Effects of diet-induced hypercholesterolemia on amyloid accumulation in ovariectomized mice

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A central hypothesis in the study of Alzheimer's disease (AD) is the accumulation and aggregation of β-amyloid peptide (Aβ). Recent epidemiological studies suggest that patients with elevated cholesterol and decreased estrogen levels are more susceptible to AD through Aβ accumulation. To test the above hypothesis, we used ovariectomized with diet-induced hypercholesterolemia (OVX) and hypercholesterolemia (HCL) diet alone mouse models. HPLC analysis reveals the presence of beta amyloid in the OVX and HCL mice brain. Congo red staining analysis revealed the extent of amyloid deposition in OVX and hypercholesterolemia mice brain. Overall, Aβ levels were higher in OVX mice than in HCL. Secondly, estrogen receptors α (ERα) were assessed by immunohistochemistry and this suggested that there was a decreased expression of ER α in OVX animals when compared to hypercholesterolemic animals. Aβ was quantified by Western blot and ELISA analysis. Overall, Aβ levels were higher in OVX mice than in HCL mice. Our experimental results suggested that OVX animals were more susceptible to AD with significant increase in Aβ peptide.

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1. Introduction

Cholesterol is considered to boost the possibility of Alzheimer's disease (AD) (Pappolla *et al*. 2003; Wolozin 2004). It has been shown in animal and cellular models to increase the production of β-amyloid (Aβ) peptide, a key pathological feature of AD (Sparks *et al*. 1994; Frears *et al*. 1999; Refolo *et al*. 2000; Shie *et al.* 2002). There is also evidence that cholesterol co-localizes with fibrillar Aβ in the amyloid plaques of transgenic mice (Burns *et al*. 2003). Even though many studies link cholesterol to Aβ production, the mechanisms by which cholesterol increases Aβ levels are still to be determined. Cholesterol homeostasis in the brain is regulated through *de novo* synthesis, with very poor transfer from the peripheral circulation due to the impermeability of the blood–brain barrier (BBB) to the lipoproteins that carry cholesterol. When one compares with cholesterol, 27-hydroxycholesterol, a product of cholesterol oxidation (oxysterol), has been shown to cross the BBB into the brain (Heverin *et al*. 2005). It may be possible that increased entrance of this oxysterol into the brain following hypercholesterolemia places the brain at risk for neurodegeneration.

Clinical studies have shown that estrogen deprivation through menopause is a risk factor in both initiation and progression of AD (Zheng *et al*. 2002). Estrogen promotes neuritis outgrowth and vascular supply to brain parenchyma to enhance neuronal regeneration and repair (Hong-Goka and Chang 2004). It also reduces oxidative damage associated with neurodegeneration. Recently, it was observed that estrogen modulates the metabolism of the amyloid precursor protein (APP) and estrogen has been implicated in the prevention of AD. In the CNS, estrogen protects neurons against Aβ. Estrogen also increases cerebral blood flow, glucose utilization and improves cholinorgic tone. It is interesting that it has been found that estrogen increases apoE mRNA and protein expression in rat astrocytes and microglia (Rena *et al*. 2000). In addition to this, physiological concentration of estrogen has been shown to decrease the

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levels of Aβ released from rodent or human primary neuronal cultures (Crystal *et al.* 1988). Estrogens have been reported to regulate hepatic LDL expression and modulate cell proliferation in different tissues (De Caterina and Massaro 2005). Increased cholesterol levels in serum and brain have also been cited as a one of the multiple factors other than familial genetic mutations that can influence Aβ peptide metabolism and accumulation in AD.

Interestingly, an epidemiological study illustrates that AD is more prevalent in countries with high dietary fat intake. Increased cholesterol concentration alters the APP processing by α-secretases and favours the production β-secretase and γ-secretase and results in the increased deposition of Aβ (George *et al.* 2004). There have been only a few studies on how the above factors affect the development of AD. Deposition of Aβ in brain parenchyma is a distinctive feature of the neuropathology of AD, and this deposition is an aging-related phenomenon. One of the best-characterized systemic metabolic changes in late life is a decline in gonadal steroid levels. Moreover, estrogen receptors within the brain have a regional distribution strikingly similar to that characteristic of Alzheimer-type brain pathology (Toran-Allerand *et al.* 1992). In addition, recent findings suggest that estrogen may be protective against AD (Henderson *et al.* 1994).

Our study aimed to investigate the effects of OVX and HCL on the production of Aβ. In this study, the mice were ovariectomized to induce estrogen deprivation and they were placed on a high cholesterol diet for 4 months. Our result shows that this induces the accumulation of Aβ in the OVX mice brain rather than the HCL mice.

2. Materials and methods

2.1 *Animals and diets*

Colony-inbred adult male Swiss White mice (30–35 g) were used in this study. A total of 40 female mice were placed on high-cholesterol diet containing 5% cholesterol, 10% fat and 2% sodium cholate and 5.2 kcal/g (Purina Test Diet No.5801C) for 4 months. All animal experiments were carried out as approved by the animal care and use committee of Central Leather Research Institute, Chennai.

