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Role of fibrillar $A\beta_{25-35}$ in the inflammation induced rat model with respect to oxidative vulnerability

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Abstract

The major pathological ramification of Alzheimer's disease (AD) is accumulation of β-Amyloid (Aβ) peptides in the brain. An emerging therapeutic approach for AD is elimination of excessive Aβ peptides and preventing its re-accumulation. Immunization is the most effective strategy in removing preexisting cerebral Aβs and improving the cognitive capacity as shown in transgenic mice model of AD. However, active immunization is associated with adverse effect such as encephalitis with perivascular inflammation and hemorrhage. Details about the mechanistic aspects of propagation of these toxic effects are matter of intense enquiry as this knowledge is essential for the understanding of the AD pathophysiology. The present work aimed to study the oxidative vulnerability in the plasma, liver and brain of the inflammation-induced rats subjected to Aβ immunization. Induction of inflammation was performed by subcutaneous injection of 0.5 ml of 2% silver nitrate. Our present result shows that the proinflammatory cytokines such as IL1α and TNFα are increased significantly in the inflammation-induced, $A\beta_{1-42}$, $A\beta_{25-35}$ treated groups and inflammation with $A\beta_{25-35}$ treated group when compared to control, complete Freund's adjuvant and $A\beta_{35-25}$ treated groups. These increased proinflammatory cytokines concurrently releases significant amount of free radicals in the astrocytes of respected groups. The present result shows that nitric oxide (NO) level was significantly higher ($P < 0.001$) in plasma, liver and brain of the rat subjected to inflammation, $A\beta_{1-42}$, $A\beta_{25-35}$ and inflammation with $A\beta_{25-35}$ injected groups when compared to control. The increased level of lipid peroxides (LPO) ($P < 0.001$) and decreased antioxidant status ($P < 0.05$) were observed in the plasma, liver and brain of inflammation-induced group when compared to control. Our result shows that significant oxidative vulnerability was observed in the inflammation with Aβ treated rats when compared to other groups. Based on our results, we suggest that immunization of AD patients with Aβ should be done with caution as the increase in Aβ could trigger the brain inflammation in uncontrollable level.

Keywords: β-*amyloid fibril, inflammation, cytokines, antioxidants, lipid peroxidation, reactive oxygen species*

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by deposition of Aβ peptides in the brain and the associated oxidative damage leads to progressive memory loss and dementia. The principal peptide component of senile plaques is β-amyloid peptide (Aβ), which, in the fibrillar form consists of 39–42 amino acid residues. Aβ peptides are proteolytically derived from the amyloid precursor protein (APP) [1]. The overexpression of mutant APP results in neuritic plaque formation and synaptic loss [2]. Neuritic plaque in AD is densely surrounded by reactive astrocytes [3].

Recently, a number of studies have shown that Aβ immunization results in a significant reduction in cerebral Aβ levels and some studies have shown improvement in cognitive deficits in APP transgenic

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(Tg) mice model. Schenk et al. [4] first reported a beneficial plaque lowering effect of active immunization against the pre-aggregated Aβ peptide mixed with complete Freund's adjuvant in a Tg mouse model of AD. This was subsequently replicated in experimenting with different Tg mice [5]. Following these reports, Lemere et al. [6] demonstrated that intranasal immunization with freshly solubilized Aβ 1–40 reduced cerebral amyloid burden in PDAPP mouse. A phase II clinical vaccination trial in which Aβ 1–42 was injected into individuals with AD has recently been terminated because of cerebral hemorrhage due to inflammation observed in some patients [7]. These side effects are implicated to Aβ toxicity and/or cell mediated autoimmunity [8].

Early results indicate that Aβ neurotoxicity in AD results from Aβ fibril induced oxidative stress [9], presumably initiated either directly by Aβ generated free radicals [10] or indirectly through intracellular production of reactive oxygen species (ROS) [11]. Oxidative stress stimulates increased vulnerability to excitotoxicity, disruption of calcium homeostasis and impaired glucose uptake, which lead to cell death and thereby contributing to pathogenesis of AD [12]. Further, there are also reports on the aggregated Aβ peptide interaction with the RAGE to give rise to the generation of ROS within the neuronal cells [11]. Antioxidants can protect neurons from Aβ toxicity *in vitro* [13], a clinical trial was performed to test the ability of vitamin E to slow down the progression of AD [14]. As many groups have focused inflammation reactions due to Aβ toxicity, the knowledge on inflammatory effect due to Aβ vaccination is scarce. Guo et al. [15] has reported that inflammation is directly involved in the initiation of amyloid deposition in the brain of Tg mice model. It has shown that anti-inflammatory drugs are decreasing the pathogenesis of AD [16].

