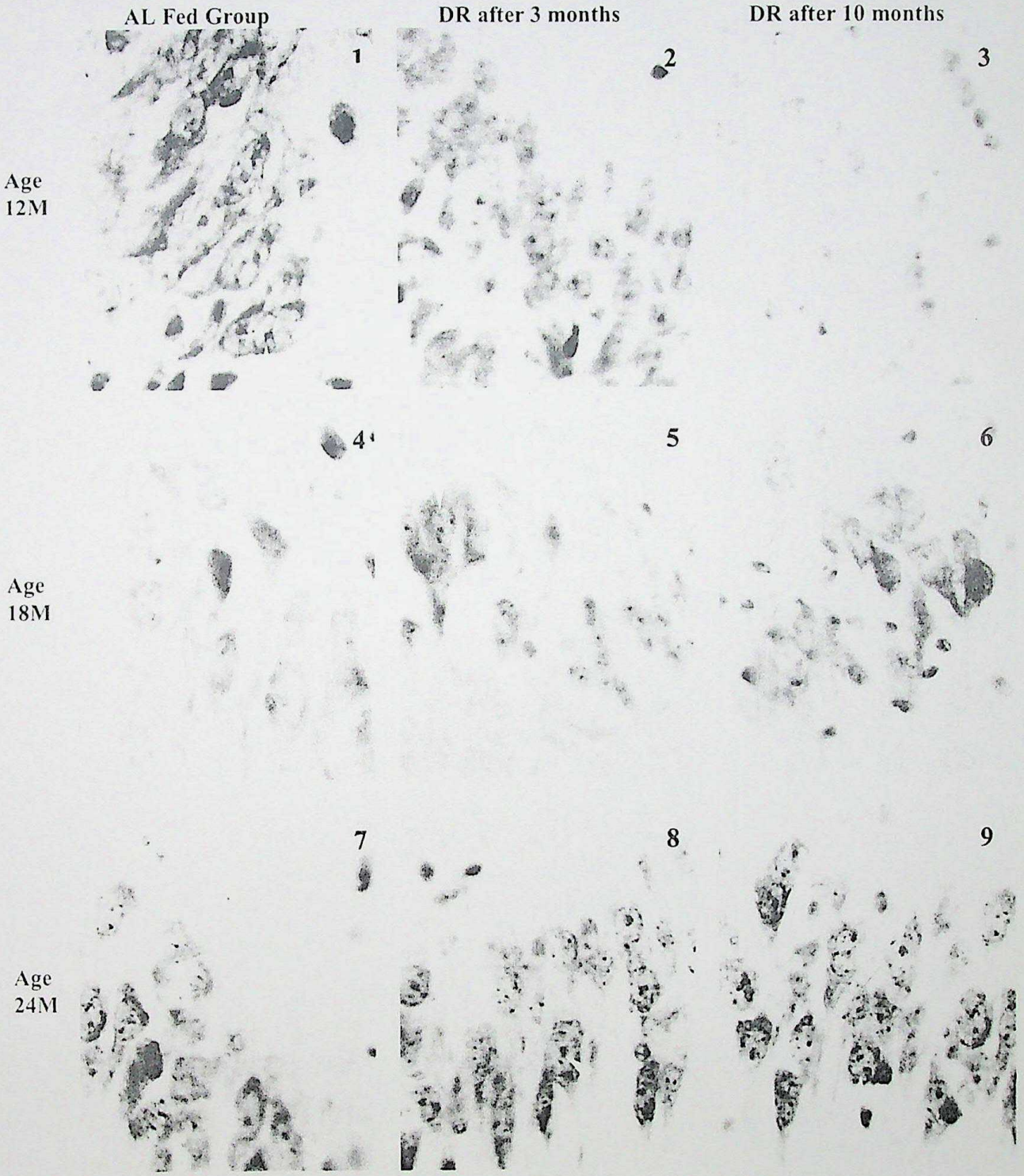
*** indicates p<0.001 i.e. highly significant

Graph No.4. Effect of dietary restriction on accumulation of lipofuscin grannules in hippocampal neurons of 18 months old mice. (Average± SD)

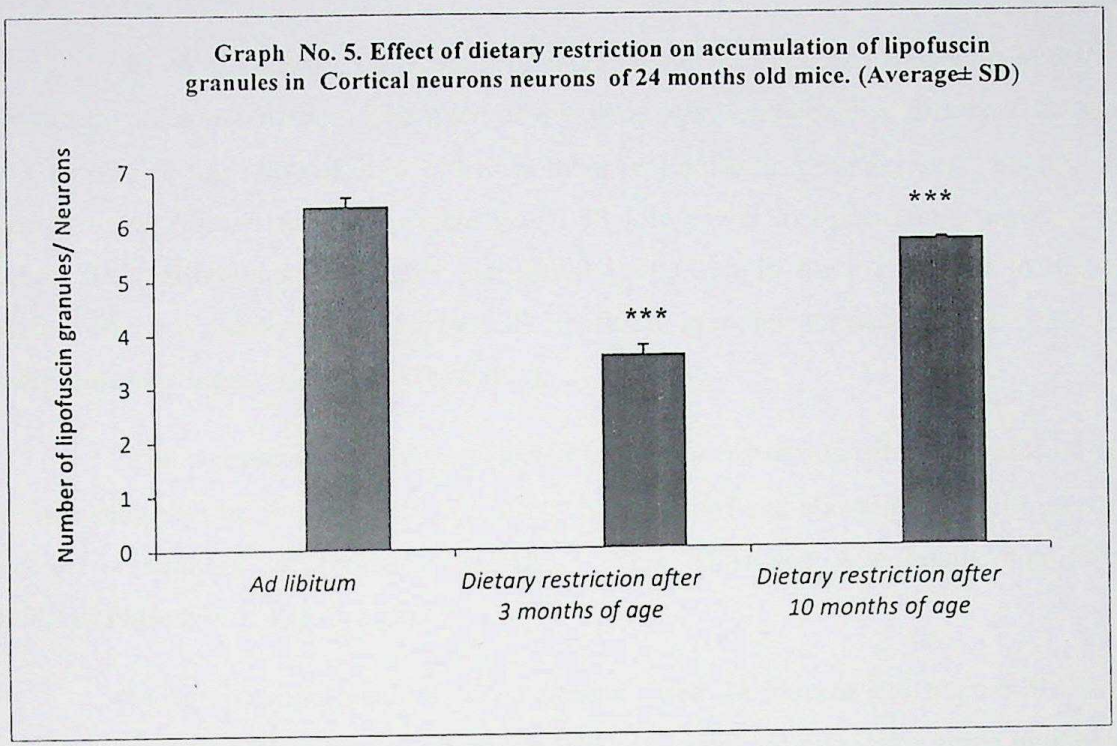


*** indicates p<0.001 i.e. highly significant

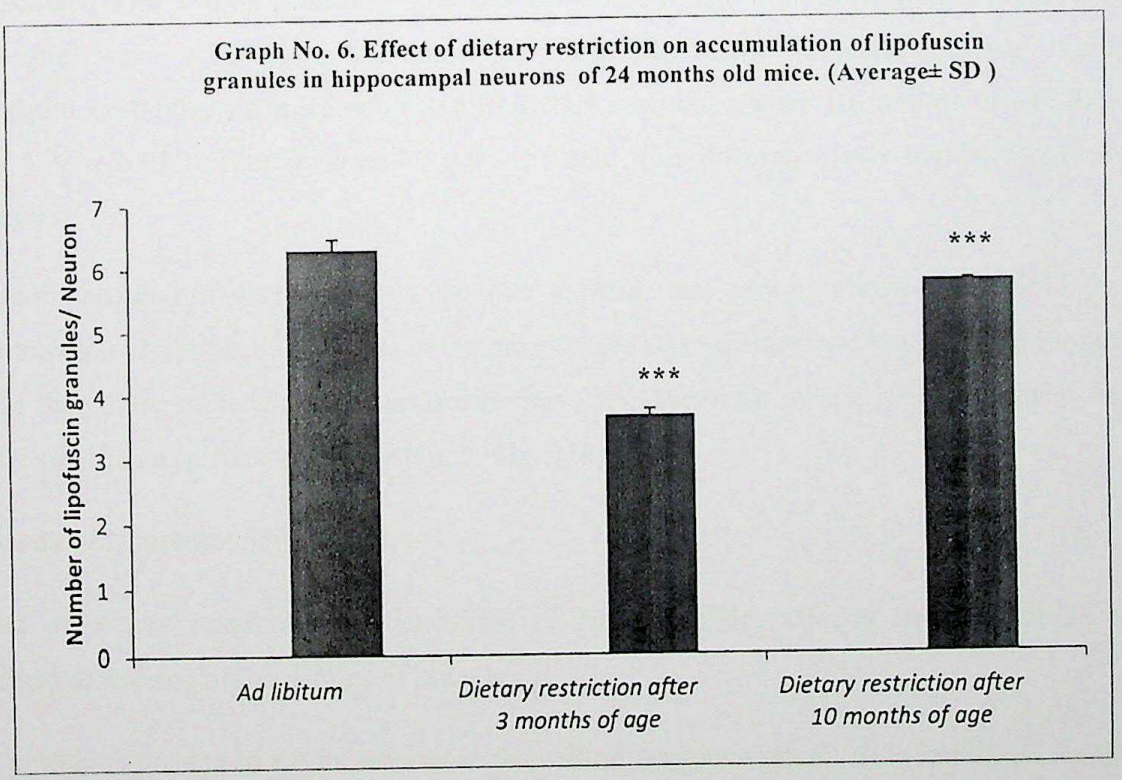
PLATE NO: 2 Results of histochemical studies on Lipofuscin granules in Hippocampus of mouse brain (1000X)



White arrow indicates Lipofuscin granules



*** indicates p<0.001 i.e. highly significant



*** indicates p<0.001 i.e. highly significant

Lipofuscinogenesis in Cerebral cortex and Hippocampus of 24 month old mice:-

The observations are presented in the Table No. 1 (Graph No. 5, 6)

In 24 months old *ad libitum* fed mice the cortical neurons showed 6.34 ± 0.16 lipofuscin granules per neuron. The mice of the same age subjected to dietary restriction after 3 months of age showed 3.56 ± 0.17 number of lipofuscin granules per neuron. As compared to *ad libitum* fed group there was 1.78 fold lower in lipofuscin granules were observed. This difference was highly significant ($p<0.001$). In mice subjected to dietary restriction after ten months of age 5.67 ± 0.02 lipofuscin granules per neuron were observed. This decrease was highly significant ($p<0.001$).

The comparison of mice subjected to dietary restriction after 3 months of age with mice subjected to dietary restriction after 10 months of age showed 1.59 fold reduced deposition of number of lipofuscin granules. This difference was highly significant ($P<0.001$). **(Plate No. 1. Fig. 7, 8, 9).**

In hippocampal neurons of *ad libitum* fed 24 months old mice 6.31± 0.11 lipofuscin granules per neuron were observed. The diet restricted mice after three months of age showed 3.66 ± 0.09 lipofuscin granules per neuron. The lipofuscin granule number in these mice was 1.72 fold lower than *ad libitum* fed mice. This difference was highly significant ($p<0.001$). In mice subjected to dietary restriction after 10 months of age there were 5.78 ± 0.01 lipofuscin granules per neuron. This difference was highly significant ($p<0.001$).

The comparison of values between the two experimental groups showed 1.57 fold less accumulation of lipofuscin granules in the mice subjected to dietary restriction after 3 months of age than those subjected to dietary restriction after 10 months of age. This difference was highly significant ($p<0.001$). **(Plate No. 2. Fig. 7, 8, 9).**

B. Study of Neurofibrillary tangles

Effect of dietary restriction on deposition of Neurofibrillary tangles in the neurons of Cerebral cortex and hippocampus of mice brain.

Tau protein is present in ample amount in the central nervous system. It is important for the microtubule assembly and stabilization. The abnormal modification of tau contributes to disruption of cytoskeleton and enhances its pathological aggregation in to Neurofibrillary tangles. (Lee *et al.*, 1988; Brion 1994)

Deposition of Neurofibrillary tangles in Cerebral cortex and Hippocampus of 12 month old mice:- The observations are presented in the Table No: 2. (Graph No. 7 and 8),

In cerebral cortex of *ad libitum* fed mice at 12 month age deposition of Neurofibrillary tangles (NFTs) was observed in 19.16 ± 2.48 % of neurons. Whereas, the animal subjected to dietary restriction after 3 months of age showed 4.46 ± 0.63% of neurons with NFTs. As compared to values of *ad libitum* fed group there was 4.02 fold less deposition of NFTs in mice subjected to dietary restriction after 3 months of age. This difference was highly significant ($p<0.001$). The animals subjected to dietary restriction after 10 months of age showed 17.08 ± 2.01 percent of neurons having deposition of NFTs. There was no any significant difference as compared to *ad libitum* fed group. **(Plate No. 3 Fig. 1, 2, 3).**

In case of hippocampus of 12 months old *ad libitum* fed mice 13.43 ± 0.87 % neurons were having deposition of NFTs. In mice subjected to 40% dietary restriction 3.7 ± 0.28 neurons were with NFTs. As compared to control group there was 3.62 fold lower depositions of NFTs in this group. This difference was highly significant ($p<0.001$). The other experimental group i.e. mice subjected to dietary restriction after 10 months of age showed 12.76 ± 1.88 % neurons having deposition of neurofibrillary tangles. As compared to control it showed similar number of neurons deposited with NFTs. **(Plate No. 4 Fig. 1, 2, 3).**

Deposition of Neurofibrillary tangles in Cerebral cortex and Hippocampus of 18 months old mice:- The observations are presented in the Table No. 2. (Graph no. 9 and 10),

The cerebral cortex of *ad libitum* fed mice showed 25.35 ± 1.67 % neurons deposited with NFTs. In mice subjected to dietary restriction after 3 months of age 9.21± 0.82 % neurons were deposited with NFTs. The number of NFTs deposited neurons was 2.75 fold lower than the *ad libitum* fed group. This difference was highly significant ($p<0.001$). The animals subjected to dietary restriction after 10 months of age showed 12 .11± 1.62 % of neurons with deposition of NFTs. As compared to *ad libitum* fed group 2.09 fold lower nuber of cells showing NFT deposition. This difference was highly significant ($p<0.001$). **(Plate No.3. Fig. 4, 5, 6).**

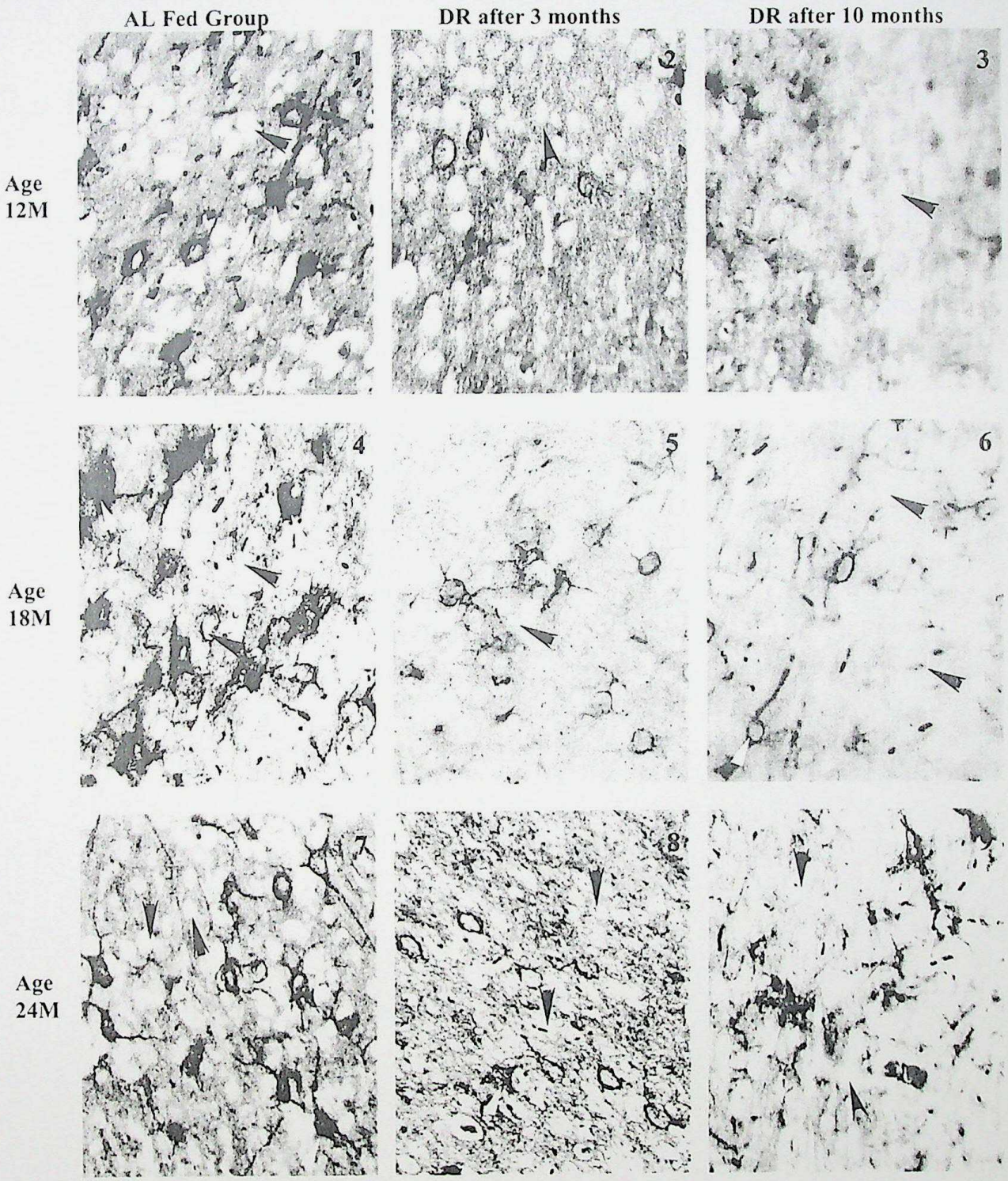
The hippocampal neurons of 18 months old *ad libitum* fed mice showed 14.55± 0.38 % neurons deposited with NFTs. In hippocampus of the same age group of mice subjected to dietary restriction after 3 months of age exhibited 4.72 ± 0.45 % neurons with NFTs deposition. As compared to values of *ad libitum* fed group three fold lower number of NFTs deposited neurons were observed. This difference was highly significant ($p<0.001$).

Table No.2:- Effect of dietary restriction on deposition of Neurofibrillary tangles in Cerebral cortex and Hippocampus of mice.

Organ	Age of animals In months	Control group (*ad libitum* fed animals) % of Neurons showing NFTs	Experimental group (Dietary restriction after 3 months of age) % of Neurons showing NFTs	Experimental group (Dietary restriction after 10 months of age) % of Neurons showing NFTs
Cerebral cortex	12months	19.16 ± 2.48	4.46 ± 0.63***	17.08 ± 2.01^{NS}
	18months	25.35 ± 1.67	9.21 ± 0.82***	12.11 ± 1.02***
	24months	30 ± 3.69	14 ± 3.78***	20.70 ± 2.76***
Hippocampus	12months	13.43 ± 0.87	3.7 ± 0.28***	12.76 ± 1.88^{NS}
	18months	14.55 ± 0.38	4.72 ± 0.45***	8.29 ± 0.93***
	24months	18.72 ± 2.74	4.83 ± 0.40***	12.61 ± 2.01***

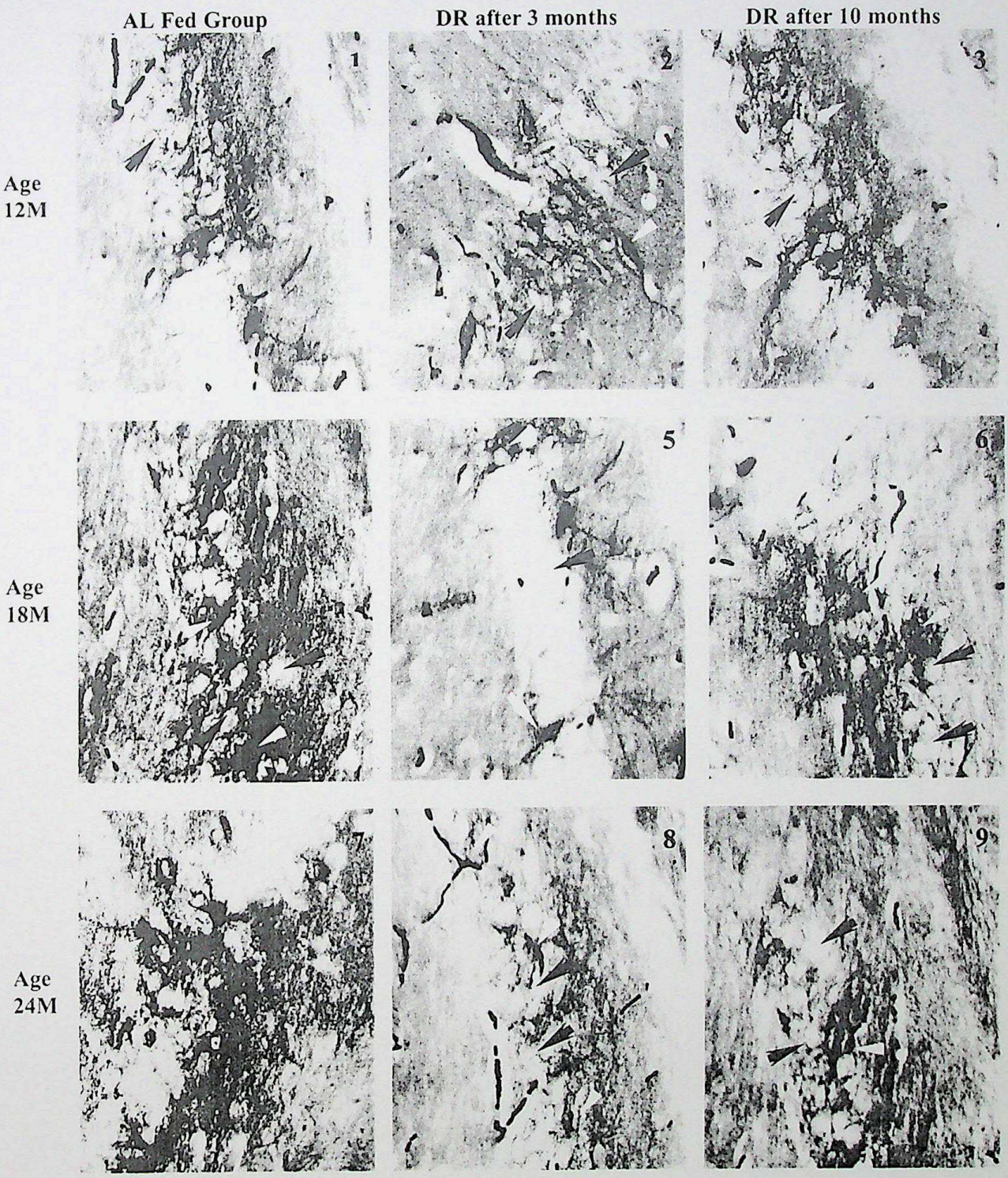
Results expressed as mean ± SD of 6 animals
*** $p<0.001$= highly significant
$p > 0.05$ NS= Non significant

PLATE: 3 Results of histochemical studies on neurofibrillary tangles (NFTs), in Cerebral cortex of mouse brain (400X)

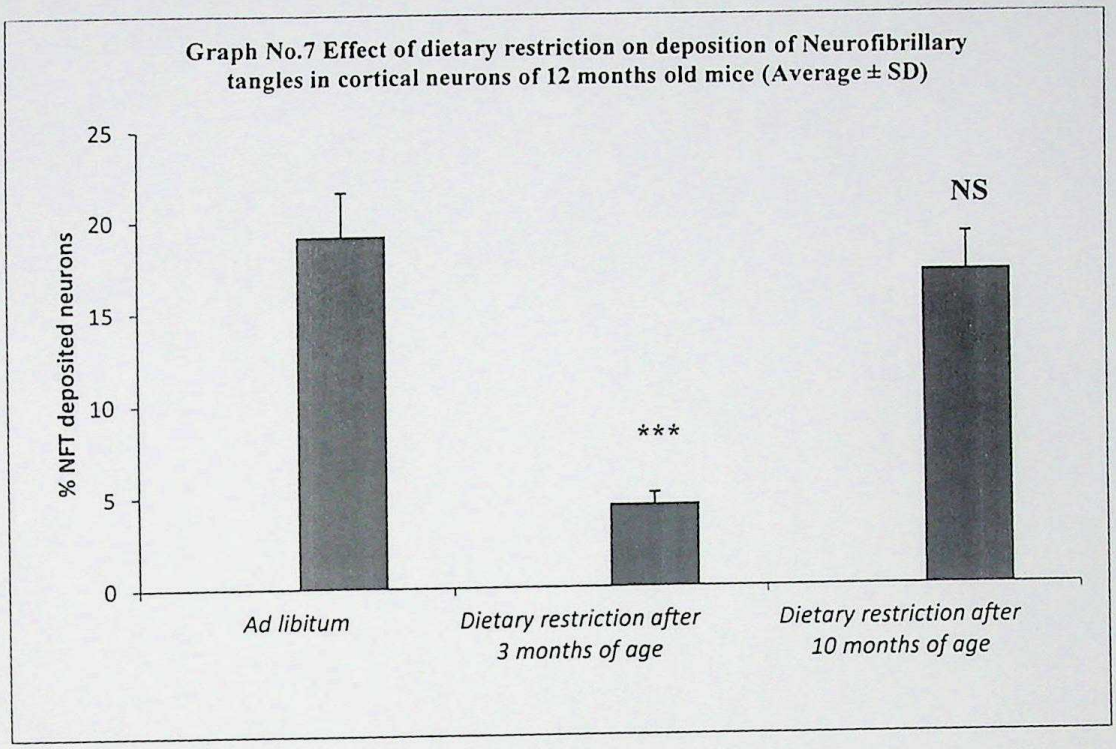


White arrow indicates deposition of NFT and Blue arrow indicates neuron without NFT

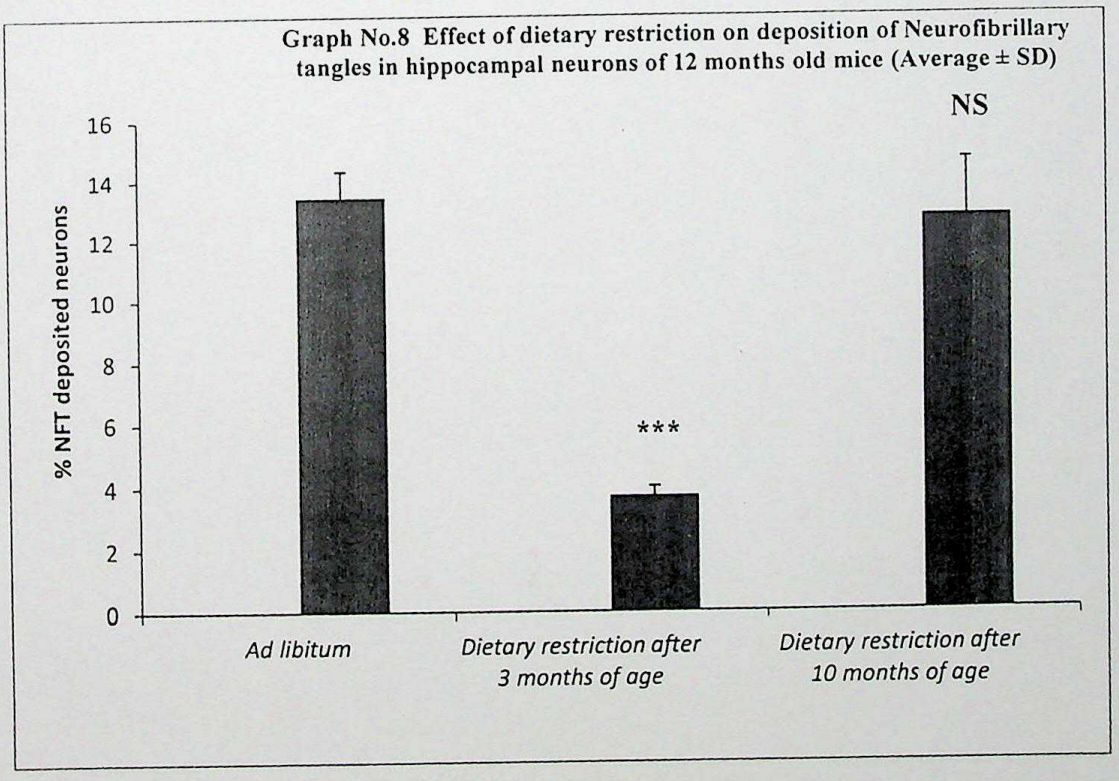
PLATE: 4 Results of histochemical studies on neurofibrillary tangles (NFTs), in Hippocampus of mouse brain (400X)