2.2 *Mouse surgery and analysis*

Female mice were obtained at 4 weeks of age and maintained. Animals were anaesthetized at 8 weeks of age using an initial dose of Ketamine (60 mg/kg) and Xylazine (8 mg/kg) i.p. A dorsolateral incision was made and the ovaries were located through visualization of the periovarian fat, prior to removal. Care was taken to ensure that the blood vessels supplying the uterus remained intact during and following this procedure. The abdominal wall was sutured with abdominal sutures. The outer skin was sutured with non-absorbable suture material. To prevent wound damage from other animals, these were caged separately for 2 days post surgery.

2.3 *Extractions and purification of Aβ*

Brain samples were removed, immediately frozen and stored at −70°C until further processing. All reagents used in extractions and purification of Aβ were obtained from Sigma unless indicated otherwise. Brain was finely minced and immediately homogenized in a buffer containing 1% SDS, 0.1 mM phenylmethylsulphonyl fluoride, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, in a homogenizer (loose-fitting pestle). The 1,1,1,3,3,3-hexafluroisopropanol (HFIP) extract of brain homogenate was loaded on Sephadex G-25 column. The fraction containing highest concentration of protein was lyophilized and dissolved in 10% formic acid (protein concentration of the extract 1 mg/mL). 20 µL of this was separated using RP-HPLC water-acetonitrile gradient system containing 0.1% TFA on a Waters HPLC (Waters, Milford, Massachusetts, USA), equipped with Shimadzu SPD 10A UV-Visible detector (Shimadzu, Kyoto, Japan). Separations were performed using Spherisorb-ODS2 25 cm× 4.6 mm, 5 µm, C_{18} (Waters, Milford, Massachusetts, USA) silica column.

2.4 *Electrophoresis and immunoblotting*

Electrophoresis separation of the proteins in the presence of SDS was performed by loading samples onto 10-well gradient gels (10–20%), followed by transfer onto 0.2 mm nitrocellulose membrane (Bio-Rad, Hercules, CA) by electroblotting. The membrane was blocked in TBS (0.05% Tween20, 150 mM Nacl, 50 mM Tris-Hcl pH 7.4) containing 3% skimmed milk and probed with primary antibody Aβ (6E10 (Senetek, Maryland heights, MO) and Erα (Santa Cruz). After those membranes were incubated with respective conjugated secondary antibody, the immunoreactivity bands were visualized using corresponding substrate.

2.5 *ELISA*

The expression of Aβ was determined in brain extract using ELISA. 200 µL fractions from the homogenates tissue were added and after subsequent washing treated with polyclonal antibody raised against the sequence of $A\beta_{25–35}$, treated in micro-plates and incubated for 24 h at 4°C. After washing with phosphate buffered saline, the micro-plates were incubated with ALP conjugated secondary-goat antirabbit IgG.

Proteins bound to antibodies were assayed by colour development using the *p*-nitrophenyl phosphate in carbonate buffer system at 405 nm (Biotek instruments EL310 Micro plate Autoreader, Winooski, Vermont).

2.6 *Congo red staining*

To identify the amyloid deposits, mouse brain sections were deparaffinized, dehydrated in differentiated alcohol solution and incubated with 1% Congo red (Sigma). Congo red staining was observed under a light microscope (Olympus, CK40 F-200 Japan).

2.7 *Immunohistochemistry*

Estrogen receptor α staining was performed as described: deparaffinization in xylene and graded ethanol; rinsing in distilled water 2×5 min; rinsing in Tris-containing buffered saline (TBS) (pH 7.6) 2×5 min; water bath pretreatment in 0.05 M Tris-HCl buffer (pH 7.6) for 30 min at 90°C; washing in TBS for 10 min; incubation in milk–TBS for 1 h at room temperature (RT); washing in salt TBS 1×5 min; incubation with a primary polyclonal rabbit anti-ERα antibody that recognizes the carboxyl terminus of the ERα (Santa Cruz, cat sc-542) diluted 1:100 in Sumi-milk (0.25% gelatine and 0.5 mL Triton X-100 and 5% of milk powder in 100 mL TBS, pH 7.6) for 1 h at RT . The next day, sections were washed in milk–TBS for 3×10 min; washed in TBS in 4% formaldehyde1×5 min; incubated with secondary biotinylated antirabbit at room temperature; IgG (Vector Laboratories, Burlingame, CA, USA) 1:200 in Sumi-milk for 1 h at RT; washed in TBS 3×10 min; incubated in Tris-HCl, hydrated and stained with thionine (0.5%); staining 0.05% 3,39-diaminobenzidine, 0.01% H_2O_2 0.3% nickel ammonium sulphate; washed in Tris-HCl 2×10 min; dehydrated in graded ethanol, cleared in xylene and cover slipped with D.P.X. mountant (MERK).

2.8 *Image analysis*

Densitometry analysis was performed using the NIH software, in order to analyse the intensity of the bands.

2.9 *Statistical evaluation*

Statistical analysis was performed with the one-way ANOVA using the SPPS software (student version 7.01). Results are presented as mean ± SD. In cases where an error bar is seen in the graph, the variation is small.