The objective of the present study is to assess the oxidant and antioxidants status in the plasma, liver and brain of the inflammation-induced rats when subject to Aβ immunization, which may be relevant to immunization along with inflammatory diseases. Our study gains importance due to the fact that it provides the first quantitative analysis of the effect of Aβ peptide on IL1α, TNFα, ROS, NO, LPO and antioxidant activity in inflammation induced rat model. The $A\beta_{25-35}$ was used for the present work because it exhibits all the biological activity of the full length Aβ and it is easy to synthesize compared to $A\beta_{1-40}$ and $A\beta_{1-42}$[17].

Materials and methods

Chemicals and animal experiments

Thio barbituric acid (TBA), Dithio bis nitrobenzoic acid (DTNB), 2′7′-diacetyl dichlorofluorescein (DCFH-DH) were obtained from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals were of analytical grade or the highest purity available. Adult male albino rats of the Wistar strain, weighing approximately 130 ± 10 g, were obtained from King Institute of Preventive Medicine, Chennai, India. They were acclimatized to animal-house conditions, were fed commercial pelleted rat chow (Hindustan Lever Ltd., Bangalore, India), and had free access to water. All the animal experiments used in this study were approved by local animal ethical committee of Central Leather Research Institute. The surgical procedures were performed in a laminar flow hood under aseptic conditions following NIH animal ethical guidelines.

The rats were divided into seven groups ($n = 6$ in each group): Group 1, received 0.5 ml of PBS pH 7.4 (control); Group 2, the inflammation was induced by single subcutaneous injection of 0.5 ml of 2% silver nitrate [18]; Group 3, single intraperitoneal injection of complete Freund's adjuvant; Group 4, single intraperitoneal injection of 25 μg scrambled $A\beta_{35-25}$ with complete Freund's adjuvant (scramble control); Group 5, single intraperitoneal injection of 25 μg $A\beta_{25-35}$ with complete Freund's adjuvant; Group 6, single intraperitoneal injection of 25 μg $A\beta_{1-42}$ with complete Freund's adjuvant; Group 7, intraperitoneal injection of complete Freund's adjuvant with 25 μg of $A\beta_{25-35}$ administered after 24 h of subcutaneous injection of silver nitrate. The dosage was fixed on the basis of information available from the report of Spooner et al. [19]. The protofibril formation was checked by light scattering method and transmission electron microscope (data not shown). After 24 h, all animals were sacrificed by cervical decapitation. Plasma, liver and brain were used for the following biochemical assay.

Synthesis and purification of $A\beta_{1-42}$, $A\beta_{25-35}$ and $A\beta_{35-25}$

The peptides $A\beta_{1-42}$, $A\beta_{25-35}$ and scramble $A\beta_{35-25}$ were synthesized by manual solid phase chemistry, using Fmoc as the protective group for N-terminal ends, and 1-Hydroxy benzotriazole (HOBt), and N,N-dicyclohexyl carbodiimide as coupling agent and pentafluorophenol activators of carboxylic ends. The peptides were cleaved from (4-Hydroxymethylphenoxy acetyl) Wang resin with trifluoromethane sulphonic acid/thioanisole/ethanedithiol/trifluoroacetic acid (1:1:1:7) and precipitated with cold ether. The composition of peptides was determined by amino acid analysis using Phenyl isothiocyanate (PITC) method. The synthesized peptides were purified by RP-HPLC as previously described [20]. The purified peptide was characterized by MALDI-TOF MS analysis. The peak corresponding to the $(M + 2H)^+$ ion for $A\beta_{1-42}$ and M^+ ion for $A\beta_{25-35}$ and $A\beta_{35-25}$ peptides were observed. The derived mass of the peptide was calculated from the MALDI spectrum to be 4515.4($A\beta_{1-42}$) and 1060.4($A\beta_{25-35}$ and $A\beta_{35-25}$) Daltons. The peptides

were then characterized by ^{1}H-NMR. All reagents used in peptide synthesis pentafluorophenol (purchased from Spectrochem Pvt. Ltd., Mumbai), N,N-dicyclohexyl carbodiimide (Sigma-Aldrich), Fmoc-AA (Novabiochem), HOBt (Sigma-Aldrich), TFA (Sigma-Aldrich) were of the purest analytical grade. The Aβ peptide fibrils were formed by solubilizing the peptide in sterile double distilled (DD) water (1 mg/ml) and incubated 24 h at 37°C [21]. The protofibrillar form thus obtained was used fresh for the present study.