White arrow indicates deposition of NFT and Blue arrow indicates neuron without NFT

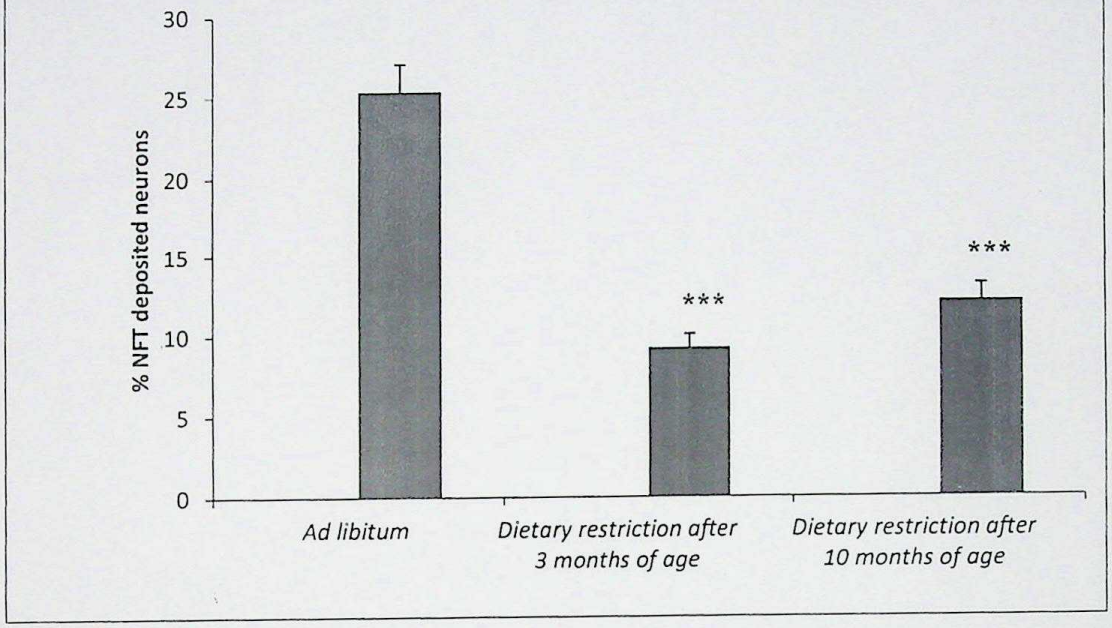


*** indicates p<0.001 i.e. highly significant
NS= Non significant



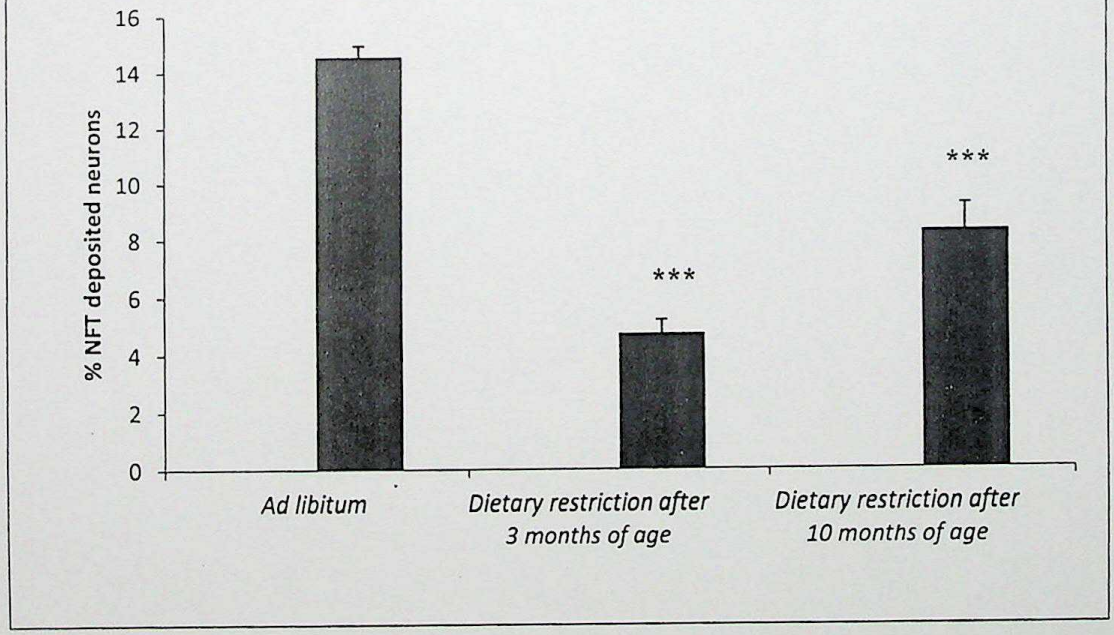
*** indicates p<0.001 i.e. highly significant NS= Non significant

Graph No. 9 Effect of dietary restriction on deposition of Neurofibrillary tangles in cortical neurons of 18 months old mice (Average ± SD)



*** indicates p<0.001 i.e. highly significant

Graph No. 10 Effect of dietary restriction on deposition of Neurofibrillary tangles in hippocampal neurons of 18 months old mice. (Average ± SD)



*** indicates p<0.001 i.e. highly significant

The animals subjected to dietary restriction after 10 months of age exhibited 8.29 ± 0.93 % neurons having NFT deposition. As compared to control there was 1.75 fold lower number of NFTs deposited neurons. This difference was highly significant ($p<0.001$). **(Plate No. 4. Fig. 4, 5, 6).**

Deposition of Neurofibrillary tangles in Cerebral cortex and Hippocampus of 24 month old mice:-The observations are presented in the Table No. 2. (Graph No. 11, 12)

The cerebral cortex of *ad libitum* fed mice showed 30 ± 1.69 % neurons deposited with NFT. In mice subjected to dietary restriction after 3 months of age 14 ± 1.78 % neurons were having deposition of NFTs. The NFTs deposition in neurons of diet restricted group was 2.14 fold lower than the *ad libitum* fed group. This difference was highly significant ($p<0.001$). The mice subjected to dietary restriction after 10 months of age showed 20.70 ± 2.76 % neurons with NFT deposition. As compared to *ad libitum* fed group there was 1.44 fold lower number in NFT deposited neuronal cells. This difference was highly significant ($p<0.001$). **(Plate No 3. Fig. 7, 8, 9)**

In hippocampus of 24 month old *ad libitum* fed mice 18.72 ± 2.74% neuronal cells were having deposition of NFT. The mice subjected to 40% dietary restriction from three months of age exhibited 4.83 ± 0.40 % neurons with NFTs. As compared to *ad libitum* fed group there was 3.87 fold less number of NFT deposited neuronal cells. This decrease was highly significant ($p<0.001$). The other experimental group i.e. animals subjected to dietary restriction after 10 months of age showed 12.61 ± 2.01% neurons deposited with neurofibrillary tangles. As compared to control group there was highly significant ($p<0.001$) lower number of neurons having NFT deposition. **(Plate No. 4. Fig. 7, 8, 9).**

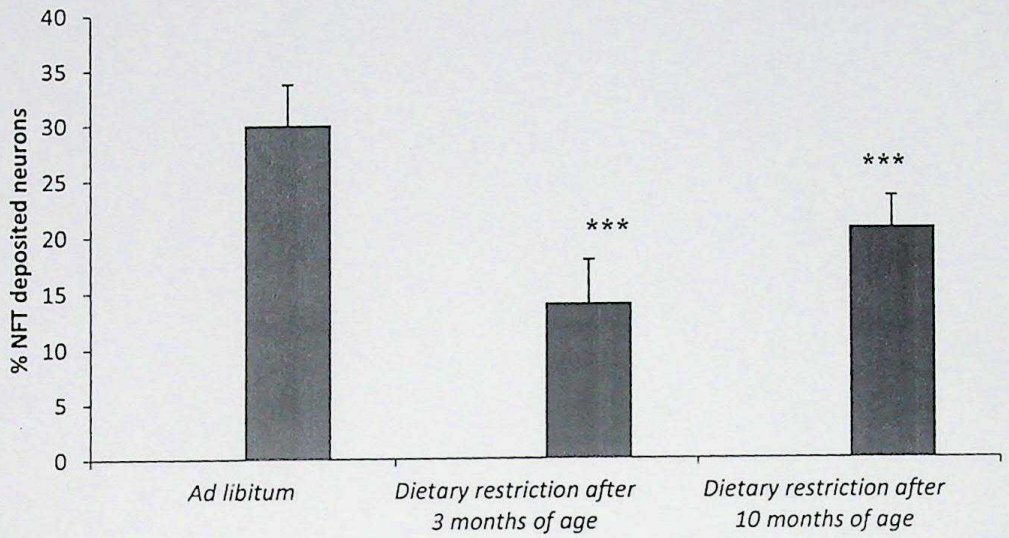
C. Study of Senile plaques

Effect of dietary restriction on deposition of Senile plaques in the neurons of Cerebral cortex and hippocampus of mice brain.

Senile plaques are deposited extracellularly in the brain of persons with Alzheimer's disease (AD). Senile plaques are formed due to aggregation of small peptides called as β- amyloid. β-amyloid is produced from amyloid precursor protein (APP). The excess formation of β-amyloid in the brain is a primary cause of AD (Zhang *et al.*, 2012).

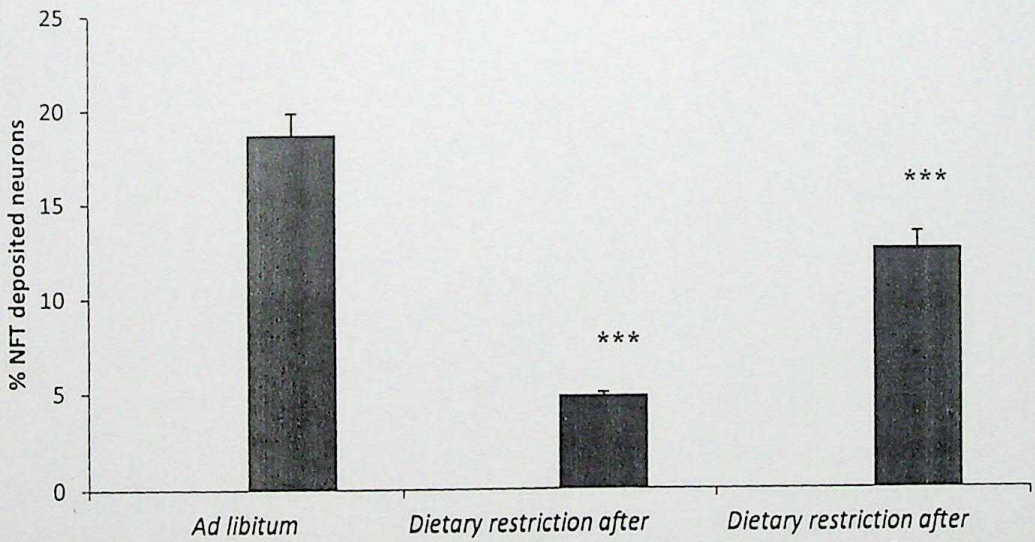
Deposition of Senile plaques in Cerebral cortex and Hippocampus of 12 month old mice:-

Graph No: 11. Effect of dietary restriction on deposition of Neurofibrillary tangles in cortical neurons of 24 months old mice. (Average ± SD)



*** indicates p<0.001 i.e. highly significant

Graph No. 12 Effect of dietary restriction on deposition of Neurofibrillary tangles in hippocampal neurons of 24 months old mice . (Average ± SD)



*** indicates p<0.001 i.e. highly significant

The *ad libitum* fed mice of age 12 months showed only one senile plaque in the Cerebral cortex region. Whereas in animals subjected to dietary restriction after 3 months and 10 months of age there were no deposition of senile plaque in cerebral cortex. **(Plate No. 5 Fig. 1, 2, 3)**

In case of hippocampal neurons of *ad libitum* fed mice 0.44 number of senile plaque deposits were observed. Whereas, in hippocampal region of mice subjected to dietary restriction no any deposits of senile plaques were seen. **(Plate No. 6 Fig. 1, 2, 3).**

Deposition of Senile plaques in Cerebral cortex and Hippocampus of 18 month old mice:-

In Cerebral cortex *ad libitum* fed 18 months old mice 1 ± 0.25 senile plaque was observed. There were no senile plaques in the cerebral cortex of diet restricted animals even after 18 months of age. **(Plate No. 5 Fig. 4, 5, 6,)**

The hippocampal regions of *ad libitum* fed mice 0.58 ± 0.14 senile plaque were observed. In case of hippocampal region of experimental group there were no any deposits of senile plaque. **(Plate No.6 Fig. 4, 5, 6,).**

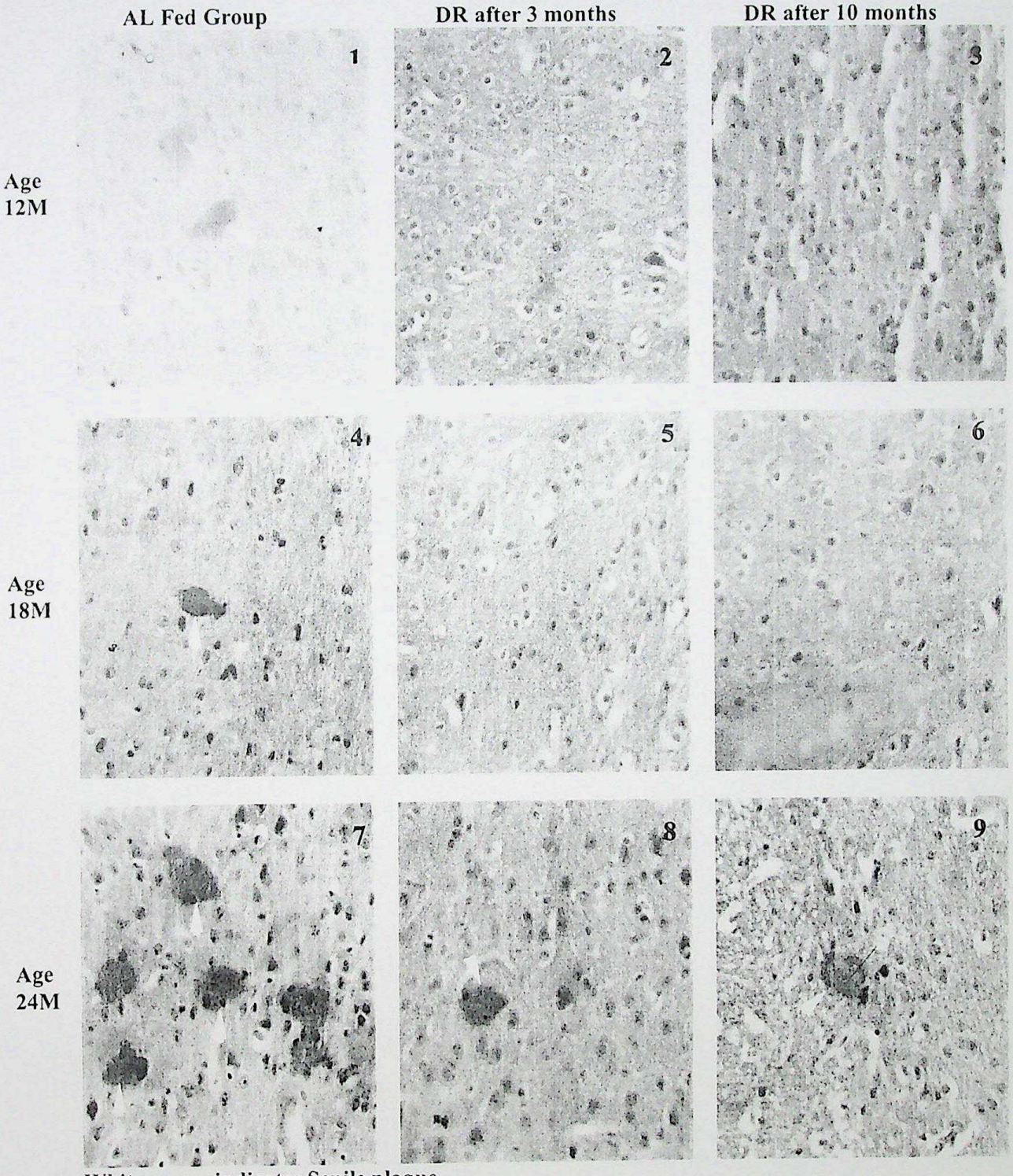
Deposition of Senile plaques in Cerebral cortex and Hippocampus of 24 month old mice:-

At 24 month age the *ad libitum* fed mice exhibited 4.73 ± 0.25 numbers of senile plaques in cerebral cortex. The mice subjected to dietary restriction after 3 months of age showed 1.43± 0.37 deposits of senile plaques in cortical region. As compared to values of control 3.30 fold reduced deposition of senile plaque was observed. This decrease was highly significant ($p<0.001$). The mice subjected to dietary restriction after 10 months of age showed 2.38 ± 1.39 number of senile plaque deposits in cortical region. The comparison of these values with the control shows two fold decrease in senile plaque deposits. This decrease was statistically significant ($p<0.01$).

The comparison of experimental group subjected to dietary restriction after 3 months of age showed 1.66 fold lower deposition of senile plaque than the mice subjected to dietary restriction after 10 month of age. **(Plate No. 6. Fig.7, 8, 9).**

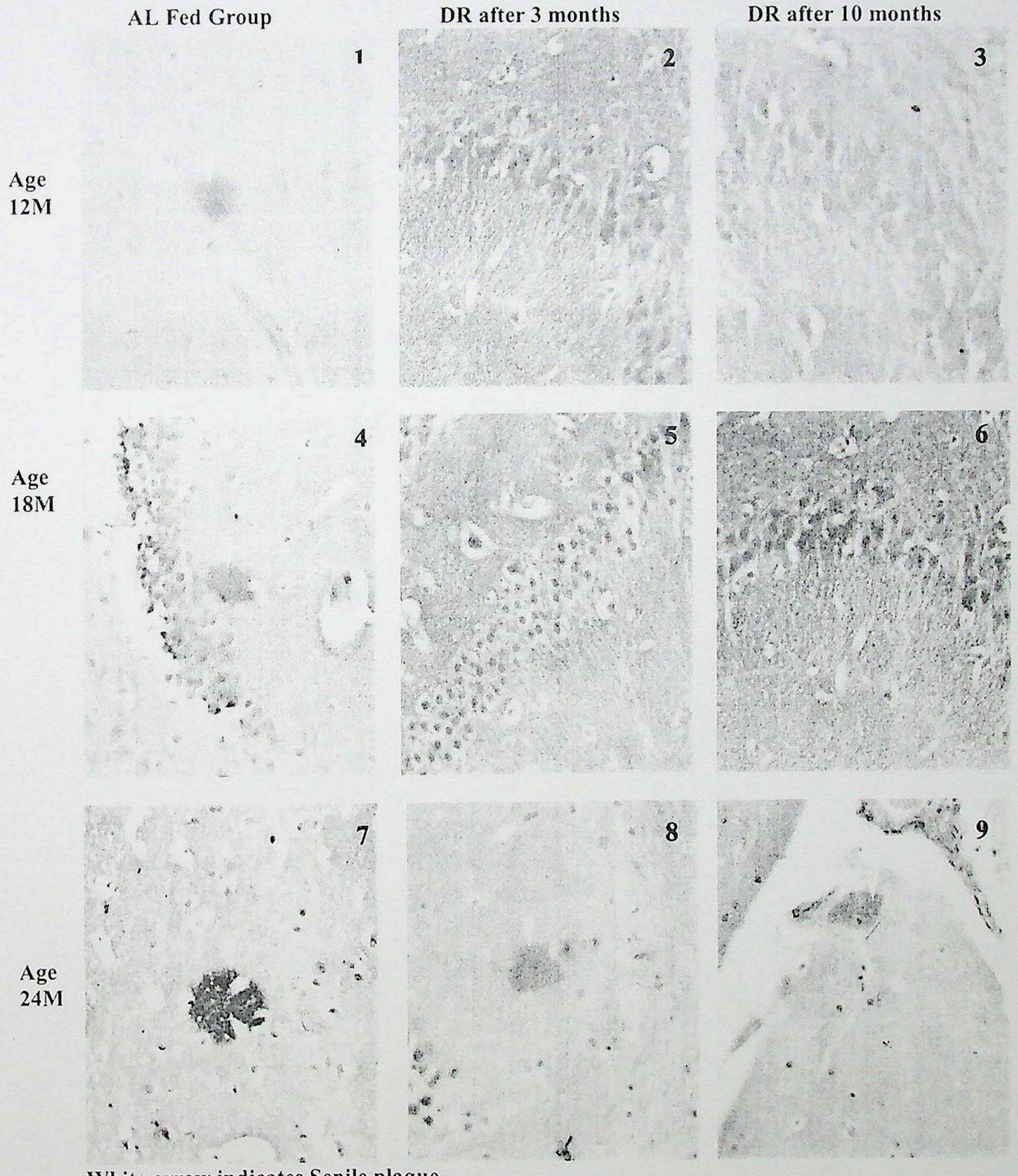
The hippocampal region of 24 month age *ad libitum* fed mice showed 0.91± 0.38 of senile plaques. Whereas, the mice on 40% dietary restriction after 3 months of age exhibited 0.49 ± 0.16 senile plaques in there hippocampal region. As compared to control, there was

PLATE NO: 5 Results of histochemical studies on Senile Plaques in Cerebral cortex of mouse brain (400X)



White arrow indicates Senile plaque

PLATE NO:6 Results of histochemical studies on Senile Plaques in Hippocampus of mouse brain (400X)



White arrow indicates Senile plaque

1.85 fold less deposition of senile plaques. This decrease was not significant. In mice subjected to dietary restriction after 10 months of age 0.69 ± 0.05 senile plaques were observed. As compared to control values the senile plaque deposit number was decreased but it was not significant. **(Plate No. 6 . Fig. 7, 8, 9).** Compared to Cerebral cortex, hippocampus exhibited less deposition of senile plaques in all the age groups.

D. Study of Nissl substance

Effect of dietary restriction on the Nissl substance in the neurons of Cerebral cortex and hippocampus of mouse.

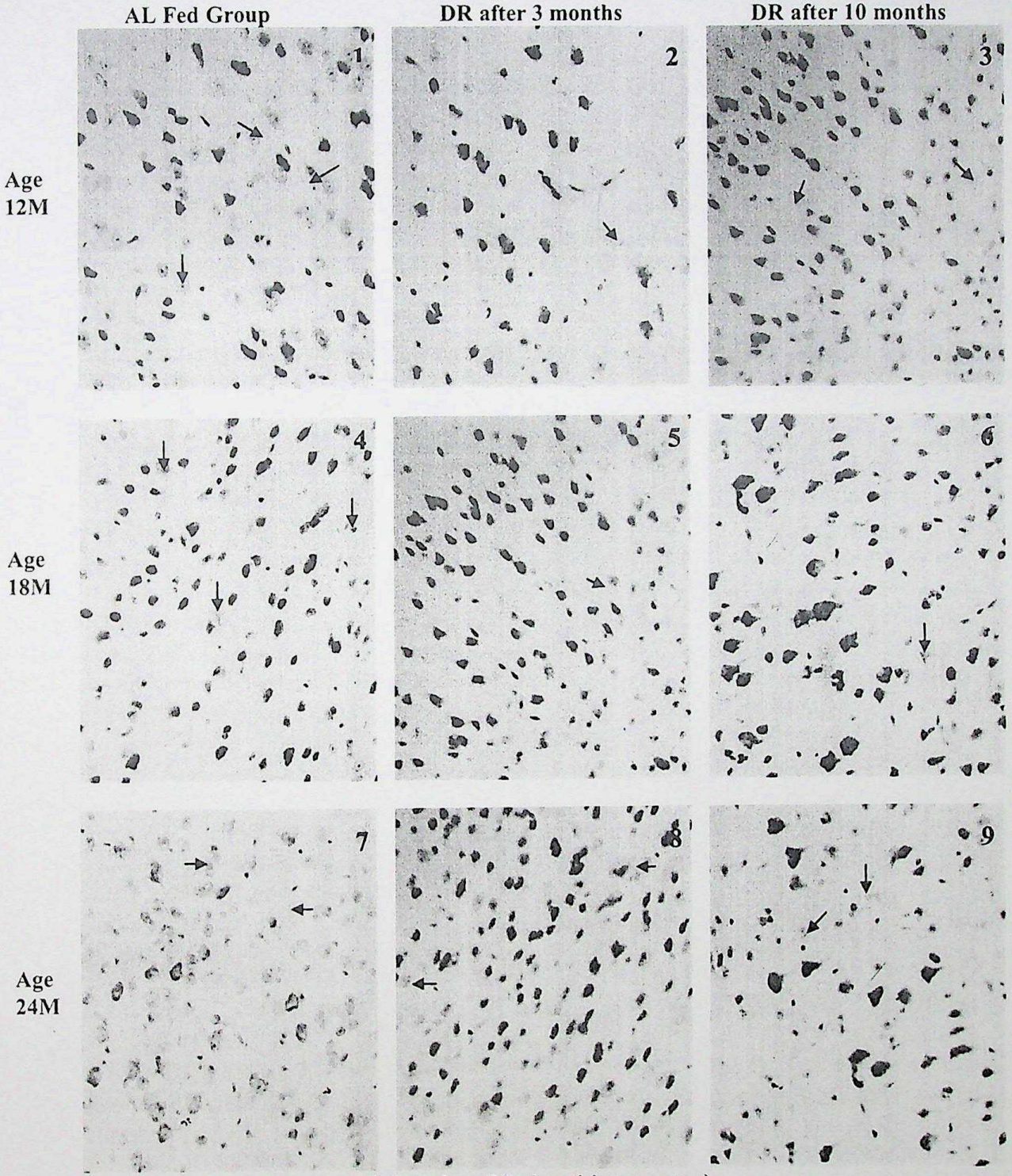
A Nissl substance is a granular body present in the cyton of neurons. These granules are Ribonucleoprotein particles i.e. complex of RNA and proteins their function is synthesis of proteins. German psychiatrist Franz Nissl, developed a method for the staining of Nissl substance. The functional efficiency of neurons is directly proportional to the abundance of Nissl substance (Scarborough 1938).

Nissl substance in Cerebral cortex and Hippocampus of 12 months old mice:- The observations are presented in the Table No.3. (Graph No. 13, 14).