3. Results

3.1 *Extraction and purification of Aβ*

Cortical areas were carefully dissected from the brain tissue were acid-lysed and chromatographed on a size exclusion column. Four discrete peaks were resolved on size exclusion column of which only the ~3–5 kDa range molecules were separated, pooled and their volumes reduced, and they were subjected to chromatography on a source phenomenox reverse phase column. A polystyrene divinyl benzene reverse phase column was employed in these experiments. Major fractions had retention times identical those of the synthetic Aβ markers. The Aβ concentration was significantly increased after the third and fourth months of both the experimental animal groups when compared to control groups (figures 1 and 2). HPLC analysis also revealed that there was a high intensity peak in the OVX when compared to HCL; fractions were similar to Aβ standards. Aβ was increased in response to OVX when compared to the HCL group. Interestingly, the amyloid deposits from OVX and HCL mice brains that were detergent-insoluble fractions were largely solubilize in HFIP.

3.2 *Western blot analysis for Aβ and estrogen receptor α*

The expression of Aβ and ER α was analysed by using Western blot. The enhanced expression of Aβ was observed in OVX (figure 3b) rather than hypercholesterolemia-induced mice (figure 3a) and the control. The formic-acid-soluble ~3–5 kDa fraction was analysed by Western blotting using 6E10 primary antibody. A strong band of approximately 5 kDa was observed. The expression of Aβ predominant in HCL in the intensity of the expression was lower when compared to OVX groups. In initial months in both the group the expression was nearly as that of control. The HCL group also follows this trend but the expression was low when compared to the OVX group in later months.

Through Western blot analysis and binding, tests were able to infer that there was a normal expression of ERα in HCL animals, like that of control. At the same time the expression of ERα was highly down-regulated in OVX groups (figures 3c and d). Intensity of staining estimated by using image analysis (Image software) is shown in table 1. Down-regulated expression of ERα confirmed at the protein level, Western blot analyses identified a signal at approximately 66k Da, which was consistent with ERα. The OVX mice showed regular intervals of faint expression of ERα, while those HCL exhibited intense signals in all months after ovariectomy. The qualitative analysis of ERα signals revealed that the amount of ERα protein expressed in OVX was lower than that in control cells, and the difference is

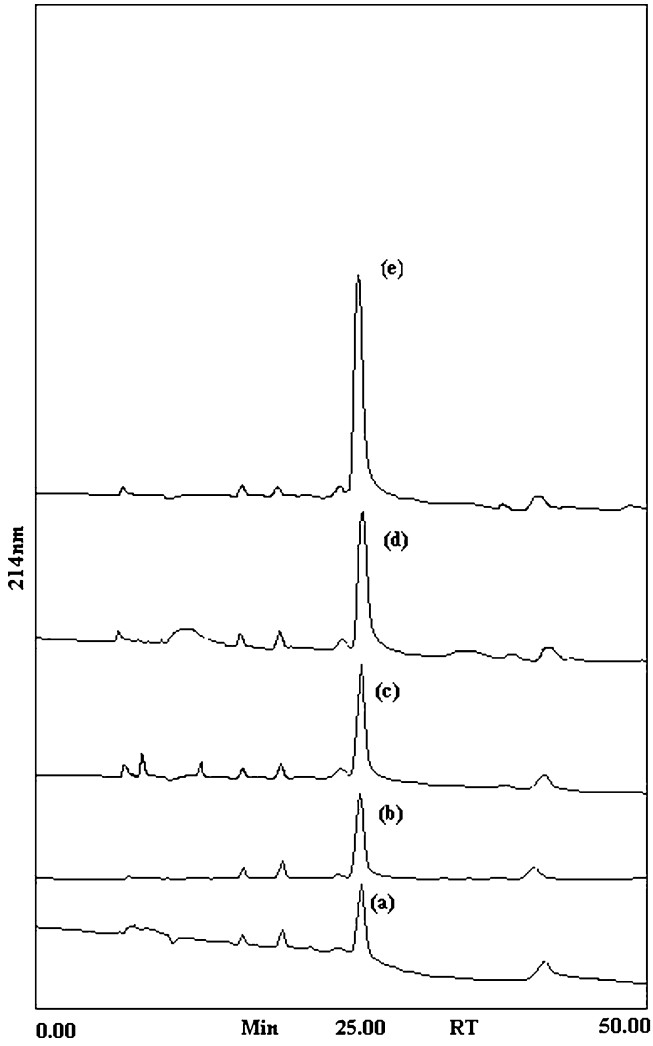

Figure 1. Aβ extract of hypercholesterolemic mice brain were identified by RP-HPLC. Eluted with 0.1% trifluoroacetic acid (TFA) in water (buffer A) and 0.1% TFA in acetonitrile (ACN) (buffer B): (**a**) control, (**b**) first month, (**c**) second month, (**d**) third month and (**e**) fourth month.