Analysis of IL1α and TNFα levels

The liver and brain of the experimental rat was quickly removed, weighed, and homogenized in ice-cold buffer containing 1% Nonidet, P-40, 50 mM Hepes, 500 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, and 5 μg/ml leupeptin (all from Sigma Chemical Co.). The homogenates were centrifuged at 3000 rpm at 4°C for 15 min, and the supernatants were removed. The IL1α and TNFα concentrations were determined by ELISA technique using specific polyclonal antibodies. After washing with phosphate buffered saline, the microplates were incubated with ALP conjugated secondary goat anti rabbit IgG. Proteins bound to antibodies were measured using *p*-nitrophenyl phosphate in carbonate buffer system at 405 nm.

Astrocytes isolation

Astrocytes were isolated as described in the procedure, which include 20% of glial and neuronal cells [22]. In brief, 100 mg of fore brains were removed from experimental animals (control and treated) and minced with scalpel in a solution containing 20 μg/ml DNase and 0.3% BSA in Hank's buffered salt solution (HBSS). This is centrifuged and incubated in a solution containing 0.025% trypsin, 0.1% collagenase and 0.3% BSA in HBSS in a water bath at 37°C. After 15 min the resulting mixer is centrifuged and the pellet is agitated 5 times with a fire-polished Pasteur pipette. It was left to settle for 4 min and the supernatant was collected. Approximately, 8.5×10^6 cells/ml were used for ROS quantification. The initial viability of astrocytes was greater than 90% as assessed by Trypan blue exclusion. Isolated astrocytes were used immediately for ROS quantification.

ROS measurement

A non-fluorescent probe, 2′7′-diacetyl dichlorofluorescein (DCFH-DH) was used to measure ROS, which is able to penetrate into the intracellular matrix and it becomes oxidized by ROS to fluorescent dichlorofluorescein (DCF), as previously described [23]. Briefly an aliquot of astrocytes (100 μl) was made up to a final volume of 2 ml in normal phosphate buffered saline (pH 7.4). A total of 500 μl aliquot astrocytes were taken to which 100 μl DCFHDA (200 μM) was added and incubated at 37°C for 30 min. Samples were then centrifuged and washed twice with PBS. Fluorescent measurements were made with excitation and emission filters set at 485 ± 10 and 530 ± 12.5 nm, respectively. All initial fluorescent values (time 0) were found to differ from each other by less than 5%. Results were expressed as percentage increase in fluorescence calculated using the following formula $[(Ft_{30} - Ft_0)/Ft_0 \times 100)]$ are the fluorescence intensities at 0 and 30 min.

This experiment is also carried out and the ROS production is observed using fluorescence microscope. The cells were viewed under FITC filter with cytovision image analysis system (Application software version 2.7). The images were captured at 40 × magnification.

Nitric oxide (NO)

The presence of nitrite, a stable oxidized product of NO, was determined as per the method described by Kim et al. [24]. Briefly, 100 μl supernatant was incubated with an equal volume of Griess reagent (1% sulfanilamide and 0.1% N-(1-napthyl) ethylenediamine dihydrochloride in 2.5% phosphoric acid). After 10 min of incubation at room temperature, the absorbance of the cromophore so formed was measured at 560 nm using a microtitre plate. Nitrite concentration in the supernatant was determined using calibration curve of sodium nitrite.

Thiobarbituric acid reactive substances (TBARS)

The method of Ohkawa et al. [25] was followed to determine the level of TBARS. This method is based on the reaction between the aldehyde products formed during the process of lipid peroxidation and thiobarbituric acid. The TBARS level is monitored by measuring the absorption at 535 nm.

Superoxide dismutase (SOD)

The SOD was assayed according to the method of Misra and Fridovich [26]. The assay is based on the inhibition of epinephrine–adrenochrome transition by the enzyme.

Catalase

The activity of catalase enzyme was determined by the method of Beers and Seizer [27]. The breakdown of hydrogen peroxide on the addition of enzyme is followed by observing the decrease in light absorption of peroxide solution in the ultraviolet (UV) region.

Glutathione peroxidase (GPx)

The GPx was determined by the method of Rotruch et al. [28]. This method is based on the reaction

between leftover glutathione in the following reaction with the dithio bis nitrobenzoic acid (DTNB) to form an intermediate, which absorbs maximally at 412 nm.

Total reduced glutathione (GSH)

The method of Moron et al. [29] was followed to determine the GSH. The method is based on the reaction of glutathione with DTNB to give an absorption at 412 nm.

Ascorbic acid

Ascorbic acid was estimated by the method of Omaye et al. [30]. The ascorbic acid is oxidized by copper to form dehydroascorbic acid and diketoglutaric acid. These products are reached with 2,4-dinitrophenylhydrazine (DNPH) to form a compound, which in the presence of concentrated H_2SO_4, undergoes rearrangement to form a product with absorbance maximum at 520 nm. The reaction is seen in the presence of thiourea to provide a reducing medium with buffer to prevent interference from non-ascorbic acid chromogen.