In cerebral cortex of *ad libitum* fed 12 months old mice, 68 ± 1 % of neurons were Nissl positive. Whereas, mice subjected to 40% dietary restriction after 3 months of age showed 88 ± 0.50 % of neurons Nissl positive. As compared to *ad libitum* fed group there was increase in Nissl positive neurons. This increase in Nissl substance in DR group was highly significant ($p<0.001$). The mice subjected to dietary restriction after 10 month of age showed 70 ± 0.28 % of Nissl positive neurons. As compared to *ad libitum* fed group Nissl positive neurons were increased. This increase was not significant. **(Plate No. 7. Fig. 1, 2, 3).**

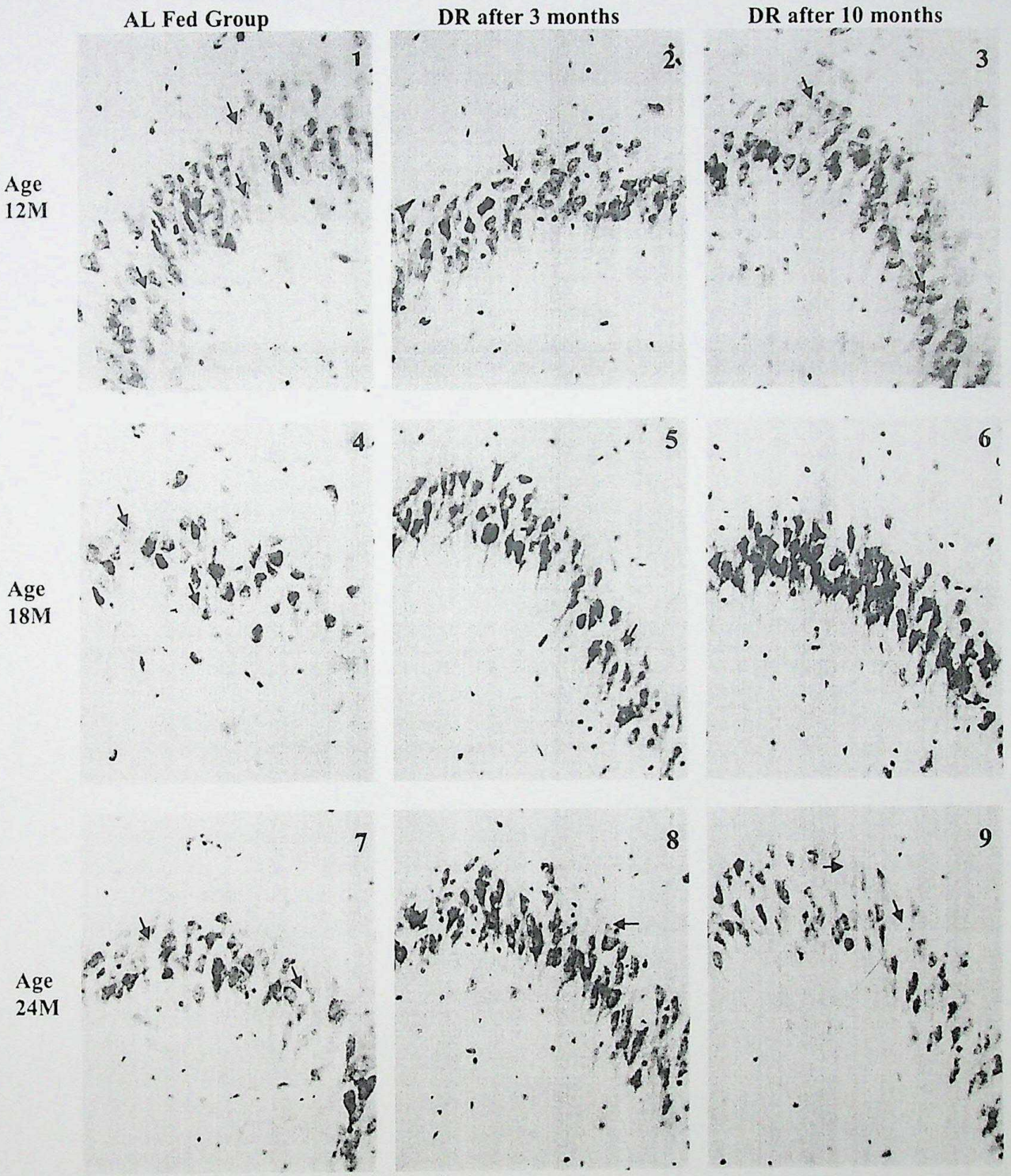
In case of hippocampus of *ad libitum* fed 12 months old mice, 81 ± 0.78 % neurons were Nissl positive. While, mice subjected to 40% dietary restriction after 3 months of age exhibited 94 ± 0.57 % of neurons Nissl positive. As compared to of *ad libitum* fed group there was increase in Nissl positive neurons. This increase in Nissl substance in DR group was highly significant ($p<0.001$). The animals subjected to dietary restriction after 10 month of age showed 87 ± 1.4 % of neurons were Nissl positive. As compared to *ad libitum* fed group Nissl positive neurons were increased. This increase was highly significant ($p<0.001$). **(Plate No. 8. Fig. 1, 2, 3).**

PLATE NO: 7 Results of histochemical studies on Nissl substance in Cerebral cortex of mouse brain (400X)



Nissl substance appears purple in colour(Nissl positive neurons)
Arrow indicates Nissl negative neurons

PLATE NO: 8 Results of histochemical studies on Nissl substance in Hippocampus of mouse brain (400X)



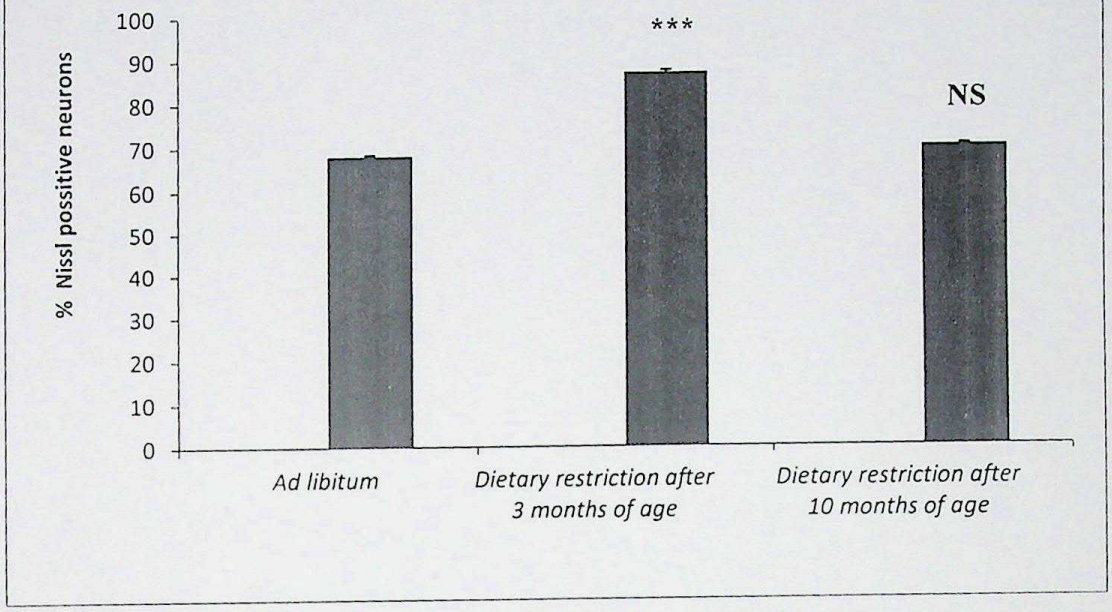
Nissl substance appears purple in colour

Table No.3:- Effect of dietary restriction on Nisslsubstance in Cerebral cortex and Hippocampus of mice.

Organ	Age of animals in months	Control group (*ad libitum* fed animals) % of Neurons showing Nissl substance	Experimental group (Dietary restriction after 3 months of age) % of Neurons showing Nissl substance	Experimental group (Dietary restriction after 10 months of age) % of Neurons showing Nissl substance
Cerebral cortex	12 months	68 ± 0.36	88 ± 0.50***	70 ± 0.28 NS
	18 months	62 ± 1	76 ± 1***	65 ± 0.57**
	24 months	57 ± 1.05	66 ± 0.63***	64 ± 0.97***
Hippocampus	12 months	81 ± 0.78	94 ± 0.57***	87 ± 1.4***
	18 months	72 ± 1	91 ± 1.5***	85 ± 0.89***
	24 months	66 ± 0.33	84 ± 0.53***	80 ± 0.36***

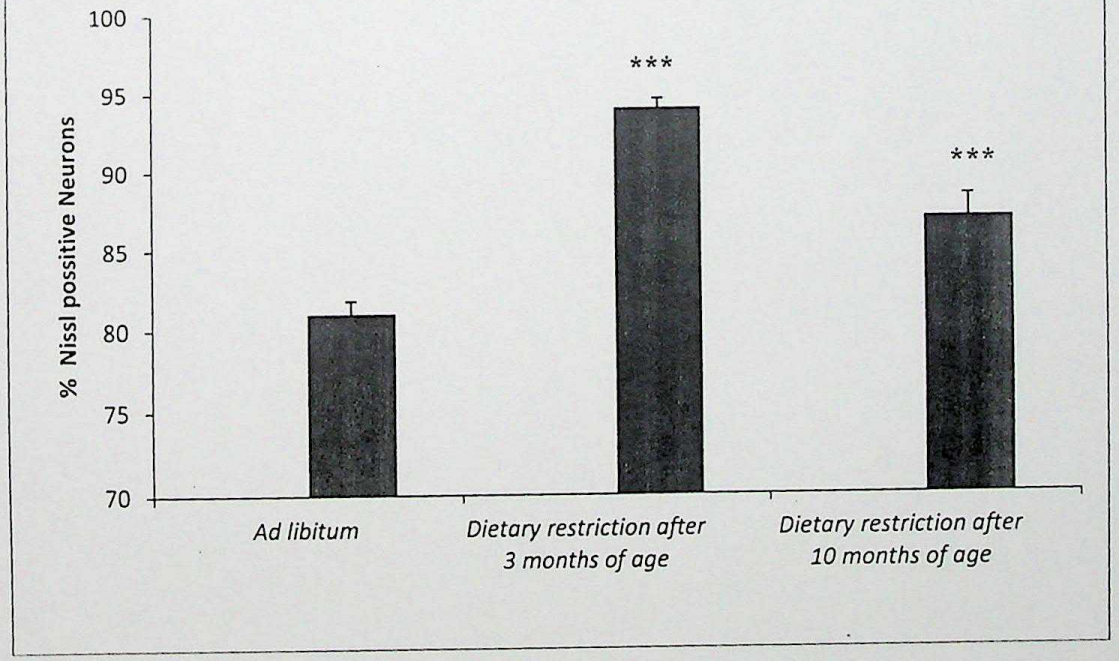
Results expressed as mean ± SD of 6 animals
*** $p<0.001$= highly significant
** $p<0.01$= Moderatelysigifivanct
$p> 0.05$ NS= Non significant

Graph No:13 Effect of dietary restriction on Nissl substance in cortical neurons of 12 months old mice. (Average ± SD)

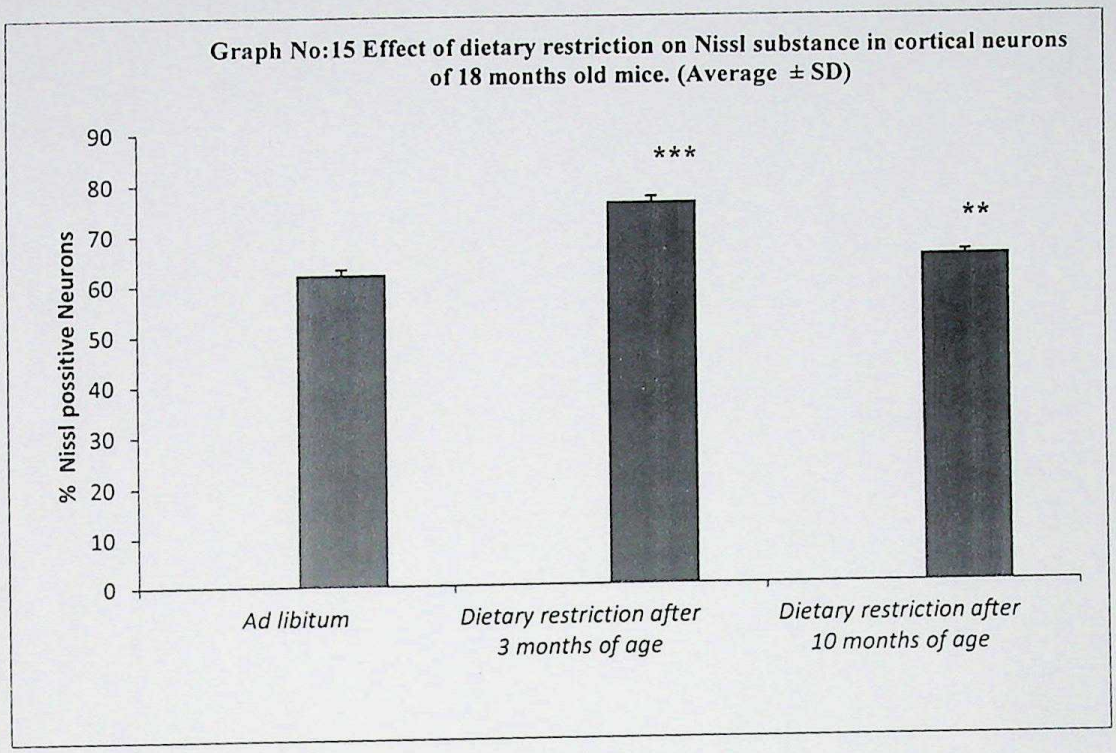


*** indicates p<0.001 i.e. highly significant
NS= Not significant

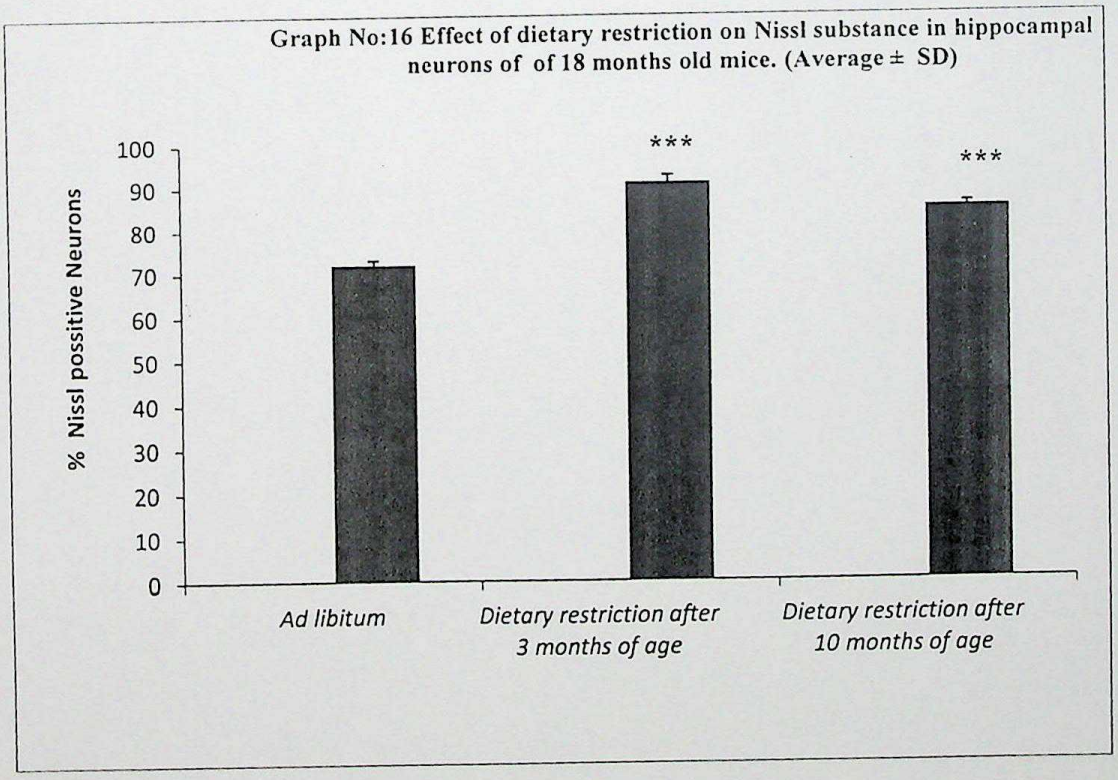
Graph No: 14 Effect of dietary restriction on Nissl substance in hippocampal neurons of 12 months old mice. (Average ± SD)



*** indicates p<0.001 i.e. highly significant



*** indicates p<0.001 i.e. highly significant
** indicates p<0.01 i.e. moderately significant



*** indicates p<0.001 i.e. highly significant

Nissl substance in Cerebral cortex and Hippocampus of 18 months old mice:- The observations are presented in the Table No.3. (Graph No. 15,16).

In cerebral cortex of *ad libitum* fed 18 month old mice, 62 ± 1 % of neurons were Nissl positive. Whereas, mice subjected to 40% dietary restriction after 3 months of age showed 76 ± 1 % of neurons Nissl positive. As compared to *ad libitum* fed group there was increase in Nissl positive neurons. This increase was highly significant ($p<0.001$). The animals subjected to dietary restriction after 10 month of age showed 65 ± 0.57 % of Nissl positive neurons. As compared to *ad libitum* fed group Nissl positive neurons were increased. This increase was highly significant ($p<0.001$). **(Plate No. 7. Fig. 4, 5, 6).**

The hippocampal neurons of *ad libitum* fed 18 months old mice, 72 ± 1 % neurons were Nissl positive. While mice subjected to 40% dietary restriction after 3 months of age exhibited 91 ± 1.5 % of neurons were Nissl positive. As compared to of *ad libitum* fed group there was increase in Nissl positive neurons. This increase was highly significant ($p<0.001$). The animals subjected to dietary restriction after 10 month of age showed 85 ± 0.89 % Nissl positive neurons. As compared to *ad libitum* fed group Nissl positive neurons were increased. This increase was highly significant ($p<0.001$). **(Plate No. 8. Fig. 4, 5, 6).**

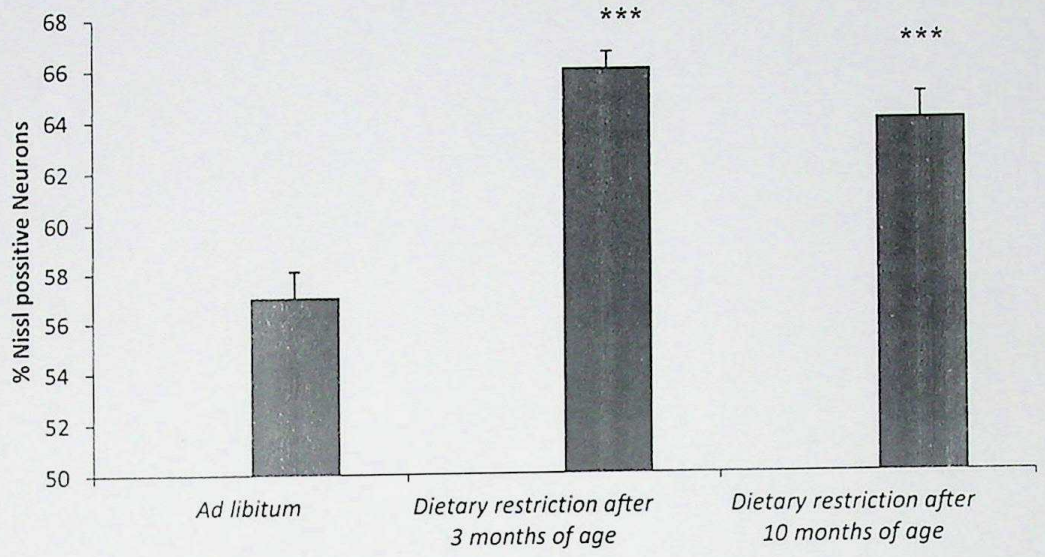
Nissl substance in Cerebral cortex and Hippocampus of 24 months old mice:-

The observations are presented in the Table No.3. (Graph No. 17,18).

In cerebral cortex of *ad libitum* fed 24 months old mice, 57 ± 1.5 % of neurons were Nissl positive. Whereas, mice subjected to 40% dietary restriction after 3 months of age showed 66 ± 0.63 % of neurons were Nissl positive. As compared to of *ad libitum* fed group there was increase in Nissl positive neurons. This increase was highly significant ($p<0.001$). The animals subjected to dietary restriction after 10 month of age showed 64 ± 0.97 % of Nissl positive neurons. As compared to *ad libitum* fed group Nissl positive neurons were increased. This increase was highly significant ($p<0.001$). **(Plate No. 7. Fig. 7, 8, 9).**

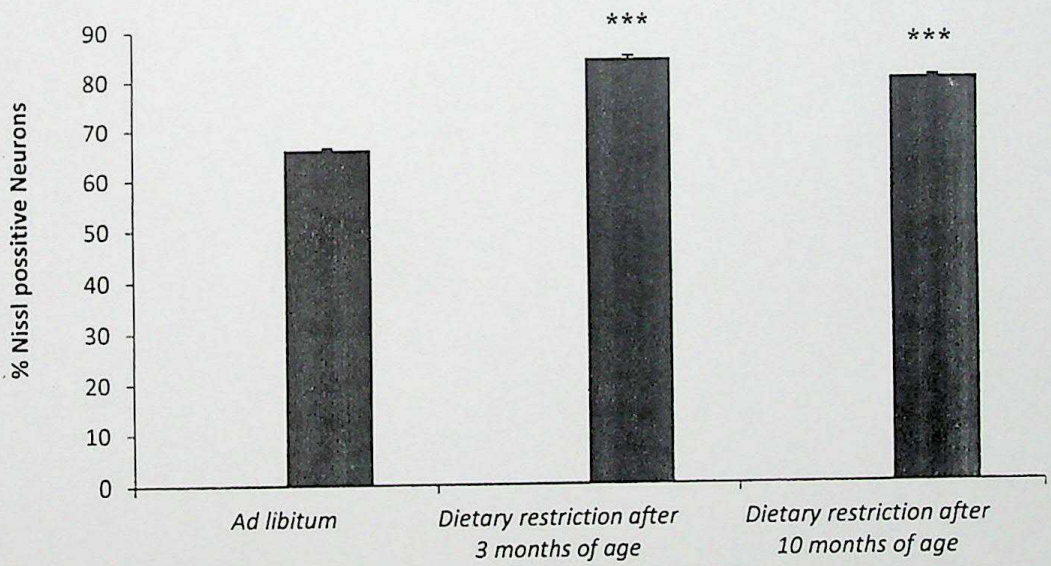
The hippocampal neurons of *ad libitum* fed 24 months old mice, 66 ± 0.33 % neurons were Nissl positive. While mice subjected to 40% dietary restriction after 3 months of age exhibited 84 ± 0.53 % of neurons were Nissl positive. As compared to of *ad libitum* fed group there was increase in Nissl positive neurons. This increase was highly significant ($p<0.001$). The animals subjected to dietary restriction after 10 month of age showed 80 ± 0.36 % Nissl positive neurons. As compared to *ad libitum* fed group Nissl positive neurons were increased. This increase was highly significant ($p<0.001$). **(Plate No. 8. Fig. 7, 8, 9).**

Graph No:17 Effect of dietary restriction on Nissl substance in cortical neurons of 24 months old mice. (Average ± SD)



*** indicates p<0.001 i.e. highly significant

Graph No:18 Effect of dietary restriction on Nissl substance in hippocampal neurons of of 24 months old mice (Average ± SD)



*** indicates p<0.001 i.e. highly significant

Electron microscopic studies

Results of Transmission electron microscopy:

- A neuron from the cerebral cortex of 18 months old *ad libitum* fed mouse exhibited abundance of heterochromatin. Cytoplasm was loaded with residual bodies. Mitochondria were swollen. Cytoplasm exhibited poor Nissl granules and RER. (Plate No.9 Fig. 1)
- A neuron from the cerebral cortex of 18 months old mouse subjected to dietary restriction after 3 months of old exhibited conspicuous nucleus with abundance of euchromatin. There were autophagosomes in various stages of digestion. Cytoplasm was abundant in Nissl substance and RER. Mitochondria appeared normal in size and shape. (Plate No.9 Fig. 2)
- A neuron from the cerebral cortex of 18 months old mouse subjected to dietary restriction after completion of 10 months of age exhibited abundance of euchromatin. Cytoplasm was heavily loaded with Nissl substance. Mitochondria appeared normal in size and form. However one swollen mitochondrion was also observed. No lipofuscin granules were evident. (Plate No.9 Fig 3)
- A neuron of the hippocampal region of 18 months *ad libitum* fed mouse exhibited conspicuous nucleus with marginal traces of heterochromatin, however there was abundance of euchromatin. Cytoplasm was fully occupied by Rough Endoplasmic Reticulum, however Golgi cisternae were not observed in inflated form. The cytoplasm was loaded with lipofuscin granules. Mitochondria were with normal size and shape without any swelling. (Plate No. 10 Fig 1). When the transverse section of axon was observed at 20,000X magnification, it exhibited deposition of senile plaque. The lumen contained secretary vesicles and mitochondria. However the microtubule supporting the axon was not evident. (Plate No. 12 Fig 1)
- A neuron of the hippocampal region of 18 months old mouse subjected to dietary restriction after completion of 3 months of age exhibited abundance of Nissl substance. Golgi cisternae were very prominent indicating secretary activity. Mitochondria were of normal size and shape without swelling and pathological contours. Nucleus was of normotexture with abundance of euchromatin. There was only one residual body observed and the contents within it were very small in size. (Plate No. 10 Fig 2). When the neuron was observed at 30,000X magnification the

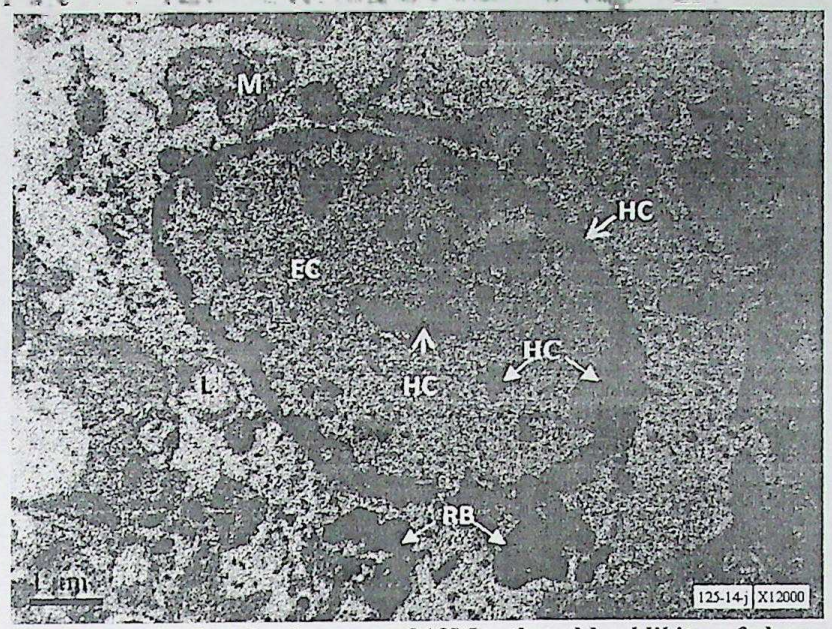


Fig 1 A neuron from the Cerebral cortex of 18Months old *ad libitum* fed mouse

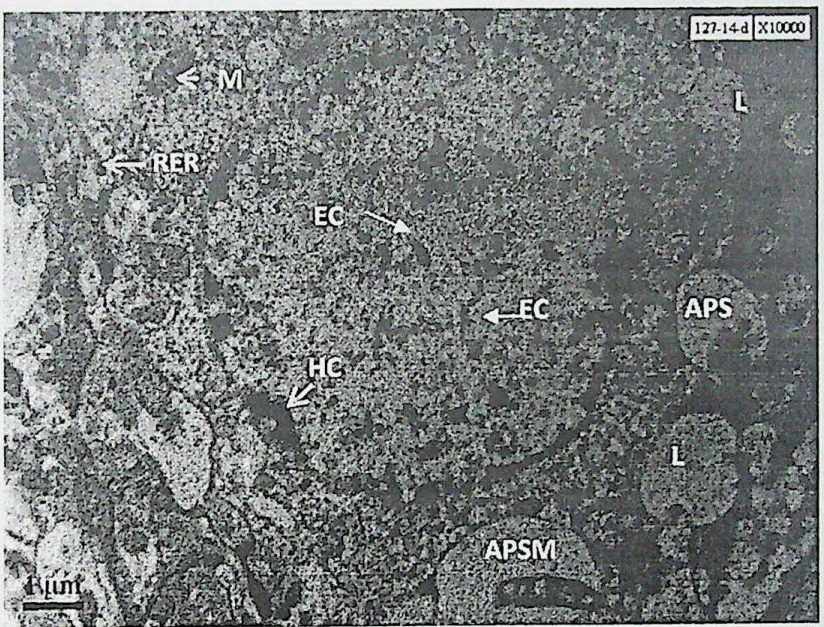


Fig 2 A neuron from the Cerebral cortex of 18Months old mouse subjected to DR after 3 months of age.