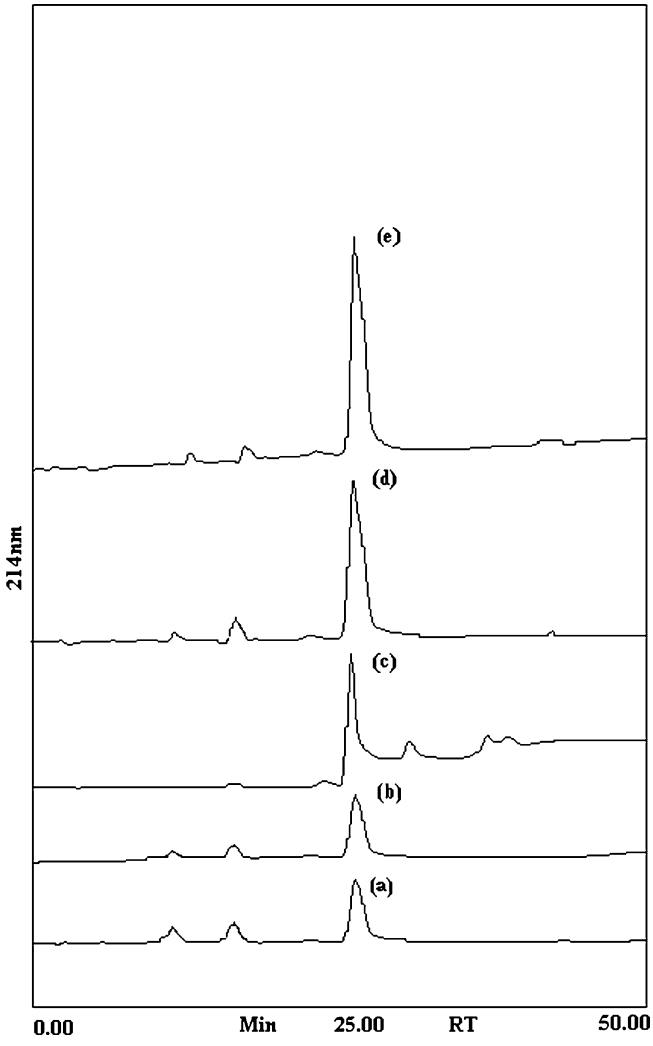

Figure 2. Reverse phase-HPLC of the major Aβ peptides present in OVX (diet-induced ovariectomized mice) animals: (**a**) control, (**b**) first month, (**c**) second month, (**d**) third month and (**e**) fourth month.

statistically significant. Intense expression of ERα was observed in the HCL group but the intensity was slightly decreased compared to control animals.

3.3 *Congo red staining*

The extents of amyloid deposition in brain and kidney tissues were analysed by Congo red staining. After Congo red staining, amyloid appears dull red and nuclei stained blue. In both the tissues staining was stronger in OVX than high-diet groups, indicating the high-degree deposition of amyloid in OVX (figures 4 and 5). Congo red birefringence suggested that amyloid deposits were in a mature fibrillar state (figure 6). Intensity of the staining was estimated semiquantitatively and is shown in (table 2).

The Congo red staining revealed that there is a condensed amyloid plaque core deposited in the cerebral vasculature in an age-specific pattern (later months) in OVX and HCL. Further, relatively higher diffused amyloid deposits were present in OVX mice than in HCL mice.

3.4 *Immunoreactivity for estrogen receptor*

ERα immunoreactive cells were highly concentrated in the control animals; it was observed that in OVX groups the expression of estrogen receptors were normal in initial

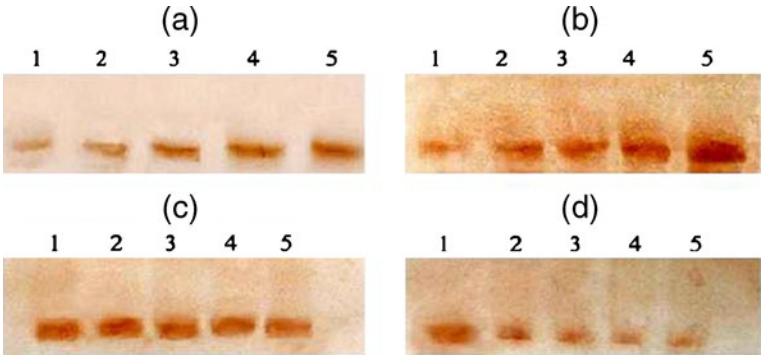

Figure 3. Immunoblotting assay for expression of Aβ, ERα: (**a**) Aβ level in diet-induced hypercholesterolemic mice brain supernatant. (**b**) Aβ Level IN OVX mice brain homogenate. (**c**) Expression of ER α in hypercholesterolemic mice brain. (**d**) Expression of ERα, in OVX animals. The densitometry analysis was performed to determine the level of ER Expression in each group (n=10) (Lane 1, control; lane 2, first month; lane 3, secondmonth; lane 4, third month; lane 5, fourth month).

months and it was highly down-regulated in later months (third and fourth month). In HCL mice the expression of estrogen receptor was normal, as that of control (figure 7). In the OVX group the intensity of ERα immunoreactivity was lower than that in the normal controls. The intensity of ERα staining was decreased and it indirectly suggests that the expression of estrogen receptor was highly down-regulated in OVX. Its down-regulation was systematic in regular month intervals. In contrast, the intensity of ERα immunoreactivity in HCL was much higher than OVX and is almost normal, as that of control animals. Intensity of the staining was estimated semiquantitatively and is shown in table 3.

3.5 *ELISA analysis for Aβ*

The expression of Aβ was analysed by ELISA. The results indicate the accumulation of Aβ increases, in months, in both the experimental groups. There was enhanced expression of Aβ in the OVX group when compared to the HCL and control groups (figure 8). ELISA analysis further suggests that Aβ expression is highly up-regulated in OVX than hypercholesterolemia. Production of Aβ via APP processing however is not the only factor that can influence the probability of Aβ deposition. Evidence indicates that factors regulating Aβ catabolism clearance and aggregation were also critical in regulating Aβ metabolism (DeMattos *et al*. 2001).

4. Discussion

The present study was designed to characterize hypercholesterolemia-associated alterations in levels of estrogen receptor and related downstream proteins that regulate Aβ production, tau phosphorylation, and cell survival in the Swiss mice with a cholesterol-enriched diet, a model system for sporadic AD. These studies demonstrated for the first time that a cholesterol-enriched diet and subsequent hypercholesterolemia on estrogen-deprived animals

Epidemiological and biochemical investigations provide increasing evidence that altered cholesterol metabolism contributes to the development of AD. The objective of the present study was to examine the links between cholesterol and amyloid metabolism from the animal model. Aβ peptides, the major component of the senile plaques in the AD brains, are the end product of the abnormal post-translational

Table 1. Western blot intensity, mean % of Aβ and Er α expression in control (C), high diet (1 to 4 months) and OVX (I to IV months) group animals

Protein intensity	C	I	II	III	IV
High Diet					
Aβ	22±0.7	26±0.9	28±0.3	30±0.2	31±0.1
ER-α	24±0.6	23±0.1	22±0.5	22±0.2	22±0.3
OVX					
Aβ	23±2.5	28±1.2	33±1.3	38±1.0	40±1.0
ER-α	23±2.1	18±1.8	17±0.2	16±0.4	16±0.5

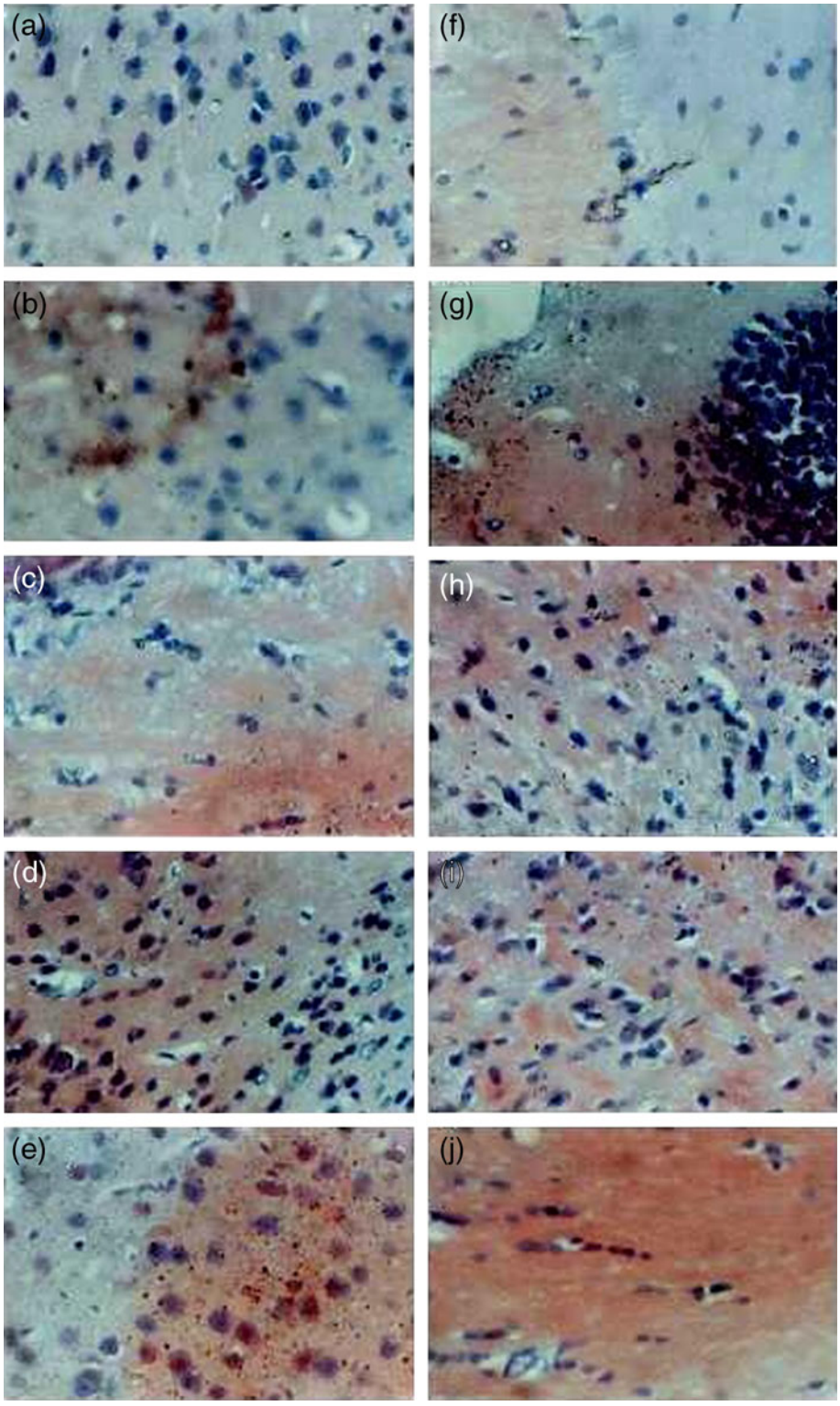

Figure 4. Amyloid staining with Congo red of diet induced mice brain (left panel). Amyloid staining with Congo red of OVX mice brain (right panel × 100).