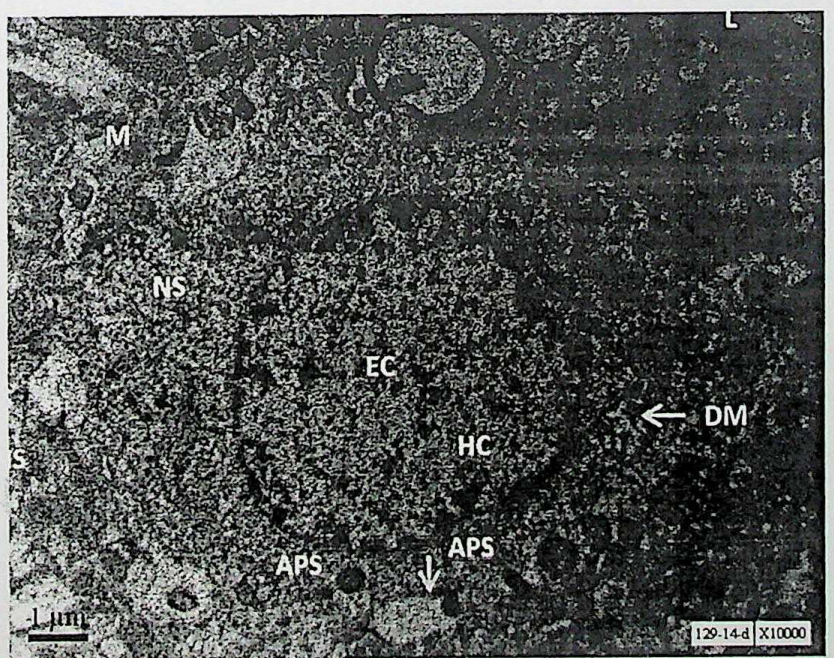


Fig 3. A neuron from the Cerebral cortex of 18Months old mouse subjected to DR after 10Months of age

Plate 10 : Ultrastructural details studied through TEM

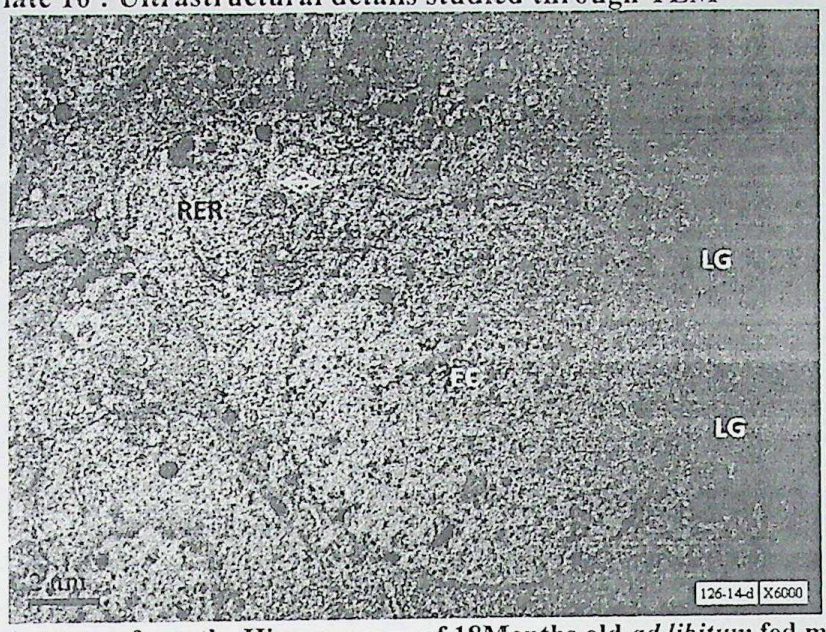


Fig 1 A neuron from the Hippocampus of 18Months old *ad libitum* fed mouse

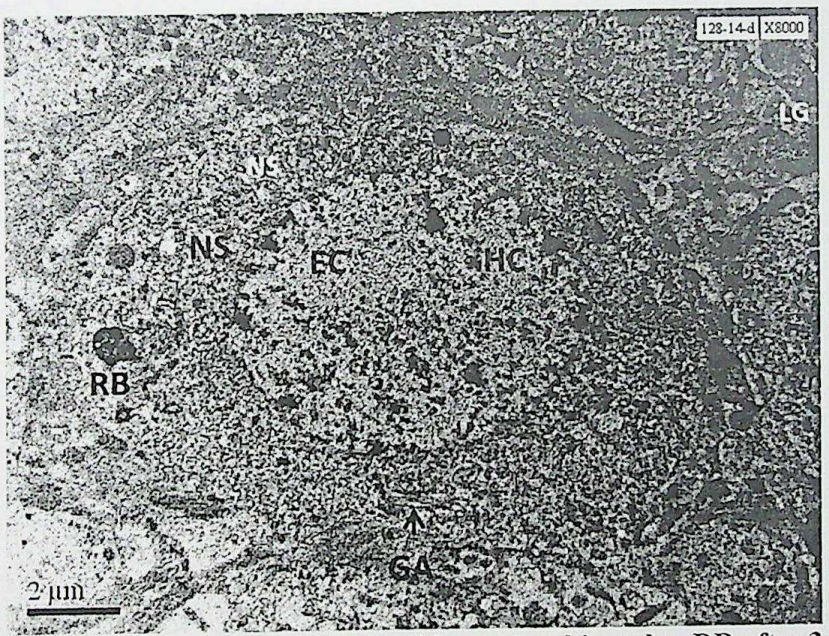


Fig:2. A neuron from the Hippocampus of 18Months old mouse subjected to DR after 3 Months of age.

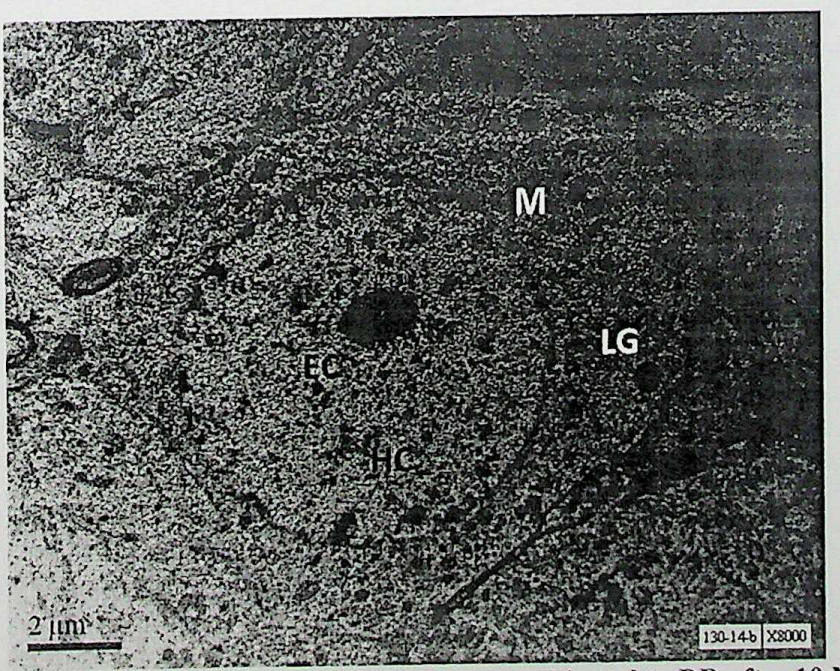


Fig 3A neuron from the Hippocampus of 18Months old mouse subjected to DR after 10 Months of age

Plate 11: Ultrastructural details studied through TEM



Fig 1 A portion of a neuron from the Hippocampus of 18Months old mouse subjected to DR after 10 Months of age

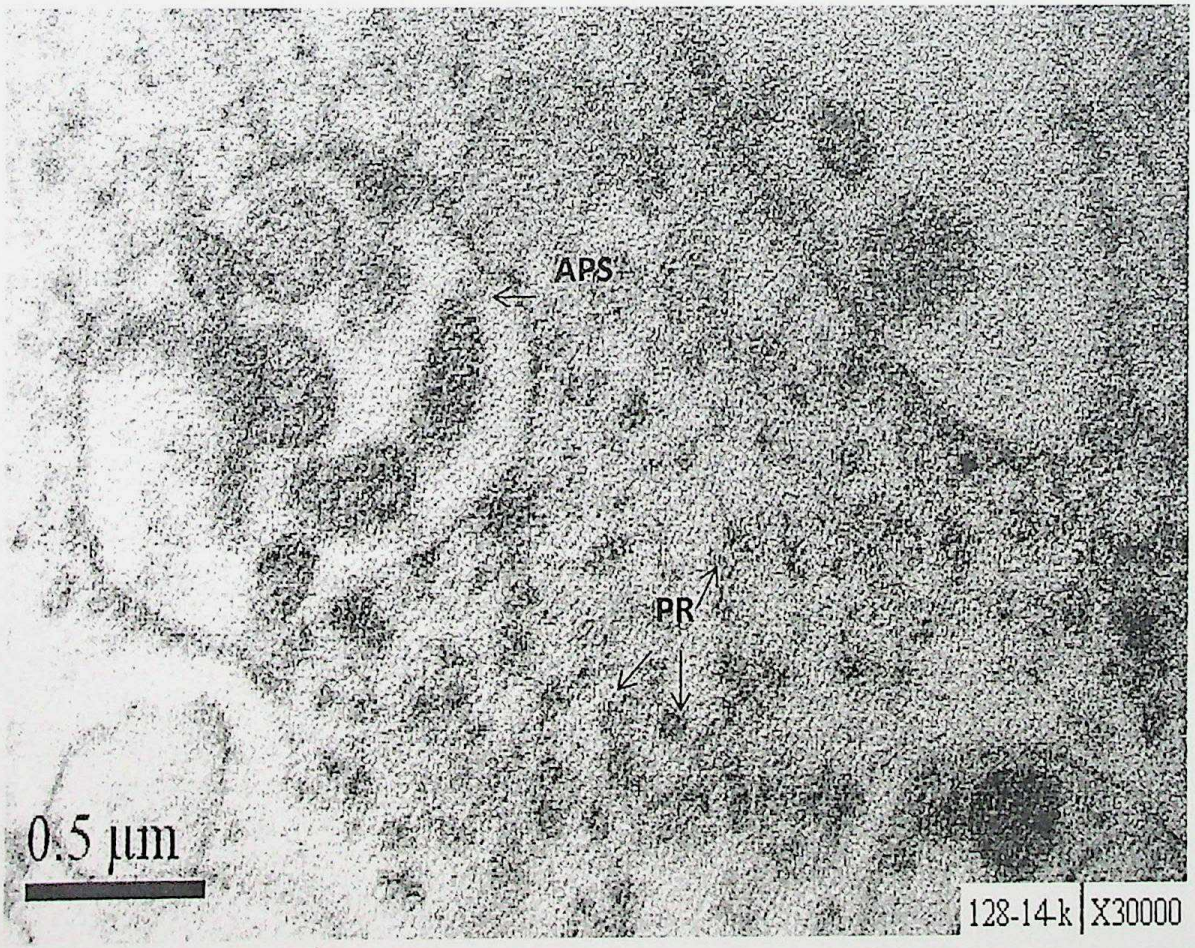


Fig 2 A portion of a neuron from the Hippocampus of 18Months old mouse subjected to DR after 3 months of age.

Plate 12: Ultrastructural details studied through TEM

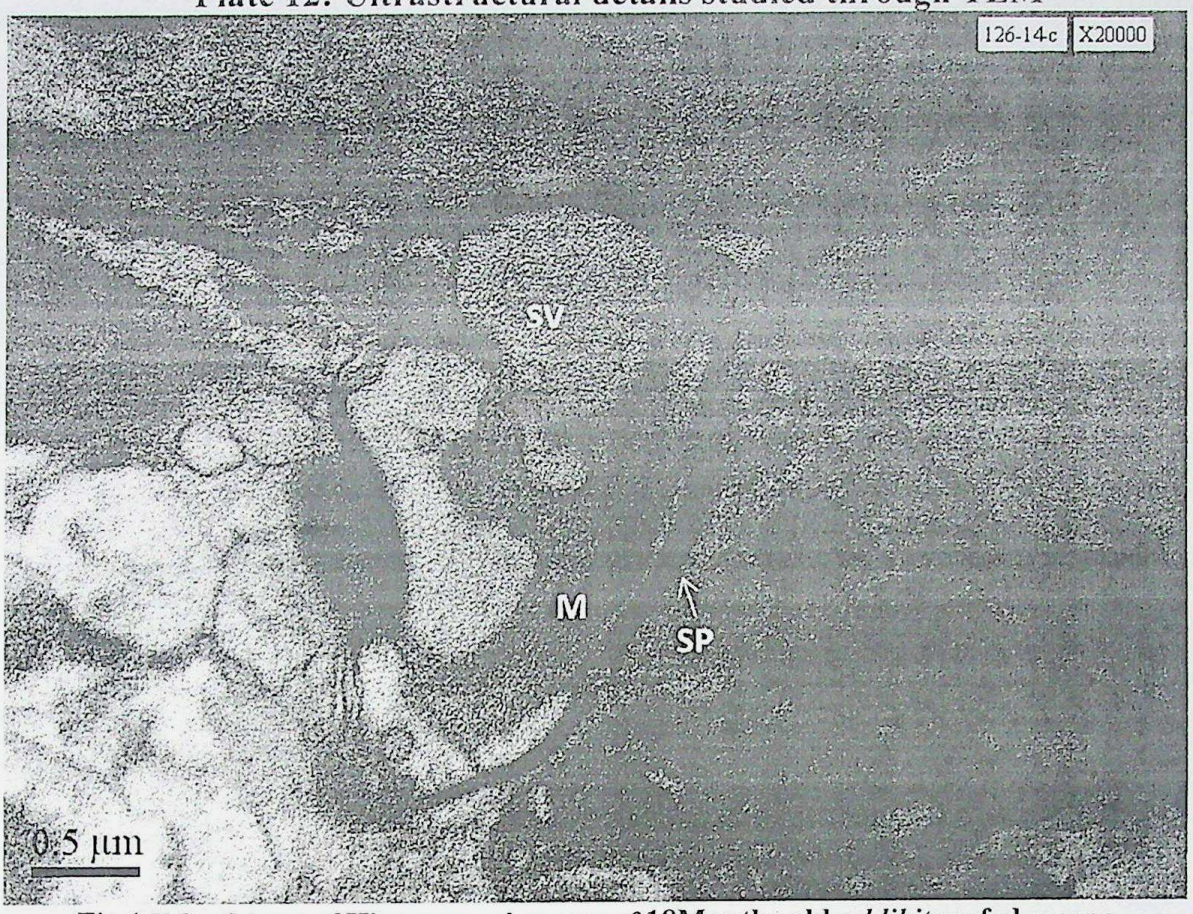


Fig 1 T.S. of Axon of Hippocampal neuron of 18Months old *ad libitum* fed mouse



Fig 2 T.S. of Axon of Hippocampal neuron of 18Months old mouse subjected to DR after 3 Months of age

Plate 13: Ultrastructural details studied through TEM

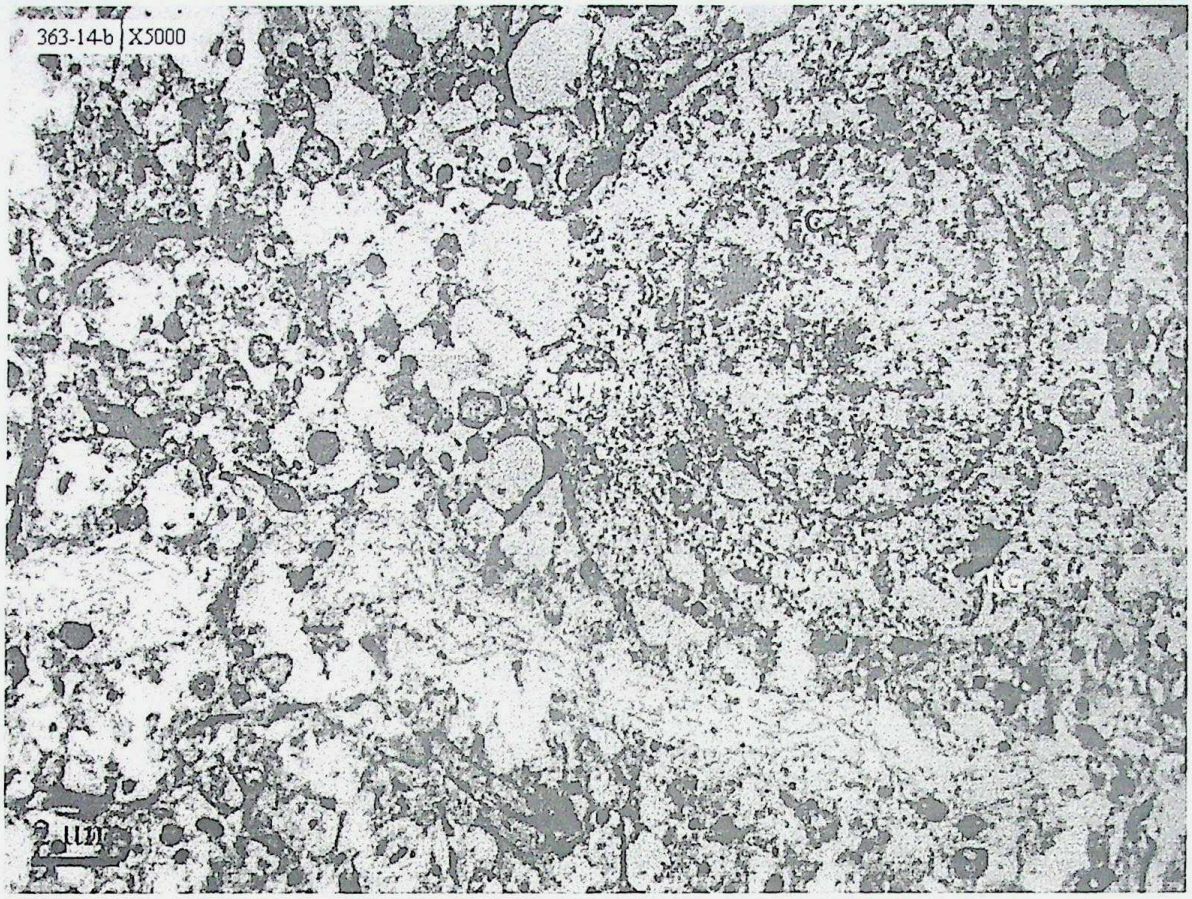


Fig 1 A neuron from the Cerebral cortex of 24 Months old *ad libitum* fed mouse



Fig 2 A neuron from the Cerebral cortex of 24 Months old mouse subjected to DR after 3Months of age

Plate 14: Ultrastructural details studied through TEM

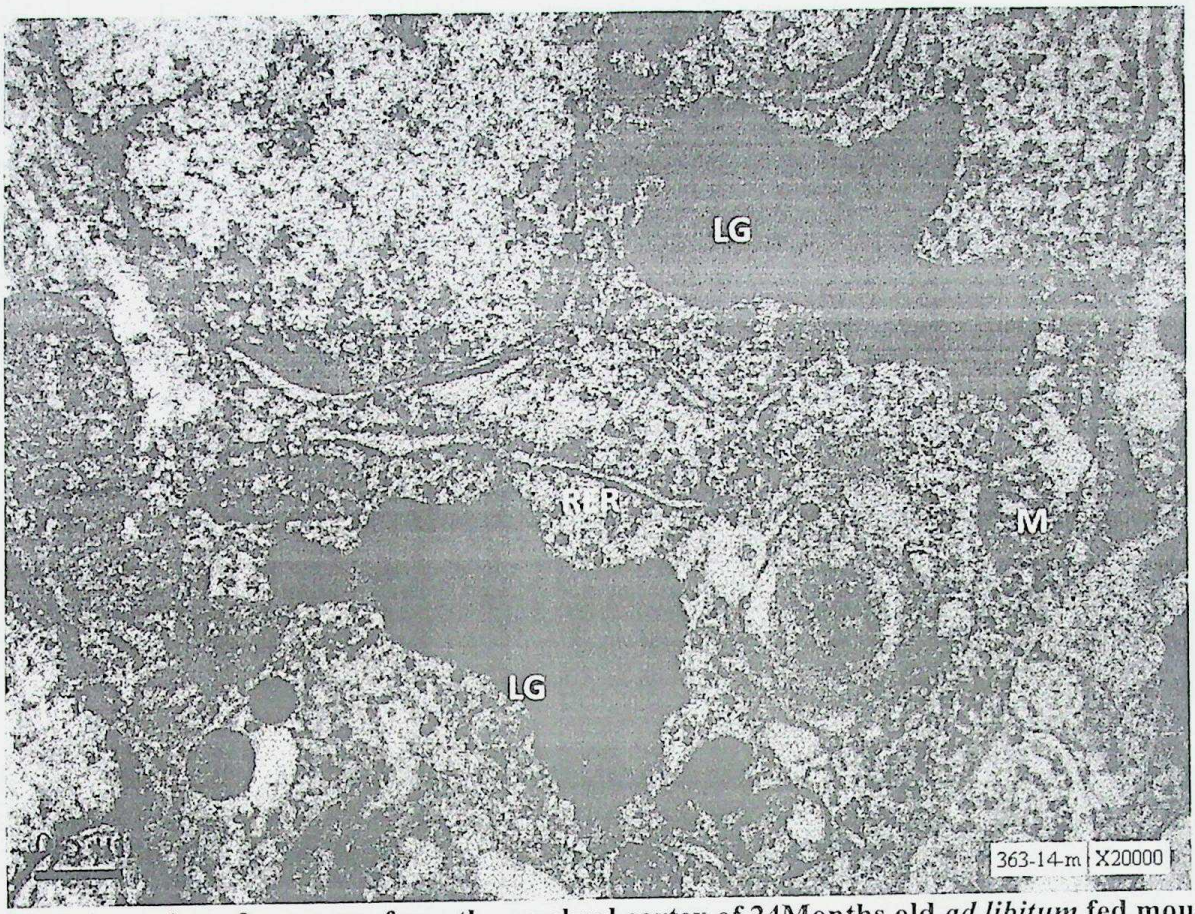


Fig 1 A portion of a neuron from the cerebral cortex of 24Months old *ad libitum* fed mouse

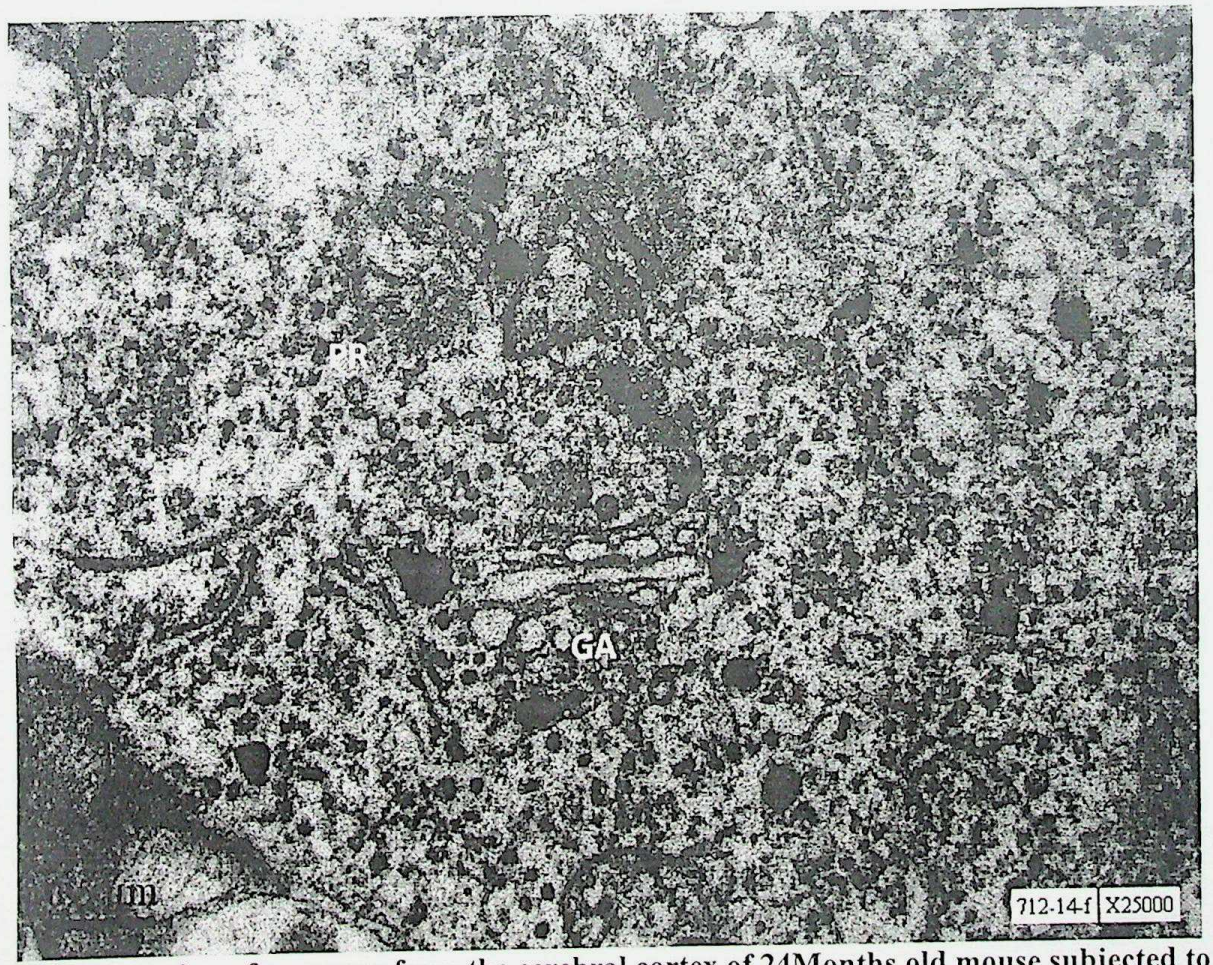


Fig 2 A portion of a neuron from the cerebral cortex of 24Months old mouse subjected to dietary restriction from 3 months of age.