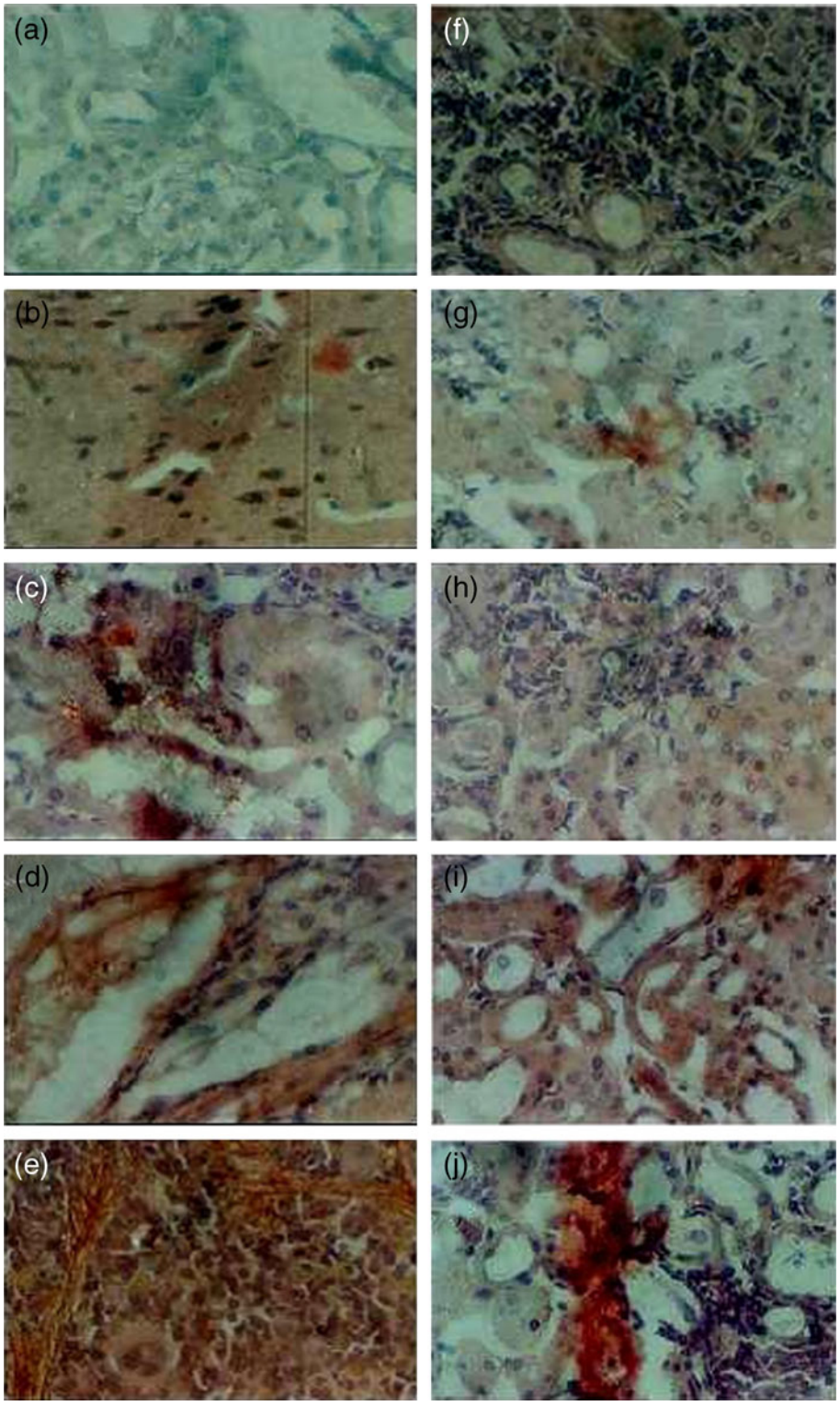

Figure 5. Amyloid staining with Congo red of diet-induced mice kidney (left panel). Amyloid staining with Congo red of OVX mice kidney (right panel × 100).

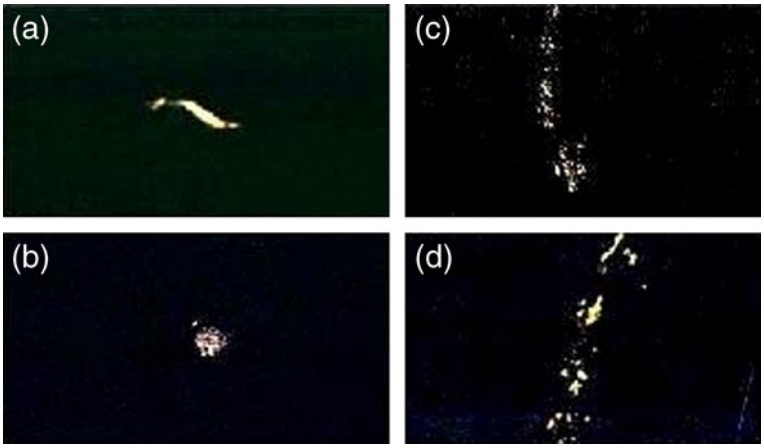

Figure 6. Congo red brain section viewed under polarized light to demonstrate the characteristic 'apple green' birefringence. (**a**) High-diet Congo red brain section × 100; (**b**) high-diet kidney Congo red section × 100; (**c**) OVX brain Congo red section × 100; (**d**) OVX kidney Congo red section × 100.

processing of its precursor, called amyloid precursor protein. The compartmentation of the amyloid precursor protein molecule within the cell membrane is regulated by the cholesterol content of the bilayers. The amyloid precursor protein molecule could be present either in or outside the membrane rafts. Any kind of process that alters the compartmentation preference of the amyloid precursor protein molecule, by transferring it to the membrane rafts, favours β- and γ-secretase cleavage, and should be recognized as an amyloidogenic process. If the BBB is intact, the brain will not be able to take up the lipoprotein particles responsible for the transport of cholesterol.