Plate 15: Ultrastructural details studied through TEM

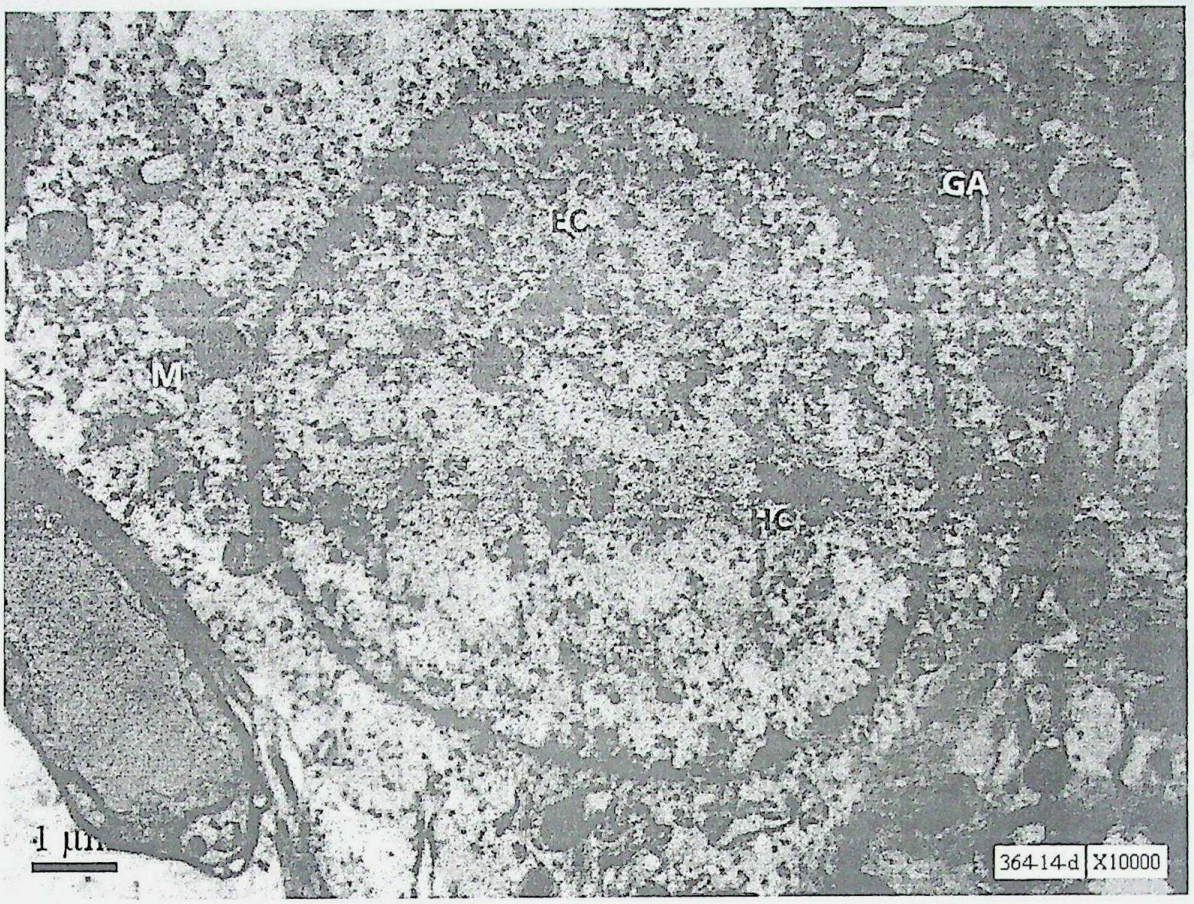


Fig 1 A neuron from the Hippocampus of 24 Months old *ad libitum* fed mouse



Fig 2 A neuron from the Hippocampus of 24 Months old mouse subjected to DR after 3Months of age

Plate 16: Ultrastructural details studied through TEM

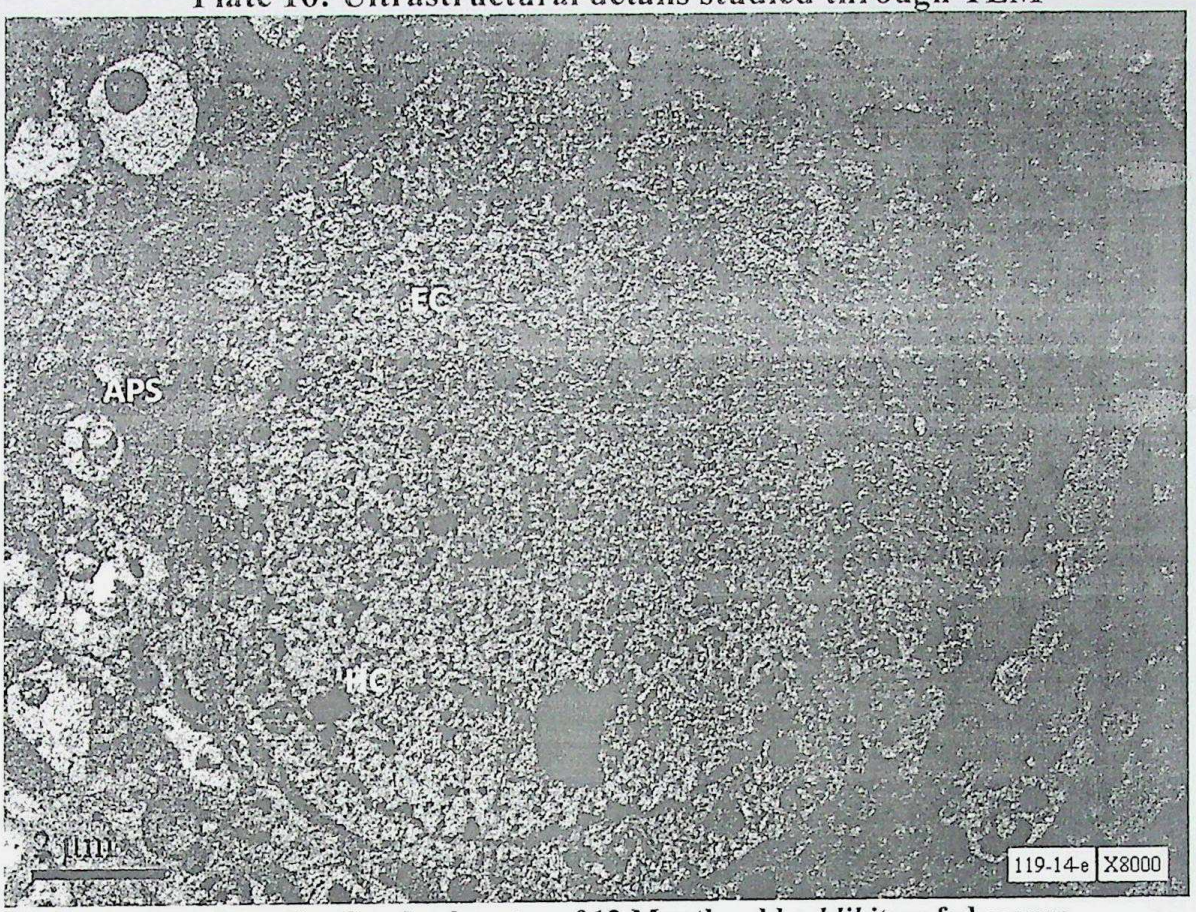


Fig 1 A neuron from the Cerebral cortex of 12 Months old *ad libitum* fed mouse

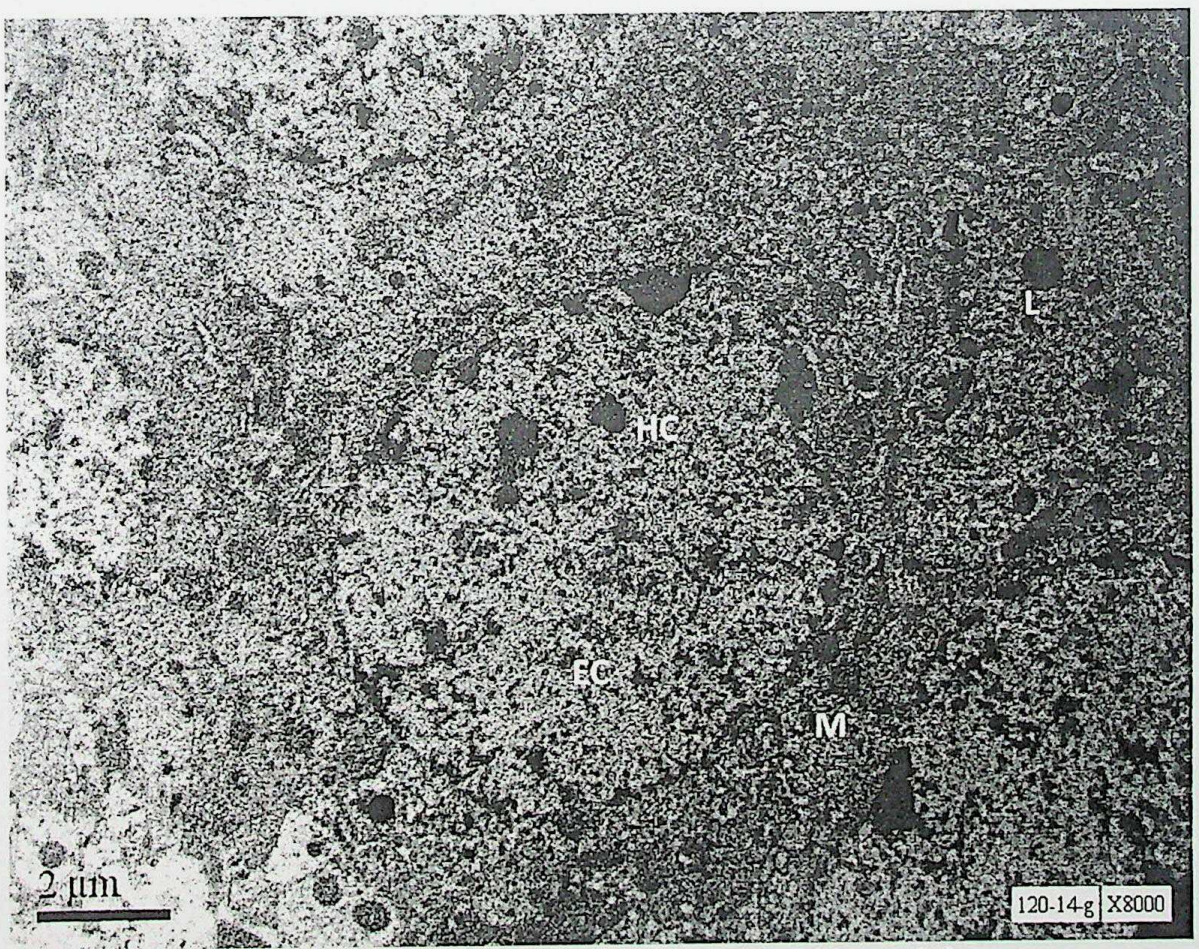


Fig 2 A neuron from the Hippocampus of 12 Months old *ad libitum* fed mouse

autophagosome containing material under degeneration was observed (Plate 11 Fig 2).

- The neuron of the hippocampal region of 18 months old mouse subjected to dietary restriction after completion of 10 months of age exhibited prominent nucleus containing predominant euchromatin and scanty heterochromatin. The cytoplasm exhibited abundance of RER and Nissl substance. Mitochondria were normal in size and shape. The neuron exhibited lipofuscin granules in it. (Plate 10 and Fig 3 Plate 17 Fig 1).
- The neuron from Cerebral cortex of 24 months old *ad libitum* fed mouse exhibited predominance of heterochromatin ; damaged mitochondria, loss of Nissl substance and accumulation of lipofuscin granules. At 20000X magnification lipofuscin granules were conspicuously seen. (Plate No. 13 and 14 Fig 1)
- A neuron from cerebral cortex of 24 months old mouse subjected to dietary restriction after 3 months of age exhibited well maintained nucleus with majority of the chromatin in euchromatin form. Cytoplasm exhibited abundance of well-maintained of RER, Nissl granules, healthy mitochondria with normal size and shape. (Plate No. 13 Fig 2)

  At 25,000 X magnification this neuron exhibited Golgi apparatus with inflated cisternae, indicating their secretary activity. There were abundance of polyribosomes and rough endoplasmic reticulum. (Plate No. 14 Fig 2)

- A neuron from hippocampus of 24 months *ad libitum* fed mouse exhibited prominent nucleus with euchromatin and heterochromatin. Lipofuscin granules were scarce. Cytoplasm exhibited few swollen mitochondria along with normal mitochondria. Golgi cisternae were also noticed. (Plate No. 15 Fig 1)
- A hippocampal neurons from 24 months old mouse subjected to dietary restriction after three months of age exhibited abundance of RER and Nissl substance, mitochondria in normal size and shape. Golgi cisternae were inflated. Lipofuscin granules were few and small in size. (Plate No. 15 Fig 2)
- A neuron from Cerebral cortex of 12 months old *ad libitum* fed mouse exhibited conspicuous nucleus with majority of euchromatin. The cytoplasm revealed RER and Nissl substance, mitochondria with normal size and shape, very low number of lipofuscin granules. (Plate No. 16 Fig 1)
- A neuron from hippocampus of 12 months *ad libitum* fed mouse exhibited conspicuous nucleus with majority of euchromatin. The cytoplasm revealed RER and

Nissl substance, mitochondria with normal size and shape, very low number of lipofuscin granules. (Plate No. 16 Fig 2)

When ultrastructural details of hippocampal neuron observed from diet restricted groups were compared to neurons from *ad libitum* fed mouse revealed that dietary restrictions maintains the cytoarchitecture of neurons to youthful condition. It promotes autophagy as evidenced by autophagosomes in these neurons.

When age dependent alterations in neurons from cerebral cortex were compared with neurons from hippocampus it was observed that the hippocampal neurons exhibit less deleterious changes and did not show dramatic alterations in cytoarchitecture which was otherwise observed in cerebrocortical neurons.Similar type of ultrastructure was noticed in the neuron of cerebral cortex after 18 and 24 months of age in diet restricted group. This indicates dietary restriction slows down the process of cellular deterioration which occurred in ad libitum fed mouse in age dependant fashion.

E. Study of lysosomal membrane integrity

Effect of dietary restriction on lysosomal membrane integrity in various regions of mice brain

Acid phosphatase is a lysosomal enzyme that hydrolyses organic phosphatase at an acidic pH (Henneberry *et al.,* 1979). The acid phosphatase activity in lysosomal and post lysosomal fraction was determined as µmols of *p*-nitrophenol/mg protein. The study of lysosomal membrane integrity was analysed by presence of acid phosphatase in lysosomes and in the cytoplasm. Appearance of lysosome specific enzyme acid phosphatase in the cytoplasm indicates release or leakage of lysosomal contents, due to damage to the lysosomal membrane. If the amount of enzyme present within the lysosomes is more and lower in the cytosol then the ratio of lysosomal to post lysosomal acid phosphatase is greater than one indicates the stable state of lysosomal membrane. If this ratio is less than one indicates leakage of the enzyme from the membrane. This suggests the loss of lysosomal membrane integrity.

Study of lysosomal membrane integrity in Cerebral cortex:-

Observations are presented in Table No.4 (Graph No. 19, 20 and 21).

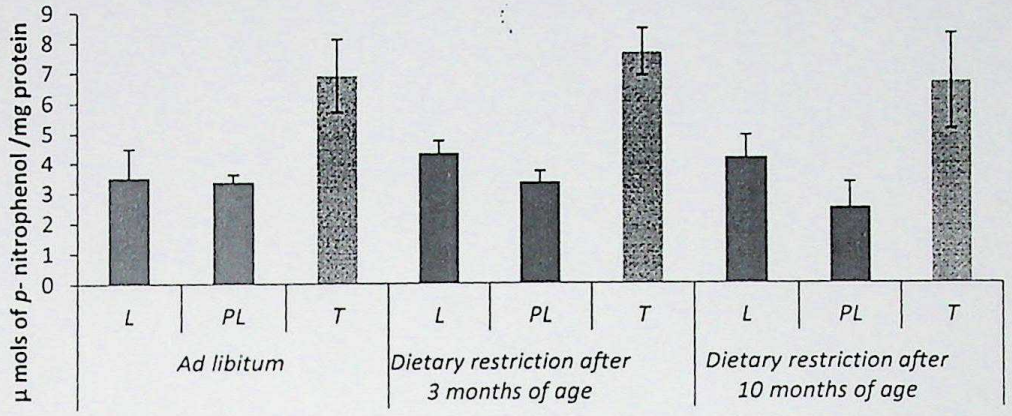
In case of cerebral cortex of *ad libitum* fed 12 months old mice the lysosomal acid phosphatase activity was 3.52 ± 0.96 µmols of *p*-nitrophenol/mg protein. The post lysosomal

Table no4:- Effect of Dietary restriction(DR) on lysosomal membrane integrity in Cerebral cortex.

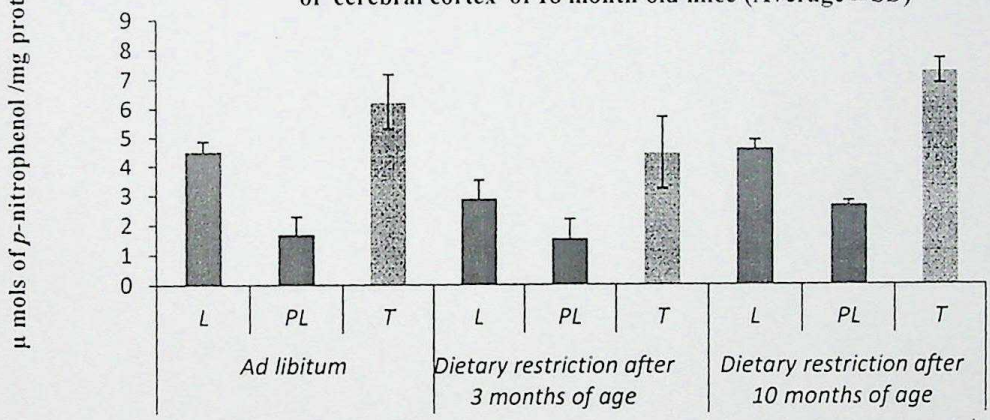
Sr.no	Experimental groups	Acid phosphatase activity (μ mols of *p*-nitrophenol generated/ mg protein)			
	12 months	**Lysosomal**	**Post lysosomal**	**Total activity**	**Ratio**
1	*Ad libitum*	3.52 ± 0.96	3.37 ±0.24	6.89± 1.2	1.04
2	DR after 3 months of age	4.32 ± 0.41	3.33 ± 0.37	7.65 ±0.78	1.29
3	DR after 10 months of age	4.16± 0.74	2.48 ± 0.83	6.66± 1.57	1.67
	18 months				
4	*Ad libitum*	4.52 ± 0.35	1.70 ± 0.58	6.22± 0.93	2.65
5	DR after 3months of age	2.88 ± 0.61	1.54±0.64	4.43 ± 1.25	1.87
6	DR after 10 months of age	4.58 ± 0.30	2.66 ± 0.12	7.24 ± 0.42	1.72
	24 months				
7	*Ad libitum*	2.06 ± 0.02	1.31 ± 0.30	3.38 ± 0.090	1.57
8	DR after 3 months of age	6.40 ± 1.04	3.29 ± 0.56	9.69 ± 1.6	1.94
9	DR after 10 months of age	2.22 ± 0.09	1.09 ± 0.0.068	3.31 ± 0.15	2.03

Results expressed as mean ± SD of 6 animals

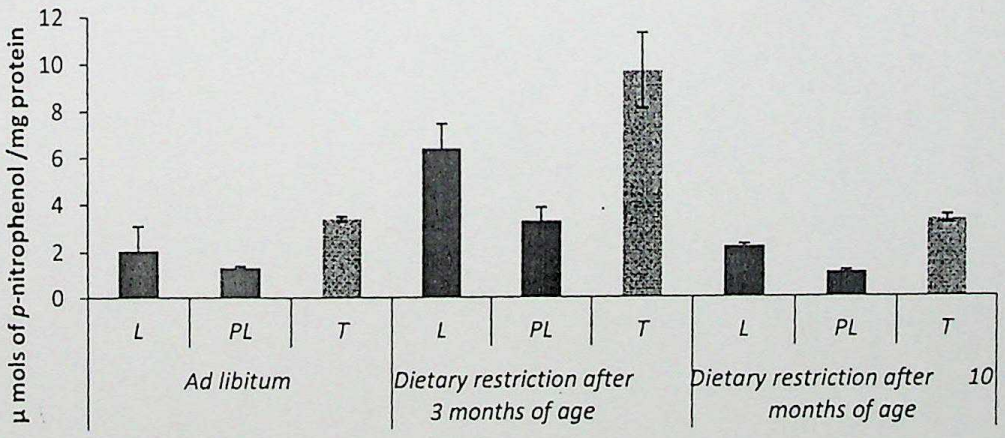
Graph No:19. Effect of dietary restriction on lysosomal membrane integrity of cerebral cortex of 12 month old mice. (Average ± SD)



Graph No: 20. Effect of dietary restriction on lysosomal membrane integrity of cerebral cortex of 18 month old mice (Average ± SD)



Graph NO:21. Effect of dietary restriction on lysosomal membrane integrity in cerebral cortex of 24month age mice. (Average ± SD)



acid phosphatase activity was 3.37 ± 0.24 µmols of *p*-nitrophenol/mg protein. The ratio of lysosomal to post lysosomal acid phosphatase was 1.041 which is greater than1. The total acid phosphatase activity was 6.89 ± 1.2 µmols of p-nitrophenol/mg protein. In mice subjected to dietary restriction after 3 months of age the lysosomal enzyme activity was 4.32 ± 0.41 µmols of *p*-nitrophenol/mg protein. The post lysosomal acid phosphatase activity was 3.33 ± 0.37 µmols of *p*-nitrophenol/mg protein. Therefore the ratio was 1.3 which is greater than 1. The total enzyme activity was 7.65 ± 0.78 µmols of *p*-nitrophenol/mg protein in lysosomal and post lysosomal fraction. In mice subjected to dietary restriction after 10 months of age the lysosomal enzyme activity was 4.16± 0.74 µmols of *p*-nitrophenol/mg protein. The post lysosomal enzyme activity was 2.48 ± 0.83 µmols of *p*-nitrophenol/mg protein. Hence, the ratio was 1.67 which is greater than 1. The total enzyme activity was 6.66 ± 1.57 µmols of p-nitrophenol/mg protein.

In case of cerebral cortex of *ad libitum* fed 18 months old mice the lysosomal acid phosphatase activity was 4.52 ± 0.35 µmols of *p*-nitrophenol/mg protein. The post lysosomal acid phosphatase activity was 1.70 ± 0.58 µmols of *p*-nitrophenol/mg protein. The ratio of lysosomal value to post lysosomal acid phosphatase was 2.65 which is greater than 1. This is greater than 1. The total acid phosphatase activity was 6.22 ± 0.93 µmols of *p*-nitrophenol/mg protein. In mice subjected to dietary restriction after 3 months of age the lysosomal enzyme activity was 2.88 ± 0.61 µmols of *p*-nitrophenol/mg protein. The post lysosomal acid phosphatase activity was 1.54 ± 0.64 µmols of *p*-nitrophenol/mg protein. Therefore the ratio was 1.87 which greater than 1. The total enzyme activity was 4.43 ± 1.25 µmols of *p*-nitrophenol/mg protein in lysosomal and post lysosomal fraction. In mice subjected to dietary restriction after 10 months of age the lysosomal enzyme activity was 4.58 ± 0.30 µmols of *p*-nitrophenol/mg protein. The post lysosomal enzyme activity was 2.66 ± 0.12 µmols of *p*-nitrophenol/mg protein. Hence, the ratio was 1.72 which greater than 1. The total enzyme activity was 7.24 ± 0.42 µmols of *p*-nitrophenol/mg protein.

In case of cerebral cortex of *ad libitum* fed 24 months old mice the lysosomal acid phosphatase activity was 2.06 ± 0.02 µmols of *p*-nitrophenol/mg protein. The post lysosomal acid phosphatase activity was 1.31 ± 0.07 µmols of *p*-nitrophenol/mg protein. The ratio of lysosomal value to post lysosomal acid phosphatase was 1.57 which greater than 1. The total acid phosphatase activity was 3.38 ± 0.09 µmols of p-nitrophenol/mg protein. In mice subjected to dietary restriction after 3 months of age the lysosomal enzyme activity was 6.40 ± 1.04 µmols of *p*-nitrophenol/mg protein. The post lysosomal acid phosphatase activity was 3.29 ± 0.56 µmols of p-nitrophenol/mg protein. The ratio was 1.94 which greater than 1. The

total enzyme activity was 9.69 ± 1.6 µmols of *p*-nitrophenol/mg protein in lysosomal and post lysosomal fraction. In mice subjected to dietary restriction after 10 months of age the lysosomal enzyme activity was 2.22 ± 0.09 µmols of *p*-nitrophenol/mg protein. The post lysosomal enzyme activity was 1.09 ± 0.06 µmols of *p*-nitrophenol/mg protein. Hence, the ratio was 2.04 which greater than 1. The total enzyme activity was 3.31 ± 0.22 µmols of p-nitrophenol/mg protein.

Study of lysosomal membrane integrity in Hippocampus:-

Observations are presented in Table No. 5 (Graph No. 22,23,24).

In case of hippocampus of *ad libitum* fed 12 months old mice the lysosomal acid phosphatase activity was 1.75 ± 0.88 µmols of *p*-nitrophenol/mg protein. The post lysosomal acid phosphatase activity was 0.56 ± 0.17 µmols of p-nitrophenol/mg protein. The ratio of lysosomal to post lysosomal acid phosphatase was 3.125 which is greater than 1. The total acid phosphatase activity was 2.31 ± 1.05 µmols of *p*-nitrophenol/mg protein. In mice subjected to dietary restriction after 3 months of age the lysosomal enzyme activity was 3.03 ± 0.33 µmols of *p*-nitrophenol/mg protein. The post lysosomal acid phosphatase activity was 1.05 ± 0.1 µmols of *p*-nitrophenol/mg protein. Therefore the ratio was 2.87 which is greater than1. The total enzyme activity was 4.09 ± 0.43 µmols of p-nitrophenol/mg protein in lysosomal and post lysosomal fraction. In mice subjected to dietary restriction after 10 months of age the lysosomal enzyme activity was 3.12 ± 0.93 µmols of *p*-nitrophenol/mg protein. The post lysosomal enzyme activity was 1.80 ± 0.58 µmols of *p*-nitrophenol/mg protein. Hence, the ratio was 1.73 which is greater than 1. The total enzyme activity was 4.92 ± 1.51 µmols of *p*-nitrophenol/mg protein.

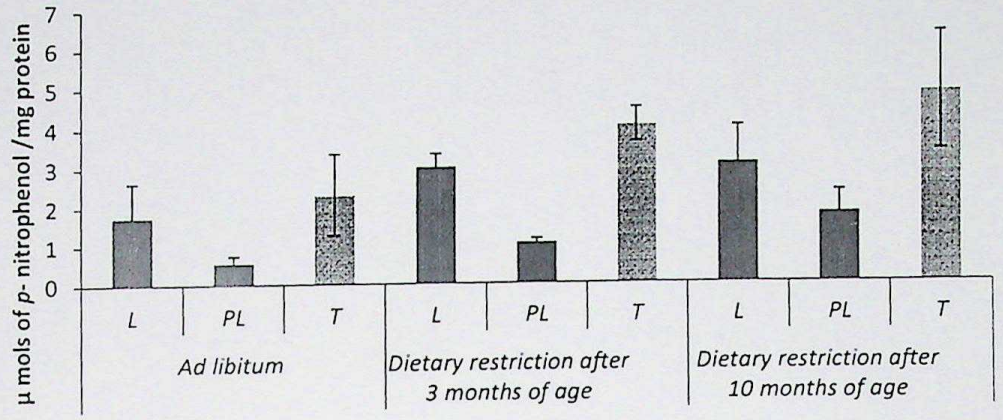
In hippocampus of *ad libitum* fed 18 months old mice the lysosomal acid phosphatase activity was 2.25 ± 0.35 µmols of *p*-nitrophenol/mg protein. The post lysosomal acid phosphatase activity was 1.17 ± 0.04 µmols of *p*-nitrophenol/mg protein. The ratio of lysosomal to post lysosomal acid phosphatase was 1.92 which is greater than 1. The total acid phosphatase activity was 3.42 ± 0.40 µmols of *p*-nitrophenol/mg protein. In mice subjected to dietary restriction after 3 months of age the lysosomal enzyme activity was 1.62 ± 0.36 µmols of *p*-nitrophenol/mg protein. The post lysosomal acid phosphatase activity was 0.33 ± 0.08 µmols of *p*-nitrophenol/mg protein. Therefore the ratio was 1.95 which is greater than 1. The total enzyme activity was 3.61 ± 0.44 µmols of p-nitrophenol/mg protein in lysosomal and post lysosomal fraction. In mice subjected to dietary restriction after 10 months of age the lysosomal enzyme activity was 3.04 ± 0.44 µmols of *p*-nitrophenol/mg protein. The post

Table no5:- Effect of Dietary restriction (DR)on lysosomal membrane integrity in hippocampus

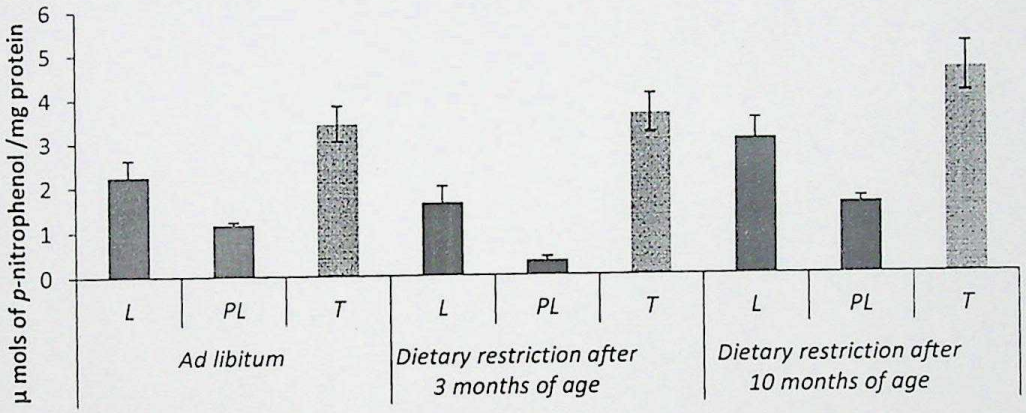
Sr.no	Experimental groups	Acid phosphatase activity (µ mols of *p*-nitrophenol generated/ mg protein)			
	12 months	**Lysosomal**	**Post lysosomal**	**Total activity**	**Ratio**
1	*Ad libitum*	1.75 ± 0.88	0.56 ± 0.17	2.31 ± 1.05	3.12
2	DR after 3 months of age	3.03 ± 0.33	1.05± 0.1	4.09 ± 0.43	2.87
3	DR after 10 months of age	3.12 ± 0.93	1.80± 0.58	4.92 ± 1.51	1.73
	18 months				
4	*Ad libitum*	2.25 ± 0.36	1.17± 0.044	3.42 ± 0.40	1.92
5	DR after 3 months of age	1.62 ± 0.36	0.33 ± 0.08	3.61 ± 0.44	1.95
6	DR after 10 months of age	3.04 ± 0.44	1.57± 0.12	4.61 ± 0.56	1.94
	24 months				
7	*Ad libitum*	3.22 ± 0.63	1.09 ± 0.026	4.31 ± 0.65	2.93
8	DR after 3 months of age	6.85± 0.21	1.61± 0.53	8.46 ± 0.74	4
9	DR after 10 months of age	5.75 ± 1.03	1.99 ± 0.24	7.74± 1.2	2.88

Results expressed as mean ± SD of 6 animals

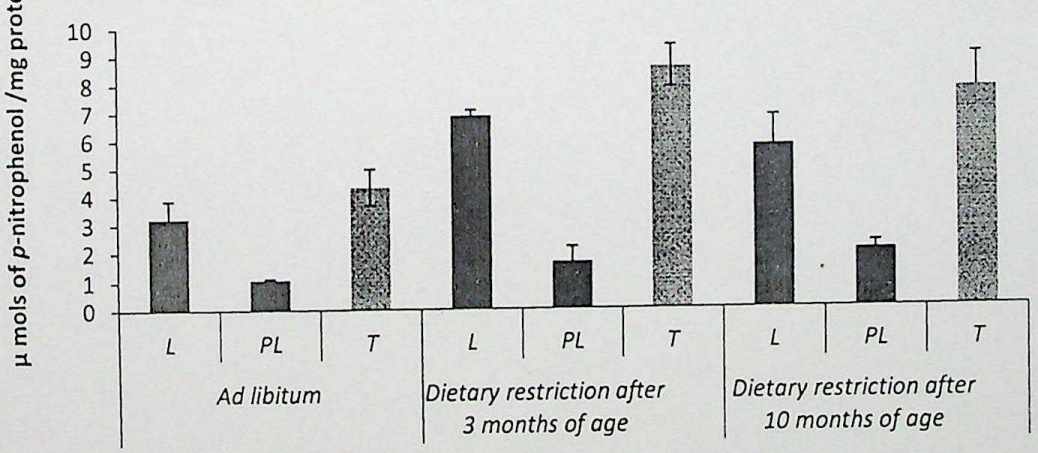
Graph No:22. Effect of dietary restriction on lysosomal membrane integrity of Hippocampus of 12 month old mice. (Average± SD)



Graph No :23. Effect of dietary restriction on lysosomal membrane integrity of Hippocampus of 18 month old mice. (Average ± SD)



Graph No :24. Effect of dietary restriction on lysosomal membrane integrity of Hippocampus of 24 month old mice. (Average± SD)



lysosomal enzyme activity was 1.57± 0.12 µmols of *p*-nitrophenol/mg protein. Hence, the ratio was 1.94 which is greater than 1. The total enzyme activity was 4.61 ± 0.56 µmols of *p*-nitrophenol/mg protein.

In case of hippocampus of *ad libitum* fed 24 months old mice the lysosomal acid phosphatase activity was 3.22 ± 0.63 µmols of *p*-nitrophenol/mg protein. The post lysosomal acid phosphatase activity was 1.09 ± 0.026 µmols of *p*-nitrophenol/mg protein. The ratio of lysosomal to post lysosomal acid phosphatase activity was 2.93 which is greater than 1. The total acid phosphatase activity was 4.31 ± 0.65 µmols of *p*-nitrophenol/mg protein. In mice subjected to dietary restriction after 3 months of age the lysosomal enzyme activity was 6.85 ± 0.21 µmols of *p*-nitrophenol/mg protein. The post lysosomal acid phosphatase activity was 1.61 ± 0.053 µmols of *p*-nitrophenol/mg protein. The ratio was 3.03 which is greater than 1. The total enzyme activity was 8.46 ± 0.74 µmols of *p*-nitrophenol/mg protein in lysosomal and post lysosomal fraction. In mice subjected to dietary restriction after 10 months of age the lysosomal enzyme activity was 5.75 ± 1.03 µmols of *p*-nitrophenol/mg protein. The post lysosomal enzyme activity was 1.99 ± 0.24 µmols of *p*-nitrophenol/mg protein. Hence, the ratio was 2.88 which is greater than 1. The total enzyme activity was 7.74 ± 1.2 µmols of *p*-nitrophenol/mg protein.

Discussion:

The cerebral cortex and hippocampus of the *ad libitum* fed mice exhibited age dependent cytological alterations such as deposition of neurofibrillary tangles, senile plaques, accumulation of lipofuscin granules and loss of Nissl substance. During the course of normal ageing, there is imbalance between formation of free radicals and their scavenging by the antioxidants (Harman 1956,1992). This results in generation of oxidative stress. The free radicals being unstable, to gain the stability hit the biomolecules and steal the electron and make the target biomolecule unstable. Such oxidatively damaged biomolecules interact with other biomolecules and crosslink. As a result, the biomolecules lose their regular functions. The functionless macromolecules are given to the lysosomes by ubiquitination (Ming *et al.*, 2015).

Over the period of time, where oxidative stress overwhelms the cell's repair or compensatory system, the cell deteriorates in its structure and ultimately leads to cell death. Among the post mitotic cells neurons experience such oxidative insults more than any other cell. This is because neurons are provided with highest oxygen supply (Halliwell 2006). It is rich in polyunsaturated fatty acids (Chen *et al.*, 2008). In cell which can rejuvenate by cell

division, there is decrease in the oxidatively damaged molecules from the cytoplasm during cell division. This is due to distribution of cytoplasm into two daughter cells. However, neurons being post mitotic and terminally differentiated cells, they don't have this opportunity. As a result the neurons experience many cytoarchitectural changes in them as they age. Such alterations are accumulation of neurofibrillary, lipofuscin granules and senile plaques.

There was age dependent accumulation of neurofibrillary tangles in cerebral cortex and hippocampus of *ad libitum* fed mice. Neurofibrillary tangles are generated due to hyperphosphorylation of a nerve cell specific protein tau, which is associated with neuronal microtubules (Iqbal *et al.*, 2010). A typical shape of a neuron is supported and maintained in that form as it is, due to tau protein and microtubules. During ageing, there is loss of this cytoskeleton due to oxidative damage to these proteins. Hyperphosphorylation of tau results in loss of microtubules. These ultimately aggregate into helical filaments in pair, so called paired helical filaments. These paired helical filaments are composed of hyperphosphorylated tau. These paired helical filaments which furthermore aggregate into neurofibrillary tangles.

One of the pathological alterations occurring in the brain during ageing is accumulation of insoluble aggregates within the neurons, called as neurofibrillary tangles (NFTs) (Delacourte 2005, Duan *et al.* 2012). In the present study, in *ad libitum* fed mice there was progressive deposition of neurofibrillary tangles in cerebral cortex and hippocampus over the age. This could be due to the damage to the microtubules. The neurons are internally supported by microtubules. Tau is a microtubule associated protein present in the neurons. It interacts with α and β tubulin to stabilize the microtubules. Tau protein is very sensitive to phosphorylation. There are 79 sites (serine and threonine) in tau which are the targets for phosphorylation (Hampel 2010). Melov *et al.* (2007) have shown that mitochondrial oxidative stress causes hyperphosphorylation of the tau. Strocchi *et al.* (2003) demonstrated that tau phosphorylating enzyme Cdk5 is stimulated by oxidative stress. Lee *et al.* (2007) revealed that GSK3β; an enzyme catalyzing the tau phosphorylation is activated during oxidative stress. Rodriguez *et al.* (2013) described that abnormal phosphorylation of tau is a consequence of oxidative stress. Free radical theory of ageing (Harman 1956, 1992) and mitochondrial lysosomal axis theory of ageing (Brunk and Terman 2002) speculate the increased oxidative stress is the cause of ageing. Therefore, the age dependent increased deposition of NFTs in *ad libitum* fed mice was the result of oxidative stress mediated tau phosphorylation. Rodriguez *et al.* (2013) further described that the oxidative stress models of

in vivo and *in vitro* studies are characterized by the presence of abnormally hyperphosphorylated tau.

In the present investigations the dietary restriction groups exhibited significantly reduced deposition of NFTs in cerebral cortex and hippocampus. However, the mice subjected to 40% dietary restriction from three months of age exhibited more beneficial effects in healthier neuronal morphology as compared to the mice in which dietary restriction was commenced after 10 months of age. Dietary restriction was found to be beneficial in reducing the oxidative stress and tau phosphorylation (Trepanowski *et al.* 2011). There is a positive correlation between oxidative stress and tau phosphorylation. (Saxena 2011, Duan *et al.* 2012). Thus the observations from the present work indicate that dietary restriction prevents or slows down the deposition of neurofibrillary tangles.

Another cytological alteration observed in the neurons of *ad libitum* fed mice was accumulation of lipofuscin granules. These are residual bodies or remnant of undigested substances within the lysosomes. All biomolecules including macromolecules have their unique span of function. Once the particular function is over it is given for degradation to the lysosomes or proteasomes. Messenger RNAs after translation are degraded. The cytoplasmic proteins including cytoskeleton proteins and enzymes also undergo lysosomal/proteasomal degradation. This process is a part of routine cellular turnover. Within the lysosomes there is complete degradation of these biomolecules into simple form. For example, proteins are degraded into amino acids, RNAs are degraded into nucleotides which in turn are used for synthesis of new macromolecules.

If the protein, lipids and other molecules entering into the lysosomes are oxidatively modified, they become resistant for lysosomal digestion (Brunk and Ericsson 1972). The oxidatively damaged/senescent mitochondria present in the lysosomes, continue to produce free radicals (Collins *et al.,* 1980). The metalloproteins during their lysosomal degradation contribute to oxidative stress by virtue of their metal ions such as Fe and Cu. The metal ions react with hydrogen peroxide to generate OH radical, the worst free radical associated with cellular damage. Therefore, the lysosomes containing such components become the source of free radicals. This causes oxidative damage to lysosomal enzymes making them inefficient to digest the lysosomal contents. In addition the oxidative damage occurs to lysosomal membrane that leads to decrease in lysosomal membrane integrity as depicted in the results of acid phosphatase activity.

Thus, lysosomes become inefficient to digest the contents present in its lumen. As a result these substances including damaged mitochondria go on accumulating in the form of residual bodies, what is called as lipofuscin granules (Brunk and Terman 2002). Thus the observed accumulation of lipofuscin granules in the cerebral cortex and hippocampus is age related cytological alteration due to oxidative damage. However, in 40% diet restricted mice there were highly significant lower accumulation of lipofuscin granules. The results suggest that 40% diet restriction is beneficial in improving the lysosomal metabolism and thereby reducing the lysosomal accumulation of cellular garbage. Diet restriction from the period of attaining sexual maturity i.e. after 3 months of age was found to be the most beneficial which was evidenced by transmission electron microscopy. The lysosomes in diet restricted group were exhibiting autophagy. Hormetic stresses induce lysosomal autophagy. Ward and Shibatani (1994) reported that the decrease in the proteolytic activity is prolonged by caloric restriction in mice and rats along with extending the life. The rate of intracellular protein degradation is slowed down during ageing (Terman and Brunk 2002). According to Van Remmen *et al.*, (1995) degradation of damaged molecules by proteolysis is enhanced by the CR. Cuervo and Dice (2000) reviewed the age dependent decline in proteolysis in various organisms such as nematodes, fruit flies and rats. Weindruch et al (1986) carried out early work on dietary restriction in mice and found that dietary restriction has antiageing effect.

In *ad libitum* fed group there was another ageing related pathological feature noticed i.e. deposition of senile plaques. The senile plaques are composed of a protein fragment termed as β amyloid. In the neurons, there is a transmembrane amyloid precursor protein involved in neurite outgrowth, synaptogenesis, neuronal protein trafficking along the axon, transmembrane signal transduction, cell adhesion either directly or through its various cleavage products (Zheng and Koo 2006). The amyloid precursor protein is cleaved by various secretases. One of the cleavage products is β amyloid that gets deposited extracellularly and affects neuronal function. In the brain of *ad libitum* fed group, there was no appearance of senile plaques at 12 and 18 months of age in both the groups. After 24 months of age the *ad libitum* fed group was exhibiting 5 senile plaques in the entire cerebral cortex, whereas, the diet restricted group (3 months onset) exhibited just one per cerebral cortex and 10 months onset group exhibited two senile plaques per cerebral cortex. This suggest that dietary restriction prevents deposition of amyloid β and hence formation of senile plaques. Qin *et al.*, (2006) demonstrated that dietary restriction or treatment of resveratrol stimulates the activation of Silent information regulator 2 (SIR2)/ SIRT1. SIRT1 in the brain of person with AD delays the formation of Aβ.

In hippocampus after 12 and 18 months there was no any senile plaque in *ad libitum* fed group so also in both the diet restricted groups. After 24 months in *ad libitum* fed group there was only one senile plaque in the entire hippocampus whereas in both the groups of dietary restriction there was not a single senile plaque in hippocampus region. These results again suggest that dietary restriction is beneficial in prevention of deposition of senile plaques. Qin *et al.*, (2006) reported that the DR prevents the Aβ plaque formation in the hippocampus and cortex. It also inactivates the neuronal apoptotic mechanism resulted due to the Aβ toxicity.

The results of cerebral cortex and hippocampus highlight that hippocampus is more protected. Pasinetti *et al.*, (2007) found that dietary restriction led to significant rise in the insulin degrading enzyme (IDE) in the brain of mouse model of Alzheimer's disease. Ferris *et al.*, (2003) found that IDE is involved in degradation of Aβ. Our findings are in accordance with the work demonstrated by Patel *et al.*, (2005) where the researchers reported the amyloid β lowering efficacy of dietary restriction in mouse models of AD amyloid neuropathology.

Thus, in light of the existing literature on dietary restriction, the beneficial effects observed in terms of absence of amyloid plaque in the present observation might be due to decreased production of APP, increased production of α secretase, down regulation of β and γ secretases, decrease in insulin and increase in insulin degrading enzyme, which *in toto* prevented the brain of these animals from the deposition of amyloid plaques.

In diet restricted group since the neurons were maintained to the heathier form (as revealed by light microscopy and electron microscopy), the production of APP might be down regulated. As a result there might be decrease in its processing by various secretases. This might have resulted in absence of deposition of amyloid plaques in diet restricted groups even after 18 months of age and significantly lower number of plaques after 24 months of age.

Ultrastructural details of neurons from mice subjected to Dietary restriction after 3 months of age when compared with neurons dietary restriction subjected from 10 months of age exhibited predominantly autophagosomes in DRASM group. This indicates from 4th month to 10th month i.e. in a span of seven month routinised the process of autophagy.

Another reason of no any deposition of senile plaque up to 18 months in diet restricted group and significantly lower number of senile plaque in this group after 24 months could be increased rate of crinophagy. It is a process of autophagy in which the

secretary vesicles instead of dispatching towards the plasma membrane, are delivered towards the lysosomes. As a result the various enzymes including β secretase and γ secretase might be delivered to lysosomes rather than secreting outside. Therefore, these enzymes might not be available in sufficient amount for amyloidosis. The β and γ secretases are involved in generation of β amyloid.

Craft and Watson (2004) demonstrated that higher levels of insulin increase the risk of deposition of β amyloid. Gasparini *et al.* (2001) found that insulin promotes the generation of extracellular amyloidogenic amyloid β peptides. 40% dietary restriction obviously reduces the normal requirement of insulin level in the circulations to a significant extent. As a result it might have contributed to the healthy cytoarchitecture in the neurons. Wang *et al.* (2005) found that 9 month dietary restriction almost completely prevented Alzheimer's disease amyloid plaque formation in cortical and hippocampal neurons, Wang *et al.* (2005) further more found that the beneficial effects of dietary restriction are due to stimulating α secretase activity that cleaves the amyloid precursor protein (APP) into secretary APP and that prevents accumulation dietary restriction resulted in of β amyloid. They found more than 2 fold elevation in the α secretase activity in the AD more model Tg 2576 an compared to ad libitum fed control Tg 2576 mice.

In the present investigations the significantly decreased amyloid plaques in cerebral cortex and hippocampus of diet restricted group might be due to stimulating α secretase action in these animals.

As the life expectancy or average life span is increased the burden of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, dementia is getting increased. Luchsinger et al (2002) described that increasing dietary intake has a potential threat of neurodegenerative diseases. Sriram *et al.* (2002) and Gustafson *et al.* (2003) found that there is a direct correlation between obesity and neurodegenerative diseases. Pasinetti *et al.* (2007) found that high caloric intake promotes B amyloidosis whereas dietary restriction is able to prevent it.

As per the observations made by Kubo, et al., (1987), Iwasaki, *et al.,* (1988), Khorakova *et al.,* (1990), Shimokawa, *et al.,* (1996) Pamplona *et al.,* (2006) Sanz *et al.,* (2006a and b) the restriction of carbohydrate or the lipid in the diet cannot protect the DNA from oxidative damage. According to Pamplona *et al.,* (2006) restricting the protein in the diet found to increase the life span by 20%. Sanz *et al.,* (2006c) Caro *et al.,* (2008) observed that the generation of ROS by mitochondria and damage to mitochondrial DNA is lowered by

40% methionine restriction in the diet. Along with life prolonging effect DR has been shown to improve the general health of an organism. According to Sohal Weindruch, (1996) aging of an organism is due to increasing the oxidative damage with age. By restricting the caloric intake the oxidative stress is lowered. According to Ingram et al., (1987), Intermittent fasting (IF) i.e. food every other day prevents the ischemic heart injury and diminish myocardial infarction (MI), cardiac remodelling by antiapoptotic and anti-inflammatory mechanisms. Logroscino *et al.,* (1996) Grant (1997), Mayeux *et al.,* (1999) reported that caloric restriction lowers the risk of Alzheimer's disease (AD), Parkinson's disease (PD) and stroke. The observations of Duan, *et al.,* (2001), Anson *et al.,* (2003), Mattson *et al.,* (2005) suggest that the risk of diseases in many systems is declined by the dietary restriction.

Dietary restriction induces mild stressful conditions on the cell. This could increase the efficiency of lysosomes to completely degrade the material delivered to them for recycling. This may occur by two mechanisms. First, 40% dietary restriction reduces rate of glycolysis and TCA by 40%. As a result there is net decrease in generation of free radicals during mitochondrial metabolism by 40% or more. Prima facie it seems to be 40% however it must be more. This is because the oxidatively damaged mitochondria and metalloproteins continue to produce increasingly large number of free radicals. Thus, in diet restricted group there must be decreased oxidative stress. This is evidenced by decreased lipid peroxidation in in the cerebral cortex and hippocampus of the diet restricted group.

Second, 40% dietary restriction might have stimulated the cell's clean up functions to the most efficient mode via stimulating the stress response pathway. In *ad libitum* feeding, there is abundance of the supply of cell's requirements through the diet. Therefore, the supply to demand ratio is very high. However, in 40% diet restricted group this need based supply is curtailed to 60% instead of 100% or more. This exerts the mild stressful condition on the cell, which stimulates autophagy and crinophagy to efficiently recycle the macromolecules.

This leads to onset of other pathways to fulfil this demand such as autophagy, crinophagy. As a result the lysosomal recycling might have occurred most efficiently, resulting into highly significant reduction in deposition of lipofuscin granules in diet restricted group than the *ad libitum* fed group. Moreover, the beneficial effects of 40% dietary restriction were found to be more in the group in which dietary restriction was commenced after attaining sexual maturity i.e. after 3 months of age.

This could be due to programming the lysosomes and secretary pathway to work in utilization of every content of the lysosome with 100% efficiency of recycling since from an early age of the cell i.e. three months. Therefore, from three months itself the neurons from the diet restricted group might have evoked a mechanism for complete utilization and degradation of the material dispatched to the lysosomes. Therefore, there must be significantly reduced number of lipofuscin granules as compared to the diet restricted group in which dietary restriction was commenced after 10 months of age.

Weindruch *et al.*, (1980, 1984) described age related increase in free radical formation and per oxidative membrane damage, age related lower activities of several inner membrane enzymes and decreased production of ATP. Weindruch *et al.*, (1986) further more claimed that the dietary restriction retards the process of ageing via mitochondrial effects.

Earlyonset of dietary restriction is giving more protection to the neurons as compared to late onset. This is obvious because the cellular damage is result of stepwise alterations in biochemical mechanisms. Therefore, if this cascade of damage is intervened in early steps, the cell gets more protection, which is observed in the present study.

Dietary restriction after 10 months of age, also gives beneficial effects in preservation of cytoarchitecture of neurons, however if early steps of molecular damage are completed then the damage can not be controlled to the extent that is observed in onset of dietary restriction after 3 months of age.

Thus findings of the present study indicate beneficial effects of dietary restriction in preservation of neuronal cytoarchitecture to youthful condition.

List of publications:

Pathan KH, Gajare KA, ***Deshmukh AA*** *(2015) Cytopathological study of neurofibrillary tangles incerebral cortex and hippocampus of swiss albino mouse: Mus musculus. J Pharma Biol. 5(4): 314-319.*

Pathan KH, Gajare KA, ***Deshmukh AA.*** *(2015). Ultrastructural study reveals that mouse hippocampal neurons are more protected than the cerebrocortical neurons from age related cytological alterations. Cell Science and Report MOJ Cell Sci Rep 2(5): 00039 DOI: 10.15406/mojcsr.2015.02.00039*

Pathan KH, Gajare KA, ***Deshmukh AA*** *(2016)Mouse cerebral cortex is more vulnerable to age related deposition of senile plaques than the hippocampus: a neuropathological study of amyloid plaques in the normal process of brain aging Acta Biomedica Scientia;3(1):32-37.*

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CYTOPATHOLOGICAL STUDY OF NEUROFIBRILLARY TANGLES IN CEREBRAL CORTEX AND HIPPOCAMPUS OF SWISS ALBINO MOUSE: MUS MUSCULUS

K.H. Pathan[1], K.A. Gajare[2] and A. A. Deshmukh[1*]

[1]Cellular Stress Response Laboratory, Cell Biology Division, Department of Zoology, Shivaji University, Kolhapur.
[2]Department of Zoology, The New College, Kolhapur, Maharashtra, India.

ABSTRACT

Age related memory deficit is commonly observed in elderly population. The strategies of drug designing for senile dementia and Alzheimer's disease are based on the advances in understanding the molecular organization of neurons and synapse, biochemistry of neuronal signaling and impulse transmission. Various anti-dementia drugs and nootropic drugs are available, such as cholinesterase inhibitors and NMDA receptor antagonists. However, patients did not get significant relief by these therapeutic strategies. Probably, maintaining the healthy cytoarchitecture of neurons may be a more promising approach in the treatment of neurodegenerative diseases. One of the neuropathological alterations is the deposition of neurofibrillary tangles (NFTs). These are aggregates of paired helical filaments that interfere in axonal transport and thus affect neuronal functions. In the present investigations we have studied the deposition of NFTs by silver staining method in the cerebral cortex and hippocampus of Swiss albino mouse *Mus musculus* after 6, 12, 18 and 24 months of age. We have observed no deposition of NFTs at 6 months of age in both the regions under study. There was progressive deposition of NFTs in cerebral cortex from 12 to 18 months and 18 to 24 months, whereas, in the hippocampal region there was gradual increase in the deposition of NFTs.

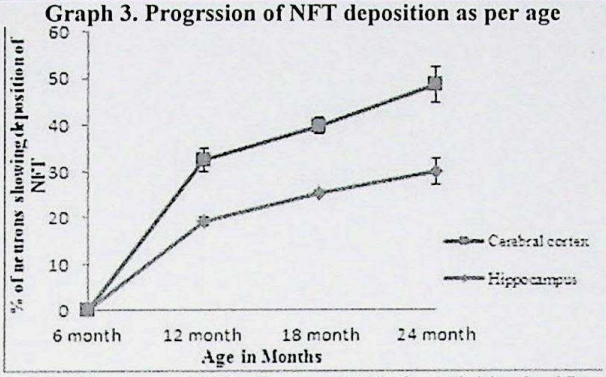
Keywords: Tau protein; Neuronal cytoskeleton; Neurofibrillary tangles (NFTs), Neurodegeneration.