Earlier studies suggest that increase in cholesterol concentration enhances the cleavage of APP by secretase and it critically depends on the lipid raft environment (Ehehalt *et al*. 2003). Significantly, these micro-domains contain several proteins important to the pathophysiology of AD including APP, Aβ, apoE, PKCα, PSI, possibly both α- and β-secretases and GPI-anchored proteins, which modulate the β-secretases cleavage of APP (Sambamurti *et al.* 1999). It is plausible that the cholesterol content of the micro-domains may be vital for the biochemical processes involved in Aβ production and accumulation (Refolo *et al*. 2000).

A number of studies have suggested that the aberrant processing of amyloid precursor protein and formation of Aβ with in the neuronal cell body may be key initial events in a series of pathological changes leading to AD (Andrea *et al*. 2001). Intracellular distribution between free cholesterol in the membrane and cholestreyl esters in the cytoplasm may be more important than total cholesterol in regulating APP processing Aβ production (Burns *et al*. 2003). Alteration of the intracellular distribution of cholesterol may also affect unknown proteins other than the secretase that control APP processing. Increase of total cholesterol does not necessarily result in elevation of Aβ production as observed in mice (Howland *et al*. 1998).

Estrogen deprivation has been implicated in the pathogenesis of AD (Manly *et al*. 2000). Postmenopausal women demonstrate an increased incidence of AD (Gandy and Duff 2000), and the level of serum estradiol is lower in women who develop AD. Epidemiological evidence suggests that postmenopausal estrogen replacement therapy (ERT) may reduce the risk or delay the onset of AD (Kawas *et al*. 1997). Because estrogen has many neuroprotective and neurotrophic proclivities (Goodman *et al*. 1996), its decline with menopause might leave the brain vulnerable to the toxic

Table 2. Semiquantitative Congo red staining intensity analysis Control (C), high diet (1 to 4 months) and OVX (1 to 4 months) group animals

	High Diet					OVX				
Congo red intensity	C	I	II	III	IV	C	I	II	III	IV
Brain	- -	+ -	+	+	+ +	- -	+ -	+	+ +	+ + +
Kidney	- -	+ -	+	+	+ +	+ -	+ -	+	+ +	+ + +