INTRODUCTION

During the process of ageing, the oxidative damage to biomolecules increases [1-3]. Brain is more susceptible to oxidative damage than any other tissue [4-5]. Tau is a microtubule associated protein, present in the central nervous system. It is involved in microtubule assembly and stabilization [7-11]. Tau is prone to phosphorylation [11].The hyperphosphorylation of tau (pTau) results in microtubule disassembly [12]. Further, the pTau aggregates into paired helical filaments. The latter ultimately deposit within the neurons as neurofibrillary tangles [13]. This causes damage to neuronal cytoskeleton and thus leads to alterations in synaptic transmission. Neurofibrillary tangles are associated with many neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and age related dementia [14-16]. Recently in our laboratory we have studied the tau phosphorylation and neuodegeneration in primary culture of mouse hippocampal pyramidal neurons [17]. In the nervous tissue, the neurons communicate with each other through synapses, where neurotransmitters are released that act as mediator for transmitting the impulse from one neuron to another [18]. In neurodegenerative diseases, this process is interrupted either due to obstructive /damaged axonal transport or problem at synaptic cleft or both [19]. Cholinesterase inhibitors function by slowing down the breakdown of acetylcholine, so that the latter is retained for larger period of time to suffice the transmission of impulse [20-21]. The excess glutamate released from damaged neurons in senile dementia and Alzheimer's disease cause opening of Ca^{++}channels and influx of Ca^{++} in the neurons, which is treated with NMDA antagonist [22]. However, these treatments are superficial and temporary solutions. There is a need to treat the root cause, such as deposition of NFTs, which are pathological hallmarks of senile dementia,

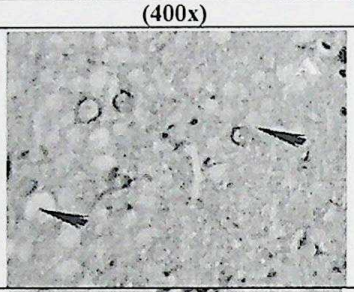
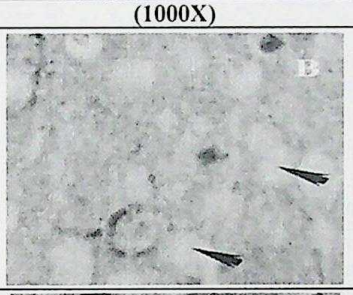
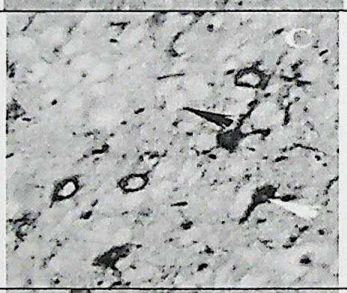
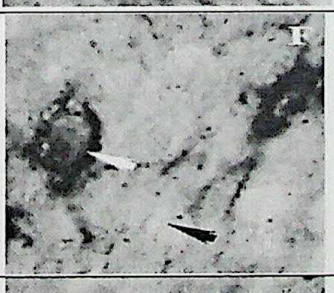
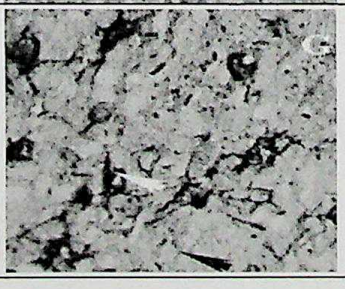
Corresponding Author:- **A. A. Deshmukh** Email:- ashish_cellbio@rediffmail.com

Graph 3. Progrssion of NFT deposition as per age



Results are expressed as mean ± SD of 6 animals *** indicates p<0.001i.e. Highly significant NS indicates Non significant

Figure 1. Histochemical studies on neurofibrillary tangles (NFTs), in Cerebral cortex of mouse brain White arrow indicates deposition of NFT and Blue arrow indicates neuron without NFT

(400x)	(1000X)	
		6 months
		12 months
		18 months
		24 months

DISCUSSION

In the present study, there was progressive deposition of neurofibrillary tangles in cerebral cortex and hippocampus over the age. This could be due to the damage to the microtubules due to increasing oxidative stress during ageing. [24-25]. As the age of an organism progresses oxidative stress also increases. Mitochondrial lysosomal axis theory of ageing [26] speculates the increased oxidative stress is the cause of ageing. Therefore, the age dependent increase in deposition of NFTs was the result of oxidative stress mediated tau phosphorylation.

Despite of therapy for senile dementia, there is no satisfactory progress in patient's compliance because the root cause remains untreated. There is need to slowdown the neurodegeneration from within. In the present investigation it is noticed that NFTs appear in 12 months of age in mice. At the age of six months, there were no NFTs. The essential life span in mouse is ten months. The essential lifespan is the time required to fulfil the Darwinian purpose of life i.e. successful reproduction [27]. The period of extended survival beyond the essential life span is the period of aging [28]. This could be a reason for absence of neurofibrillary tangles at six months of age and appearance by twelve months of age. During aging, the ability of cells to repair and maintain the molecules gets shrinked [29].

In the cerebral cortex, there was 32% and 58% increase in NFTS from 12 months to 18 months and from 12 months to 24 months respectively, which is in accordance with Holliday and Rattan [29].

While, in hippocampus there was no significant rise in NFTs upto 18months, which was increased by 39% after 24months of age. Thus, there is need to target NFTs for the treatment of neurodegenerative diseases. Karakaya *et al.* speculated the treatment options for taupathies [30]. The hyperphosphorylated tau can be degraded by Heat shock protein HSP 90 [31-32]. The drug development to treat various neurodegenerative diseases is aimed to treat free radical induced oxidative damage, disturbances of calcium homeostasis and excitatory amino acid function [20].

The mice studied in the present investigation were symptomatically nonpathological and apparently healthy. We have studied the deposition of NFTs during normal course of aging. If this is the scenario of NFT deposition, one can envisage the neuropathology of AD and related dementia in the context of NFTs.

CONCLUSION

Thus the present study gives neuropathological conceptualization of NFTs deposition in mouse brain during aging.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST: None

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Figure 2 Histochemical studies on neurofibrillary tangles (NFTs), in Hippocampus of mouse brain. White arrow indicates deposition of NFT and Blue arrow indicates neuron without NFT

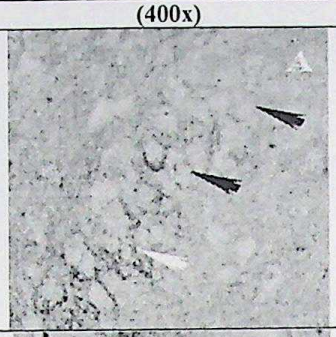
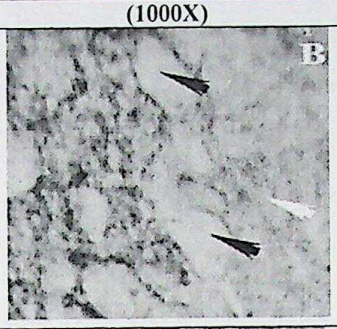
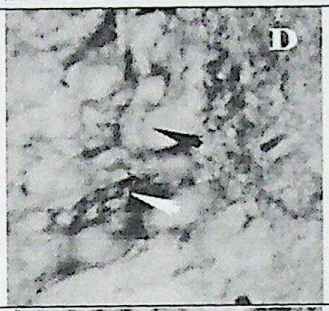
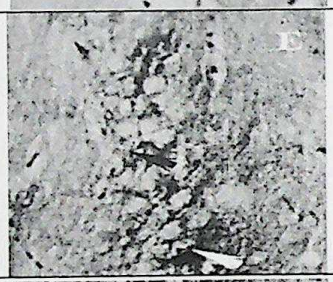
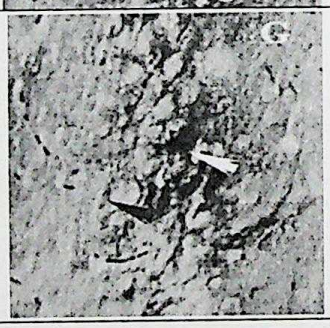
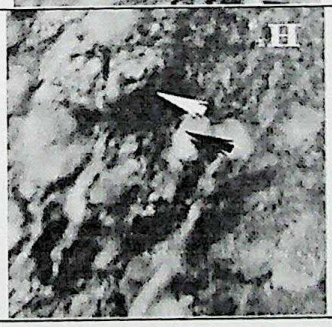
(400x)	(1000X)	
		6 months
		12 months
		18 months
		24 months

Table 1 Deposition of Neurofibrillary tangles in the cerebral cortex and the hippocampus of mouse

Age group	Cerebral cortex % of Neurons showing neurofibrillary tangles	Hippocampus % of Neurons showing neurofibrillary tangles
6months	--	--
12 months	19.16 ± 2.48	13.43 ± 0.87
18 months	25.35 ± 1.67***	14.55± 0.38^{NS}
24 months	30 ± 3.69***	18.72 ± 2.74 ***

Results are expressed as mean ± SD of 6 animals *** indicates p<0.001i.e. Highly significant, NS indicates Non significant

Ultrastructural Study Reveals that Mouse Hippocampal Neurons are More Protected than the Cerebrocortical Neurons from Age Related Cytological Alterations

Abstract

Neurons are post mitotic cells. They are more vulnerable to oxidative damage because of high content of unsaturated fatty acids and high oxygen consumption per unit weight. As the age of an organism progresses, the oxidative damage to molecules also increases which leads to increase in oxidatively modified molecules in the cell. Damage to membrane lipids causes alterations in the membrane functions. In the present study we have assessed the oxidative damage to membrane lipids in the form of nM of malondialdehyde (MDA) in cerebral cortex and hippocampus of 12, 18 and 24 month old male albino mice. We found that the degree of oxidative damage was higher in cerebral cortex than the hippocampus. This indicates that hippocampus is more resistant to oxidative damage than cerebral cortex. We have also studied the ultrastructure of cerebrocortical and hippocampal neurons. We found that the RER, Golgi apparatus and mitochondria were well organized in hippocampal neurons, whereas in cerebrocortical neurons, these cell organelles exhibited degenerative changes at 24 months of age. Nucleus of cerebrocortical neurons exhibited predominantly heterochromatinised form, whereas in hippocampal neurons it was in Euchromatin form.

Keywords: Malondialdehyde (MDA); Oxidative damage; Lipid peroxidation; Ultrastructural neuropathology

Research Article

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Pathan KH[1], Gajare KA[2] and Deshmukh AA[1]*

[1]*Cellular Stress Response Laboratory, Cell Biology Division, Department of Zoology, Shivaji University, Kolhapur (MS), India*

[2]*Department of Zoology, The New College, Kolhapur (MS), India*

***Corresponding author:** Deshmukh AA, Cellular Stress Response Laboratory, Cell Biology Division, Department of Zoology, Shivaji University, Kolhapur (MS), India, Tel: +91-231-2609249, +919822230905; Fax: 0231-2691533; Email: ashish_cellbio@rediffmail.com

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Abbreviations: ROS: Reactive Oxygen Species; LP: Lipid peroxidation; PUFAS: Polyunsaturated Fatty Acids; MDA: Malondialdehyde; HHE: 4-hydroxy- 2-hexenal; HNE: 4-hydroxy-2-nonenal; AD: Alzheimer's disease; TBA: Thiobarbituric acid;

Introduction

The "free radical theory of aging" proposed by Harman postulates that free radicals cause damage to cellular macromolecules and thereby deteriorates cellular functions [1]. The brain is more susceptible to oxidative stress due to high content of unsaturated fatty acids; high oxygen consumption per unit weight; high content of lipid peroxidation key ingredients (iron and ascorbate); and the scarcity of antioxidant defenses systems [2]. Oxidative damage to the brain leads to dementia. Even smaller impairment in the neural tissue increases the susceptibility to neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis [3]. Neurodegenerative diseases are characterized by decline in the normal antioxidant defense mechanisms that leads to deleterious oxidative changes [4]. Since, mitochondria are involved in redox reactions associated with cellular respiration, they are the source of Reactive Oxygen Species [ROS] [5-6]. Superoxide radicals are generated during mitochondrial metabolism get dismutated to hydrogen peroxide [7] which react with ferrous ions to generate most dangerous hydroxyl radicals [8]. The free radicals cause oxidative damage to mitochondrial DNA and affect mitochondrial metabolism [9-10]. The damaged mitochondria produce less ATP and more free radicals thereby increases the oxidative stress [11]. The free radicals react with membrane lipids and cause oxidative damage [12-13]. Except ribosome's, all the cell organelles are membrane bound. Thus, membranes of virtually all cell organelles are potentially targets of oxidative damage. Damage to membrane structure affects ion transporters, signal transduction, membrane potential and membrane permeability which ultimately perturb cellular metabolism that eventually lead to cell death [14]. Oxidative damage to proteins cause structural modifications such as protein cross linking, change in three dimensional structure of proteins [15-16]. This leads to loss of functions of the proteins. Oxidatively modified biomolecules become resistant to lysosomal degradation and get accumulated in the lysosomes as intracellular debris called as lipofuscin granules [17-18]. ROS also cause oxidative damage to DNA resulting into genome instability and affect transcription [19-20]. ROS and mitochondria are involved in pathogenesis of neurodegenerative diseases [21]. Lipid peroxidation (LP) is the oxidative modification in the polyunsaturated fatty acids (PUFAS) [22]. During this process, large numbers of toxic byproducts are generated which alter the cellular functions and ultimately affect the cell survival [23].

The byproducts of lipid peroxidation are chemically reactive aldehydes. They possess the neurotoxic activity e.g. Malondialdehyde (MDA), acrolein, 4-hydroxy- 2-hexenal (HHE), and 4-hydroxy-2-nonenal (HNE) [24-25]. The free radical-mediated MDA production causes damage to the proteins as well as DNA in the brain [26]. ROS contributes to Alzheimer's disease (AD). The persons with AD has shown increased levels of

thiobarbiturate reactive substances in deceased brain regions as compared to age matched control [27]. Age associated increase in oxidative stress, factors like accumulation of amyloid in Alzheimer's disease and iron deposition in Parkinson's disease enhance the lipid peroxidation process and generation of 4-hydroxy-2-nonenal (HNE) [28-29]. The lipid peroxidation studies are more important in AD [30-31].

In the present investigation, we have studied the age related alterations in the MDA and Ultrastructural changes in cerebrocortical and hippocampal neurons at 12, 18 and 24 months.

Materials and Methods

In the present investigations, male Swiss albino mice *Mus musculus* were used. The experiments were carried out with due permission of Institutional animal ethics committee in accordance with CPCSEA guidelines. The mice were provided with drinking water and laboratory animal feed (Pranav Agro industries, Sangli) *ad libitum* and maintained in animal house six animals per cage and 12 hrs light and 12 hrs dark cycle. The animals were sacrificed after 12, 18 and 24 months of age. Six animals were sacrificed after stipulated age. The brain was dissected on prechilled platform, cerebral cortex and hippocampus were separated and, used for further studies.

Measurement of lipid peroxidation

cerebral cortex and hippocampus of six animals were used after attaining the age of 12, 18 and 24 months. Malondialdehyde (MDA) is the most common end product of lipid peroxidation [32-33], which was estimated spectrophotometrically by thiobarbituric acid reaction [34]. In this reaction one molecule of malondialdehyde reacts with two molecules of Thiobarbituric acid (TBA) at boiling temperature to produce red colored MDA-TBA complex which was read spectrophotometrically at 532nm. The cerebral cortex and hippocampus were homogenized in reaction mixture containing potassium phosphate buffer pH 7.0 (75mM), ascorbic acid (1mM) and ferric chloride (1mM) at the concentration of 2mg/ml. 0.2ml of the homogenate was mixed with 1ml of 20%TCA and 2ml of 0.67%TBA and kept in boiling water bath for 10 min. After cooling, the optical densities were read at 532nm.

Statistical analysis

The results were processed as arithmetic mean ± standard deviation of six animals and level of significance was analyzed by 'unpaired Student t test'. The levels of MDA in the hippocampus were compared with the levels of MDA in the cerebral cortex at all the ages.

Transmission electron microscopic studies

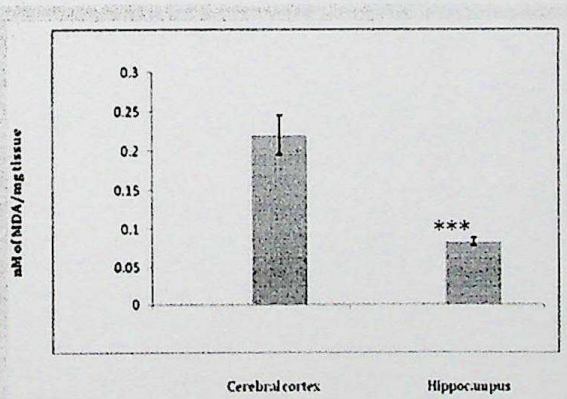
Transmission Electron Microscopy was carried out at the department of Electron Microscopy, Histopathology Unit, Jaslok hospital and research Centre, Mumbai. Approximately 1mm X 1mm pieces of frontal cortex and dentate gyrus region of hippocampus were fixed in 3% glutaraldehyde for 24hrs at 4°C, and washed twice with 0.1M cacodylate buffer pH 7.4, each wash was for 30min. The pieces were transferred to 1% Osmium tetroxide for 2hrs, washed with 0.1M cacodylate buffer pH 7.4 for 20 min. then dehydrated through 50%, 70%, 90% ethanol, for 30min in each grade (15 min + 15 min two changes of each grade) and finally in absolute ethanol for one hour (two changes of 30min). The dehydrated pieces were transferred to propylene oxide for 30min and passed through 1:1 propylene oxide and araldite (two changes of 15min), 1:3 propylene oxide and araldite (two changes of 15min) and finally embedded in araldite in Beem capsules and kept for drying at 80°C for 12hrs.

After trimming the araldite blocks, semi-thin sections of 1micron thickness were cut on Leica-UC7 Ultra microtome followed by ultrathin sectioning of 80nm. Each ultrathin section was collected on 300mesh copper grid (Tabb Laboratories Equipment Ltd England) and stained with 12.5% uranyl acetate for 40min followed by 0.25% lead citrate for 5min. After drying, the sections were observed using Jeol-100S transmission electron microscope at 80KV voltage.

Results and Discussion

Levels of MDA in nM/mg tissue in various regions of mouse brain expressed as Arithmetic mean ± SD of six animals

In cerebral cortex of 12 month old mice 0.22 ± 0.025 nM of MDA/mg tissue was observed. While the hippocampal MDA level was 0.083 ± 0.005 nM/mg tissues. In cerebral cortex of 18 month old mice the MDA levels were 0.23 ± 0.014 nM of MDA/ mg tissue. While in hippocampal region the MDA level was 0.14 ± 0.003 nM/ mg tissues. The MDA levels in cerebral cortex of 24 month old mice were 0.24 ± 0.015 nM/mg tissue. In hippocampal region the MDA level was 0.15 ± 0.01 nM/mg tissue (Graph 1-3).

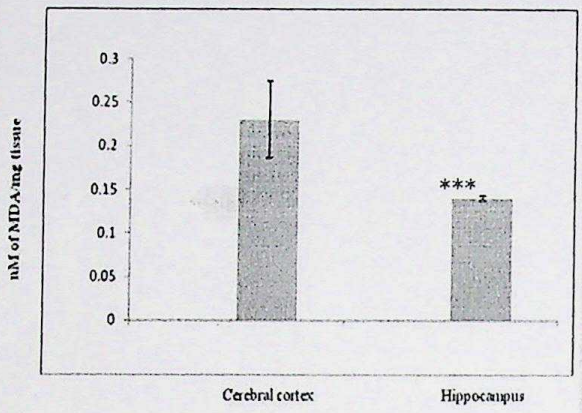


Graph 1: Lipid peroxidation in Nm of MDA/ mg tissue in cerebral cortex and Hippocampus of 12 month old mice (Arithmetic mean ± SD of six animals) *** indicates $p < 0.001$ i.e. highly significant.

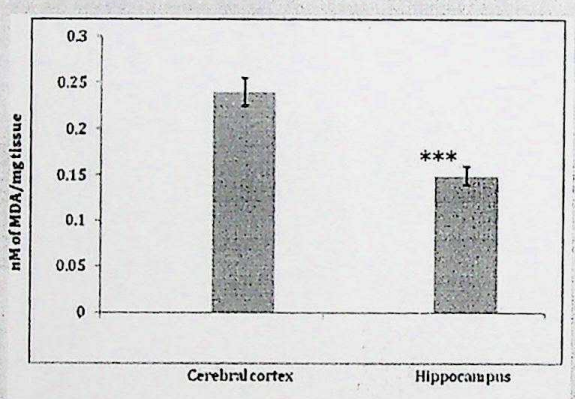
Reults of electron microscopic studies

A neuron from cerebral cortex of 12 months old mouse exhibited conspicuous nucleus with prominent euchromatin. The cytoplasm revealed RER, Nissl substance, mitochondria, autophagosome and few lipofuscin granules (Figure 1).

Axons from cerebral cortex at age 12 months showed depostion of electron dense material, well organised neurofilaments (Figure 2).



Graph 2: Lipid peroxidation in Nm of MDA/ mg tissue in cerebral cortex and Hippocampus of 18 month old mice (Arithmetic mean ± SD of six animals) *** indicates $p < 0.001$ i.e. highly significant.



Graph 3: Lipid peroxidation in Nm of MDA/ mg tissue in cerebral cortex and Hippocampus of 24 month old mice (Arithmetic mean ± SD of six animals) *** indicates $p < 0.001$ i.e. highly significant.

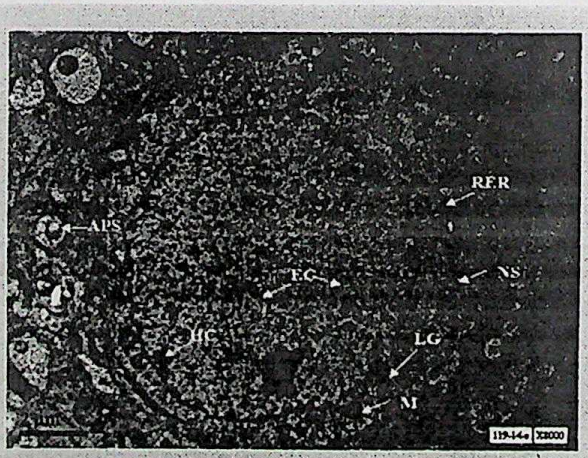


Figure 1: A neuron from the cerebral cortex of 12 Months old mouse. EC-Euchromatin, HC-Heterochromatin, APS-Autphagosome, LG-Lipofuscin granules, M- Mitochondrion, NS- Nissl substance.

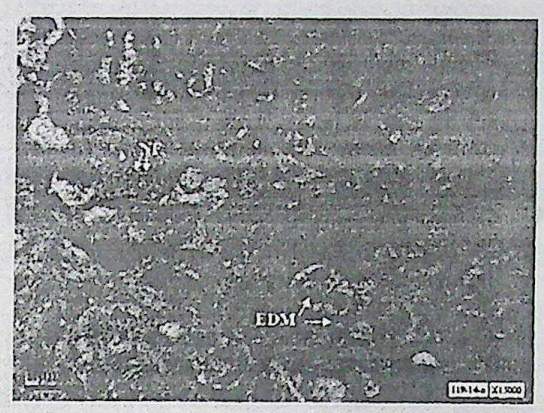


Figure 2: T. S. of axons from cerebral cortex of 12 months old mouse showing neurofilaments at 15000X. NF- Neurofilament, EDM- Electron dense material.

A neuron from hippocampus of 12 months old mouse exhibited conspicuous nucleus with prominent euchromatin. The cytoplasm revealed RER, Nissl substance, mitochondria, Golgi cisternae and few lipofuscin granules (Figure 3).



Figure 3: A neuron from the Hippocampus of 12 Months old mouse. EC-Euchromatin, HC-Heterochromatin, M- Mitochondria, NS- Nissl substance, LG-Lipofuscin granules, RER- Rough Endoplasmic Reticulum, GA- Golgi apparatus.

Axons from hippocampus at age 12 months show well organised neurofilaments, functional mitochondria and secretary vesicles (Figure 4).

A neuron from the cerebral cortex of 18 months old mouse exhibited abundance of heterochromatin. Cytoplasm was loaded with residual bodies. Mitochondria were swollen. Cytoplasm exhibited poor Nissl granules and RER (Figure 5 & 6).

Axons from cerebral cortex at age 18 months show depostion of external deposits, disorganised neurofilaments and scarce secretary vesicles. One axon shows damaged mitochondrion (Figure 7).

A neuron of the hippocampal region of 18 months old mouse exhibited conspicuous nucleus with marginal traces of heterochromatin and abundant euchromatin. Cytoplasm was fully occupied by Nissl substance and Rough Endoplasmic Reticulum, however Golgi cisternae were not observed in inflated form. The

cytoplasm was loaded with lipofuscin granules. Mitochondria were with normal size and shape without any swelling. (Figure 8).

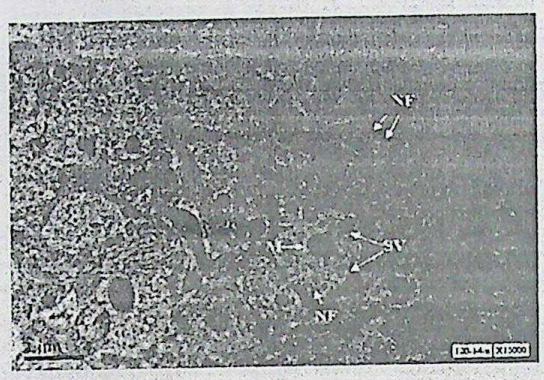


Figure 4: T.S. of axons from Hippocampus of 12months old mouse showing neurofilament at 15000X. NF- Neurofilament, SV- Secretory Vesicles, M- Mitochondria.



Figure 5: A neuron from the cerebral cortex of 18Months old mouse. EC-Euchromatin, HC-Heterochromatin, M- Mitochondria, RB- Residual bodies.

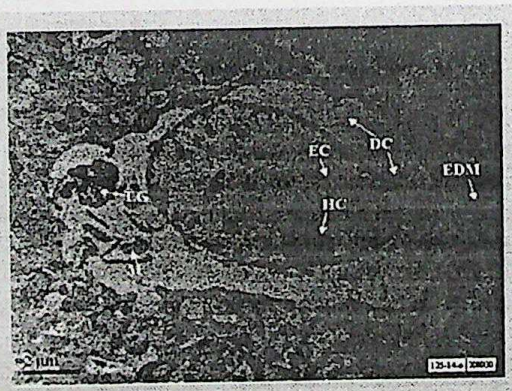


Figure 6: A neuron from the cerebral cortex of 18Months old mouse.

EC-Euchromatin; HC-Heterochromatin; M- Mitochondria; LG-Lipofuscin granules; DC- Degenerating cytoplasm; EDM- Electron dense material

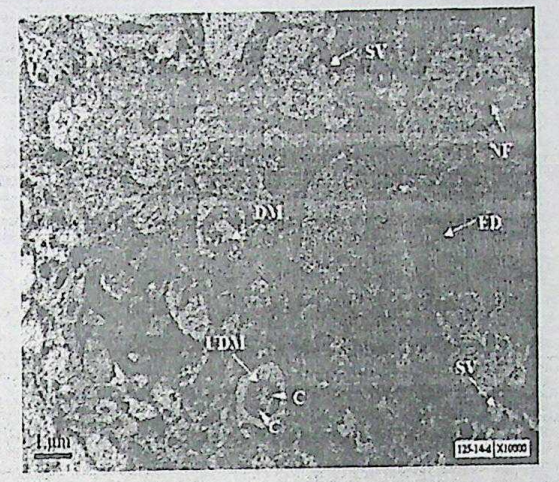


Figure 7: T.S. of axons from cerebral cortex of 18 months old mouse showing irregularity of neurofilaments and damaged mitochondria at 15000X. NF- Neurofilaments, DM- Damaged mitochondrion, ED- External deposits, SV- Secretory vesicles,UDM- Undamaged mitochodrion, C-crista.

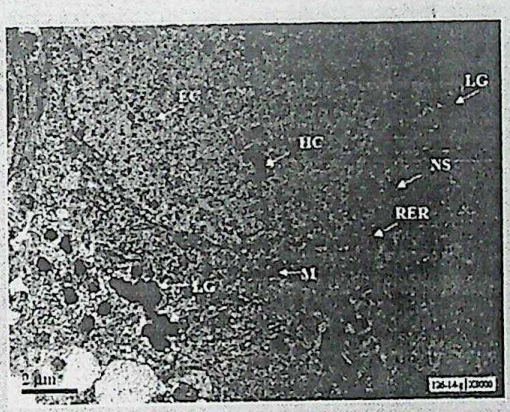


Figure 8: A neuron from Hippocampus of 18months old mouse. EC-Euchromatin, HC-Heterochromatin, M- Mitochondria, LG-Lipofuscin granules, NS-Nissl substance, RER-Rough Endoplamic Reticulum.

Axons from hippocampus at age 18 months show no depostion of electron dense material, but little irregularaity in neurofilaments (Figure 9).

Two neurons from cerebral cortex of 24 months old mouse exhibit predominance of heterochromatin, nucleus shows early stages of fragmentation; one neurons shows formation of nuclear lobes (neuron in the right side of the panel) , whereas the other neuron shows advanced nuclear fragmentation (neuron in the left side of the panel). Both the neurons show loss of cytoplasm, damaged mitochondria, loss of Nissl substance and accumulation of lipofuscin granules (Figure 10). At 20000X magnification lipofuscin granules were conspicuously seen (Figure 11).

Axons from cerebral cortex at age 24 months show extracellular deposits, axonal rupture from inside and complete loss of regularity of neurofilament architecture (Figure 12).

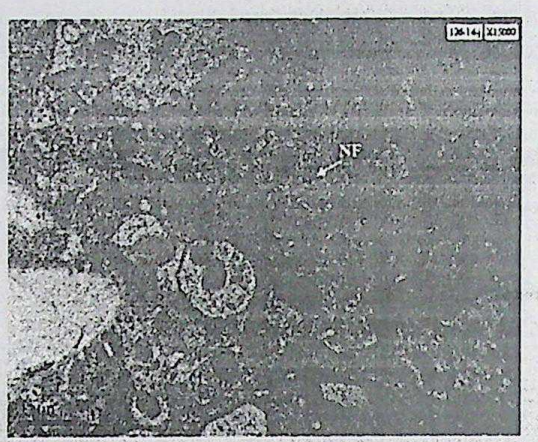


Figure 9: T.S. of axons from Hippocampus of 18months old mouse at 15000X showing irregularity of neurofilaments. NF- Neurofilament



Figure 10: A neuron from cerebral cortex of 24months old mouse showing Nucleus undergoing formation of lobes an initial step of nuclear fragmentation at 8000X. EC-Euchromatin; HC-Heterochromatin; M- Mitochondria; LG- Lipofuscin granules; NF-Nuclear fragmentation; NLB- Nuclear lobe.

A neuron from hippocampus of 24 months old mouse exhibits prominent nucleus with euchromatin and heterochromatin. Lipofuscin granules were scarce. Cytoplasm exhibits few swollen mitochondria along with normal mitochondria and Golgi cisternae (Figure 13).

Axons from hippocampus at age 24 months show beginning of deposition of extracellular aggregates (Figure 14) and an axon in the left side of the panel shows rupture.

The degree of lipid peroxidation was expressed as nM of MDA/mg tissue. There was no significant difference in the levels of MDA in the cerebral cortex at the age of 12, 18 and 24 months. In the hippocampus, there was highly significant difference ($p<0.001$) in the levels of MDA at the age of 12 and 18 months as well as 12 and 24 months. The cerebral cortex exhibited high levels of MDA as compared to hippocampus. There was highly significant difference in the levels of MDA at all ages in the hippocampus when compared to cerebral cortex of respective ages. The cerebral cortex is concerned with perception of stimuli, recognition, planning, short term memory [35] which get declined during aging. Hippocampus is associated with long term memory which persists even in senescent condition [36]. In the present investigation, the MDA levels were higher in cerebral cortex than in the hippocampus. This pattern was consistent in 12, 18 and 24 months old mice. These results are correlated with decline in neuronal functioning during aging which may be due to difference in the fatty acids composition in the various regions of the brain.

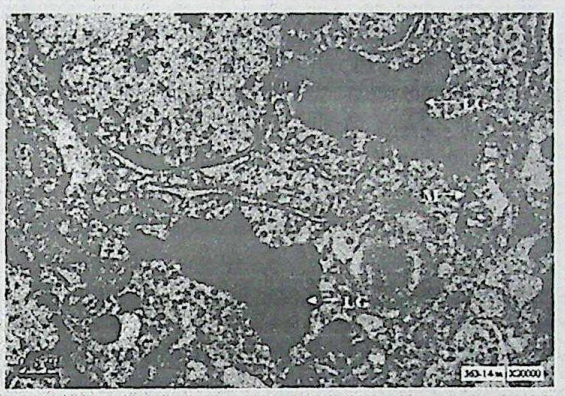


Figure 11: A portion of a neuron from the cerebral cortex of 24Months old mouse.
LG-Lipofuscin granules, M-Mitochondrion.



Figure 12: T.S. of axons from cerebral cortex of 24 months old mouse showing deposits from exterior at 25000X. ED- Extracellular deposits; AR-Axonal rupture from inside; NF- Neurofilament.

There was no significant increase in the extent of lipid peroxidation in the cerebral cortex from 12 to 18 months and from 18 to 24 months. This might be due to maximum damage that might have occurred at the age of twelve months. This scenario was altogether different in the hippocampus. The

maximum oxidative damage that occurred in the hippocampus at the age of 24months was 37% lower than in the cerebral cortex at the same age. This indicates that hippocampus is more protected. This could be due to involvement of hippocampus in adult neurogenesis [37-38]. As new neurons are generated in the hippocampus, they are equipped with more antioxidant defense than the older cerebrocortical neurons.

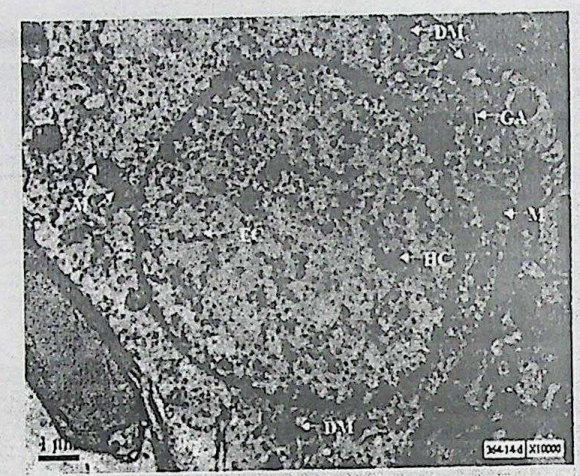


Figure 13: A neuron from the Hippocampus of 24 Months old mouse. EC-Euchromatin; HC-Heterochromatin; M- Mitochondria; DM-Damaged mitochondria; GA- Golgi apparatus.

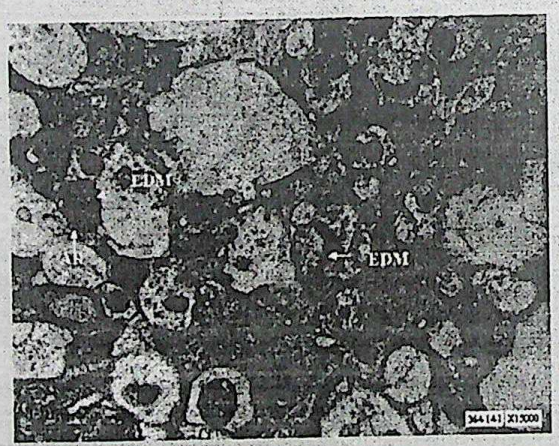


Figure 14: T.S. of axons from Hippocampus of 24 months old mouse showing deposition of electron dense material. EDM; Electron dense material; AR- Axonal rupture.

The Ultrastructural details also reveal that the hippocampus is protected than the cerebral cortex. This is evidenced by loss of cytoplasmic contents such as mitochondria, RER, ribonucleoprotein particles in cerebrocortical neuron. Nucleus shows heavily heterochromatinised region, which suggest transcriptional inactivation. A neuron from hippocampus shows nucleus with predominant euchromatin and scanty heterochromatin suggesting the transcriptional activity of the neuron. Cytoplasm is studded with ribonucleoprotein particles, abundance of RER and mitochondria.

In the transverse section, axons of cerebrocortical neurons of age 18 months show intra-axonal electron dense material which may be beneurofibrillary tangles or aggregates of phosphorylated tau. Recently we have demonstrated tau phosphorylation in the neurons undergoing degeneration in culture [39]. Axons of hippocampal neurons exhibit well organized neurofilaments. Some axons also show presence of secretary vesicles and undamaged mitochondria. At the age of 24 months, axons of cerebrocortical neurons show heavy extracellular deposition, and few axons show rupture. Whereas, axons of hippocampal neurons show beginning of extracellular deposition and rupture. This suggests that hippocampal neurons are more protected than cerebrocortical neurons at advanced age also. Recently we have also demonstrated that cerebral cortex is more vulnerable to deposition of neurofibrillary tangles than hippocampus [40]. This may be due to involvement of adult hippocampus in neurogenesis as described by Eriksson et al. [41]. The results of the present study give biochemical and ultrastructural evidence to the neuronal plasticity in the hippocampus.

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MOUSE CEREBRAL CORTEX IS MORE VULNERABLE TO AGE RELATED DEPOSITION OF SENILE PLAQUES THAN THE HIPPOCAMPUS: A NEUROPATHOLOGICAL STUDY OF AMYLOID PLAQUES IN THE NORMAL PROCESS OF BRAIN AGING

K.H. Pathan[1], K.A. Gajare[2] and A.A. Deshmukh[1*]

[1]Cellular stress response laboratory, Cell Biology Division, Department of Zoology, Shivaji University, Kolhapur (MS) India 416004.
[2]Department of Zoology, The New College, Kolhapur (MS) India 416012.

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ABSTRACT
The amyloid β (Aβ) is a protein, deposited on the neuronal plasma membrane in the patients of Alzheimer's disease (AD). It is considered as the hallmark of AD. It is generated due to the abnormal processing of amyloid precursor protein (APP). It exists in the isoforms like Aβ40 and Aβ42 that get deposited in the brain as senile plaques. Deposition of senile plaques leads to dementia. Enormous literature speculates the neuropathology of amyloidosis in AD. However, there are hardly any reports speculating the deposition of amyloid plaques in the normal process of aging. In the present investigation, we have studied the deposition of senile plaques by Congo red method in the cerebral cortex and hippocampus of Swiss albino mouse *Mus musculus* of 12, 18 and 24 months of age. As compared to 12 and 18 months, at 24 months of age the cortical and hippocampal region showed a highly significant increase in the deposition of senile plaques. Among the cerebral cortex and hippocampus, the cerebral cortex showed higher deposition of senile plaques. These results demonstrate the progressive deposition of senile plaques in the normal course of aging, i.e. in the absence of neurological disorder such as AD.

INTRODUCTION

Alzheimer's disease (AD) is a neurological disorder in which patients experience serious dementia i.e. loss of memory. This is marked by the extracellular deposition of β- Amyloid (Aβ) and intracellular aggregation of neurofibrillary tangles in the neurons [1]. Aβ is produced from the membrane bound Amyloid Precursor Protein (APP). The APP is involved in the formation of synapses and their repair [2]. The "amyloid cascade hypothesis" [3-4] states that abnormal metabolism of Amyloid Precursor Protein (APP) in the brain is a primary event in the pathogenesis of AD. The Aβ40 and Aβ42 are the most common isoforms of amyloid beta that are produced as monomers. These monomers aggregate into dimers, trimers, oligomers, protofibrils and fibrils to form amyloid plaque [5]. Aβ oligomers are toxic forms of the Aβ peptide [6]. The amyloid β interacts with neurons and glial cells, and triggers the inflammatory process resulting in oxidative stress [7]. The above cited literature emphasizes amyloid deposition in AD. Per se, there are seldom reports describing the process of amyloid deposition in the normal process of aging. The present investigation is an attempt to understand the extent of deposition of senile plaques in the cerebral cortex and

Corresponding Author

A.A. Deshmukh
Email: - ashish_cellbio@rediffmail.com

hippocampus of mouse at 12, 18 and 24 months of age in the absence of neurological disorder such as Alzheimer's or Parkinson's disease.

MATERIAL AND METHODS

For the present investigation Swiss male albino mice *Mus musculus* were used as an experimental animal. The work was carried out with the due permission of the Institutional Animal Ethics Committee. The animals were reared in the departmental Animal house and pelleted animal chaw (Pranav Agro Industries, Sangli) and water were provided *ad libitum*. The animals were exposed to 12hr-12hr light dark cycle. Animals were sacrificed by deep ether anesthesia after 12, 18 and 24 months of age. Six animals were used in each age group. The brain was gently excised on prechilled platform and longitudinally cut into two equal halves. The longitudinal halves were fixed in neutral buffered formalin for 24 hrs, washed under running tap water for 24 hrs and dehydrated through ascending grades of ethanol viz. 30%, 50%, 70%, 90% and 100% each for 2hrs duration. Then the halves were cleared in 1:1 ethanol-xylene mixture and finally into xylene for 15 min, finally embedded in molten paraffin wax at 60°C for 90 min, initial wash was about 30 min followed by impregnation for 60min. The rectangular paraffin blocks were made, trimmed and cut into 6µm thick sections. The paraffin sections were spread on the micro slides, which were further used for histochemical studies.

Histochemical studies of β amyloid

Staining of β amyloid was carried out by modified Congo red method described by Puchtler *et al.* [8]. Sections were deparaffinized and hydrated through descending grades of ethanol and were stained with Mayer's haematoxylin for 10 min. Then sections were rinsed in distilled water and treated with 80% ethanol saturated with NaCl followed by staining with Congo red for 1 hour at 37°C. Thereafter sections were dehydrated rapidly in 90% and absolute alcohol and cleared in xylene and mounted in D.P.X. and observed under microscope. The amyloid positive areas were measured in the micrometer square (μm^2) under oil immersion lens i.e. at 1000X magnification.

Statistical analysis

The results were calculated as the arithmetic mean of six animals ± standard deviation. To study the level of significance in three age groups, the amyloid positive areas from 18 months were compared with 12 months and area of amyloid plaques from 24 months were compared with 18 months in both the regions of the brain. To know the level of significance among the cerebral cortex and the hippocampus, the areas of amyloid plaques from the both the regions were compared with each other at respective ages.

RESULTS AND DISCUSSION

The photomicrographs of cerebral cortex and hippocampus are displayed in figure 1and 2 respectively. The cerebral cortex of 12 months old mice showed 382.0 ± 10.81 μm^2 area positive for senile plaques (Figure 1A); whereas, the hippocampal region showed 337.66 ± 9.81 μm^2 area positive for senile plaques (Figure 2A). In the cerebral cortex of 18 months old mice (Figure 1B)the amyloid positive area was 672.0 ± 22.2 μm^2, while in the hippocampal region it was 429.5 ± 21.51 μm^2 (Figure 2B). At the age of 24 months in the cerebral cortex, the amyloid positive area was 2990.33 ± 88.22 μm^2 (Figure 1C) and in the hippocampus it was 1418.66 ± 84.83 μm^2 (Figure 2C). There was age related increase in the amyloid positive area in both the cerebral cortex and the hippocampus from12 to 18 months and from 18 to 24months. This increase was highly significant ($p<0.001$). When an amyloid positive area in the hippocampus was compared with the amyloid positive area in the cerebral cortex, it is observed that cerebral cortex contains greater amyloid positive area. This difference was highly significant at $p<0.001$. At the age of 24 months the amyloid positive area in the hippocampus was 50% smaller than the amyloid positive area from the cerebral cortex.

The results demonstrate age related increase in the amyloid positive area in the mouse cerebral cortex and the hippocampus. Under pathological conditions APP is metabolized by the amyloidogenic pathway and gets accumulated due to the reduction in the clearance of Aβ peptides [9]. However, the present study depicts that amyloid deposition also occurs in nonpathological condition. Rhein and Eckert (2007) described that the accumulation of Aβ in the brain causes mitochondrial dysfunction, increased oxidative stress, abnormal neuroinflammatory response, decreased neuroplasticity and increased tau phosphorylation that finally leads to neuronal death [7]. Recently we found the abnormal mitochondria and degenerative ultrastructural changes in the cerebrocortical neurons in mice during aging [10].

The age related increase in the deposition of amyloid plaques in the cerebral cortex and hippocampus observed in this study may be due to declined autophagic clearance of amyloid β in the neurons of cerebral cortex and hippocampus. Dice [11] and Martinez-Vicente *et al.* [12] found that there is an age related decline in lysosomal proteolysis. Another reason may be altered processing of amyloid precursor protein at an advanced age.

Table 1. Showing the Amyloid positive area in μm^2 in the cerebral cortex and hippocampus of mouse brain. Results are expressed as mean ± SD of 6 animals.

Age of mouse	Amyloid positive area stained by Congo red in μm^2	
	Cerebral cortex	Hippocampus
12 months	382.0 ± 10.81	337.66 ± 9.81 ^{b***}
18 months	672.0 ± 22.2 ^{a***}	429.5 ± 21.51 $^{a*** b***}$
24 months	2990.33 ± 88.22 ^{a***}	1418.66 ± 84.83 $^{a*** b***}$

a*** indicates p<0.001 i.e. highly significant when area of amyloid plaques from 18 months were compared with 12 months and area of amyloid plaques from 24 months were compared with 18 months. b*** indicates p<0.001 i.e highly significant when area of amyloid plaques from hippocampus were compared to area of amyloid plaques from cerebral cortex at respective ages

Figure 1. Photomicrographs exhibiting the deposition of Congo red positive Senile Plaques in the mouse cerebral cortex

(400X) **(1000X)**

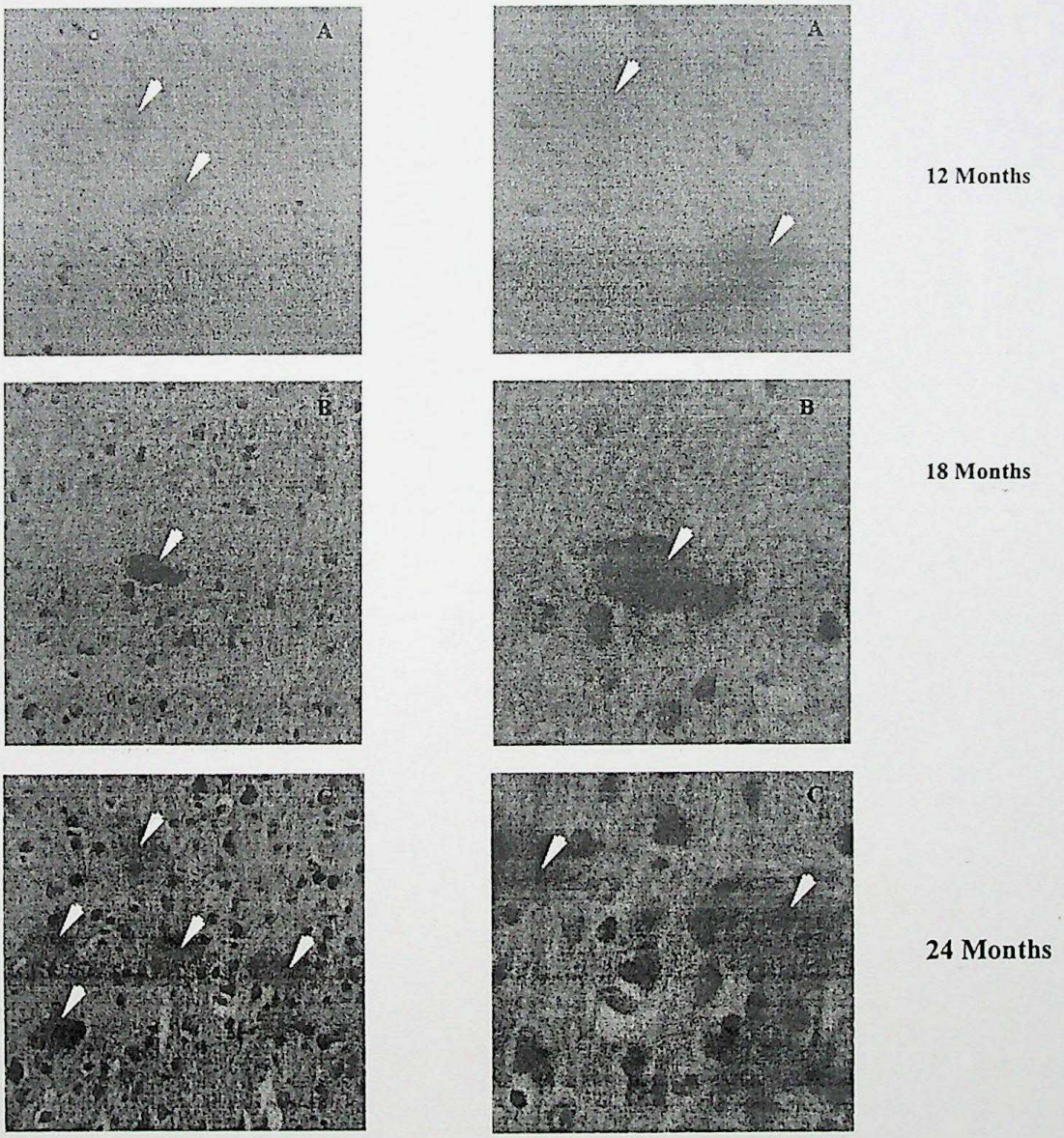
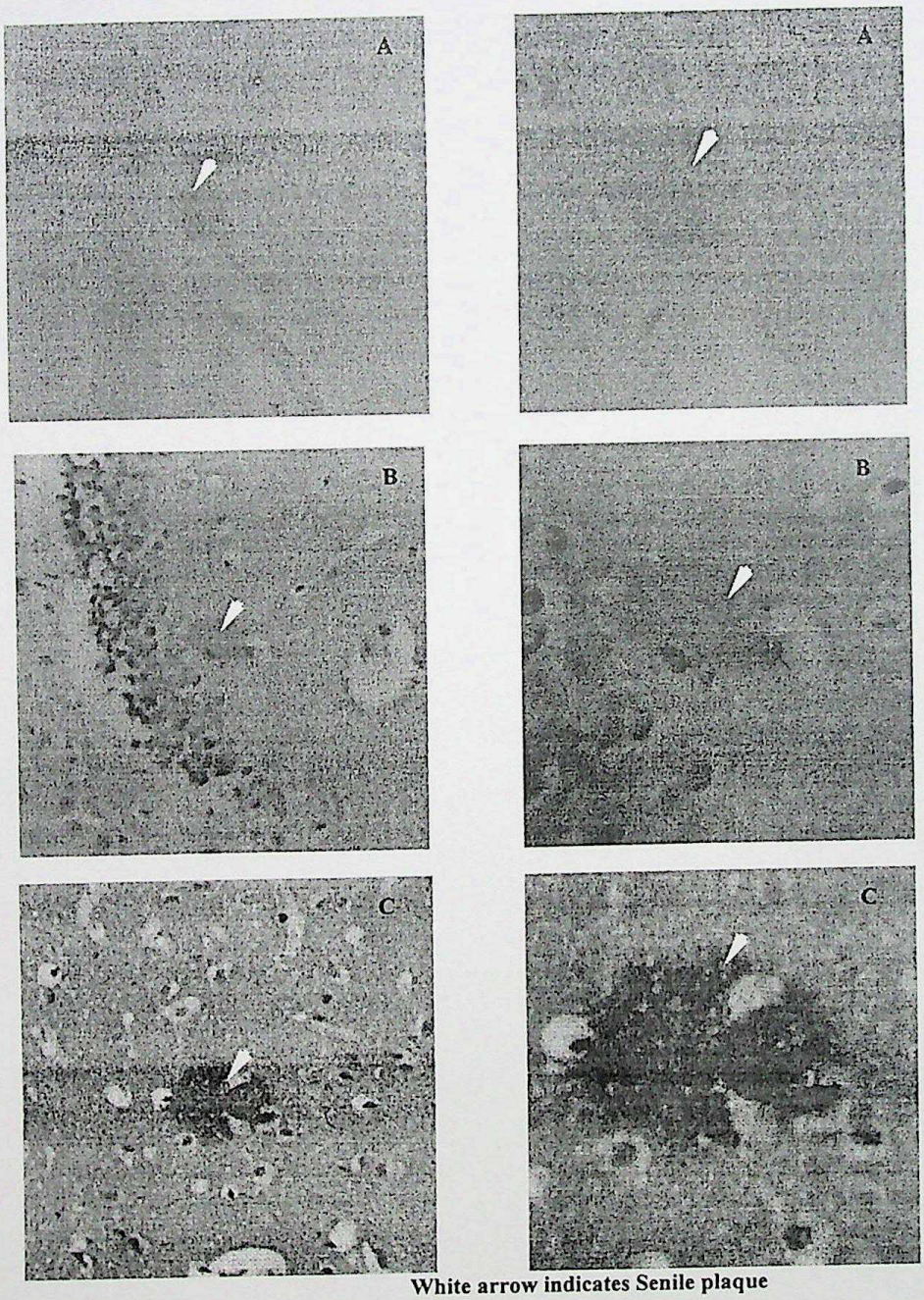
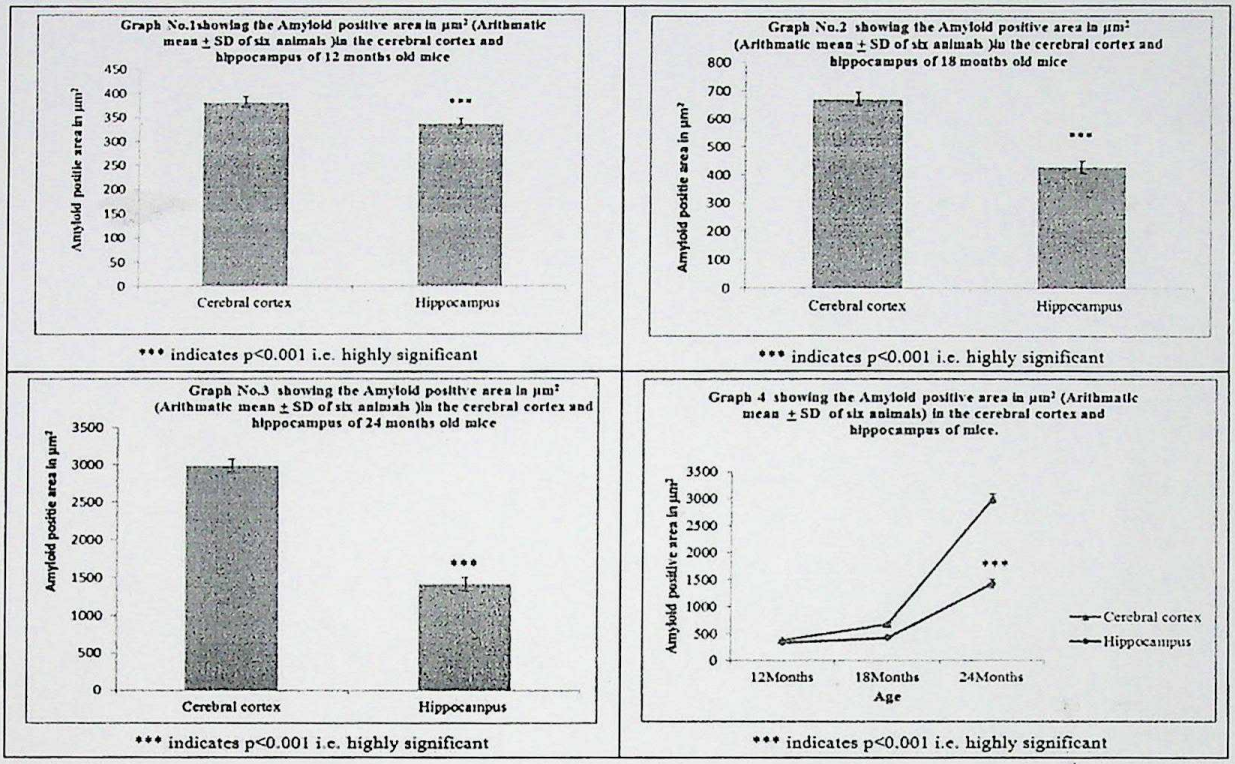


Figure 2. Photomicrographs exhibiting the deposition of Congo red positive Senile Plaques in the mouse hippocampus (400X) (1000X)



White arrow indicates Senile plaque



Mattson [13] and Tanzi *et al.* [14] found that increase in Aβ levels is due to the altered proteolytic processing of the amyloid precursor protein (APP) and decline in the lysosomal clearance. The increasing number of Aβ peptides forms the oligomers of amyloid beta that leads to neuronal toxicity via generating oxidative stress [15]. Pathan *et al.* [10] demonstrated the age related increase in the lipid peroxidation in the cerebral cortex and the hippocampus of mice. Neurons are susceptible to damage as revealed by Bele *et al.* (2015) in primary culture of mouse hippocampal pyramidal neurons [16]. In our earlier study we found that the cerebral cortex exhibits more aggregation of neurofibrillary tangles than the hippocampus [17].

The findings of the present study further strengthen these observations which may be involvement of hippocampus in adult neurogenesis and thus the cells may be occasionally rejuvenating unlike cerebrocortical neurons. Aβ is a suspected culprit in causing dementia [18]. Age related dementia is a common problem in the elderly, which may be due to a gradual increase in the deposition of amyloid plaques during aging.

CONCLUSION

The present findings unravel one of the reasons of senile dementia and emphasize the progressive deposition of Aβ during advancing age.

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CONFLICTS OF INTEREST: None

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