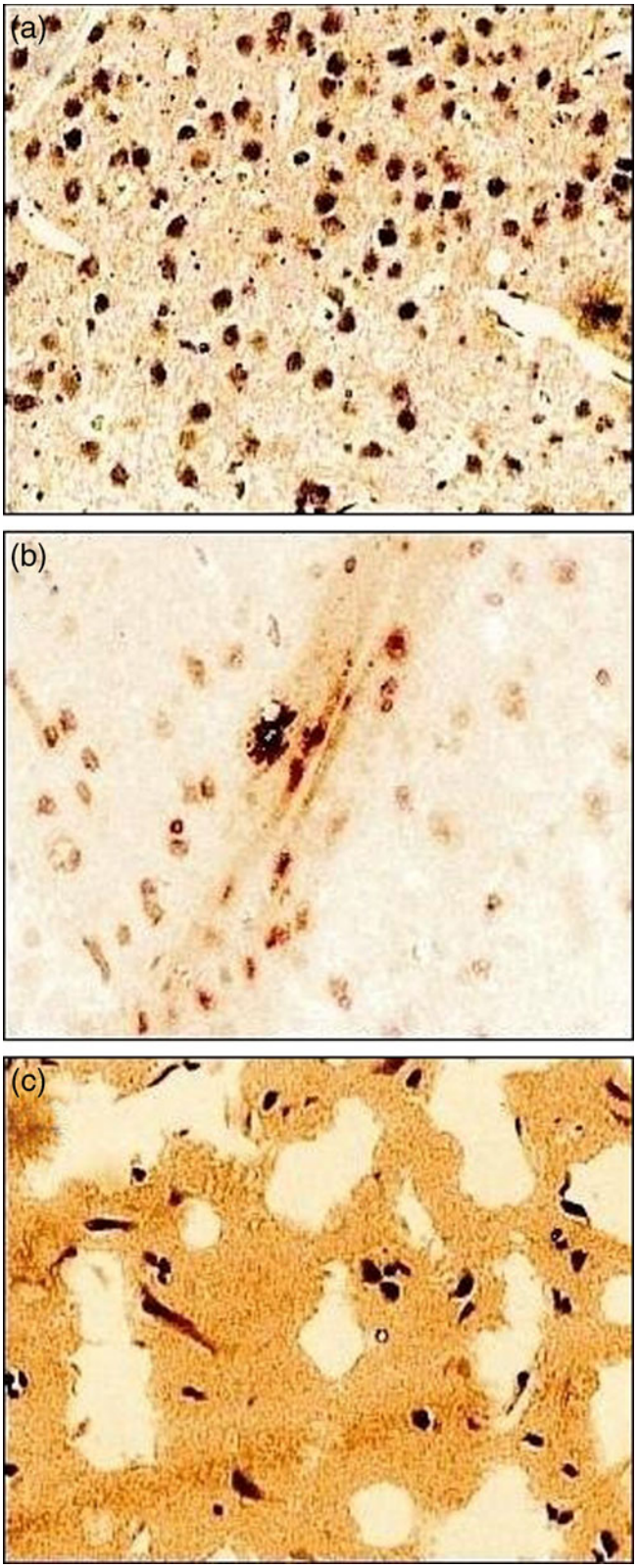

Figure 7. Immunohistochemistry staining for ERα in (**a**) control × 100; (**b**) high diet × 100 and (**c**) OVX × 100

Table 3. Staining intensity of Er α

Group	Intensity
Control	+ + +
High diet	+ +
OVX	+ -

Note: (+++), strong; (++), moderate; (+), weak; (+ −) very weak; (−) absent.

influence of elevated levels of Aβ or inflammatory proteins such as seen in AD (Brinton *et al*. 2000). Estrogen's ability to attenuate the progress of AD (Duka *et al*. 2000) may be due to its ability to reduce the activation of microglia. Estrogen's ability to protect vulnerable neuronal populations within the AD brain may be due to the presence of estrogen receptors on neurons and glia (Mufson *et al*. 1999). A recent *in vitro* study demonstrated that 17-β estradiol could dose-dependently prevent the activation of primary cultures of rat microglia and reduce the expression of inflammatory proteins (Vegeto *et al*. 2001). Given the apparent neuroprotective actions of estrogen within the brain of female humans at risk for AD, the present study investigated estrogen's ability to influence accumulation of Aβ within the brain of female mice. The co-localization of estrogen receptors in the basal forebrain, cerebral cortex and hippocampus with the receptors for nerve growth factor, as well as with nerve growth factor itself, is consistent with the idea that growth factors might play a role in mediating the effects of estrogen in the central nervous system (Miranda *et al*. 1993).

The decreases in estrogen receptor α immunoreactivity in OVX group suggest that depletion of estrogen down-regulates the expression of ERα in cellular and extracellular localizations. Earlier studies also supports to our finding (Jesmin *et al*. 2003). At the same time immunoreactivity in HCL and control groups showed no significant

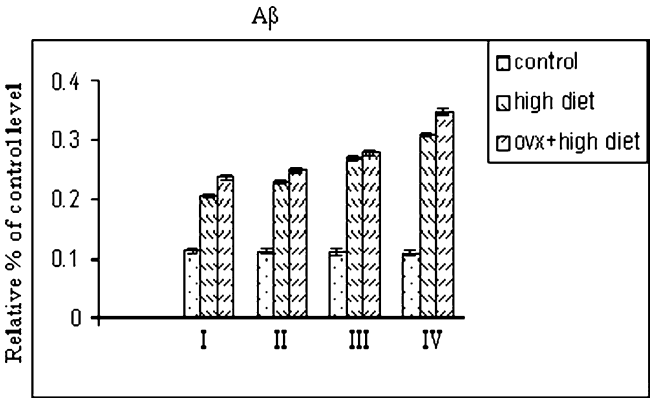

Figure 8. Quantitation of Aβ by ELISA in OVX, high-diet induced and control mice brain.

change in the intensity. This suggests that decrease deposition in hypercholesterolemia-treated groups might be due to the up-regulation of estrogen receptors in these groups. Importance has been recognized of inflammatory mechanism in the pathogenesis of neurodegenerative disease such as AD. Microglia, the resident of macrophages of brain, are pathologically activated in AD.

These studies suggest that deprivation of estrogen and hypercholesterolemia raised the accumulation of Aβ expression in brain. This result also reveals that deprivation of estrogen inhibits phagocytosis of Aβ so as the Aβ clearance is reduced. This study may be useful as some therapeutic approach may exist for estrogen-deprived condition, a drug as an agonist. In conclusion the present study suggests that hypercholesterolemia and estrogen deprivation raised accumulation of Aβ in ovariectomized mice animal models. Ovariectomized with diet-induced hypercholesterolemia, which is known to produce chronic inflammation and associated systemic amyloidosis, triggers Aβ accumulation in the mouse brain.

HPLC method was used to show the enhanced synthesis of Aβ fragments in 1–4 months of ovariectomized mice model. The amyloid fibrils were identified with Congo red staining to test the presence of green birefringence characteristic of amyloid when viewed under the light microscope using polarized light, and this was further confirmed by Western blotting and ELISA analysis. In this article we also discussed estrogen receptors. The decrease in estrogen receptor α immunoreactivity in OVX group suggests that depletion of estrogen down-regulates the expression of estrogen receptor α in cellular and extra-cellular localizations. These results indicate that the hypercholesterolemia and estrogen deprivation raise the risk of development of accumulation of Aβ than hypercholesterolemia-induced ovariectomized mice.

These animal models may usefully, and certain therapeutic approaches exist for certain forms of amyloidosis that offer encouragement that it may also be possible to develop logical treatment approaches for the pathogenesis of AD.

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