Protein content

The protein content of the plasma and tissue homogenate was determined by the method of Lowry et al. [31] using bovine serum albumin as standard.

Statistical analysis

Results were statistically evaluated using one-way analysis of variance (ANOVA) for repeated measurements. Values of $P < 0.05$ were considered to be significant.

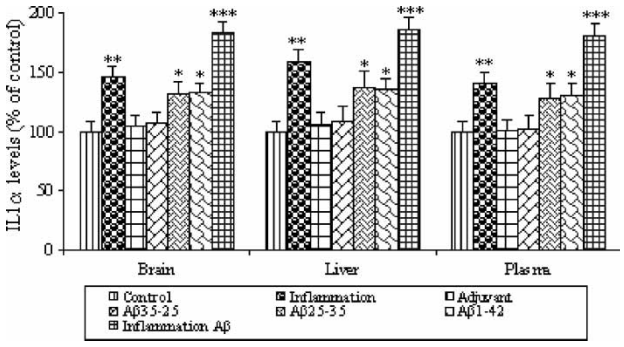

Figure 1. Variation of IL1α content in the brain, liver and plasma of PBS, silver nitrate, adjuvant, $A\beta_{35-25}$, protofibrillar form of $A\beta_{25-35}$, $A\beta_{1-42}$ and silver nitrate along with $A\beta_{25-35}$ protofibril treated group of rats. The values were expressed as a percentage of IL1α content relative to control. Each data point represents the mean of six animals. $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$ with respect to the value in the corresponding control.

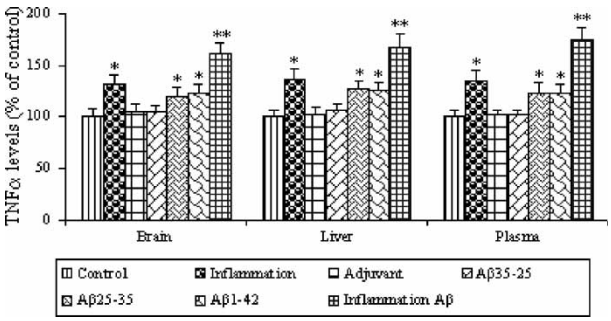

Figure 2. Variation of TNFα content in the brain, liver and plasma of PBS, silver nitrate, adjuvant, $A\beta_{35-25}$, protofibrillar form of $A\beta_{25-35}$, $A\beta_{1-42}$ and silver nitrate along with $A\beta_{25-35}$ protofibril treated group of rats. The values were expressed as a percentage of TNFα content relatively to control. Each data point represents the mean of six animals. $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$ with respect to the value in the corresponding control.

Results

Inflammation can activate macrophage or monocytes directly, initiating a cytokine cascade in the inflammatory process as well as immunological responses [32]. Stimulated monocytes release a broad spectrum of cytokines, which can induce the subsequent cytokine cascade. Our present result shows that the proinflammatory cytokines such as IL1α and TNFα produced by monocytes are significantly high in the inflammation-induced group, $A\beta_{1-42}$, $A\beta_{25-35}$ treated groups and inflammation with $A\beta_{25-35}$ treated group when compared to different control used in this work (Figures 1 and 2).

These proinflammatory cytokines can initiate and maintain the acute phase of the inflammatory responses with the concurrent release of free radicals [33]. One possible toxic mechanism of AD can be viewed as the accumulation of such free radicals during inflammation [34]. In the present study the intracellular ROS concentration was significantly higher in the astrocytes of inflammation-induced group, $A\beta_{1-42}$, $A\beta_{25-35}$ treated groups and inflammation with $A\beta_{25-35}$ treated group when compared to controls (Figures 3 and 4). However, our result shows that there were no significant

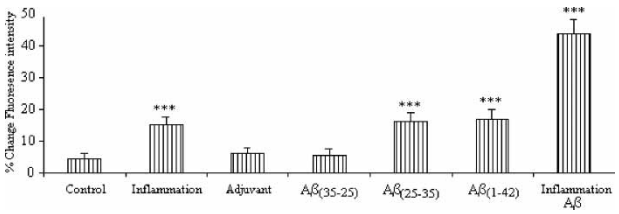

Figure 3. Spectrofluorimetric quantification of ROS levels in the astrocytes extracted from rats treated with PBS, silver nitrate solution, adjuvant, $A\beta_{35-25}$, protofibrillar form of $A\beta_{25-35}$, $A\beta_{1-42}$ and silver nitrate along with $A\beta_{25-35}$ protofibril treated group of rats. The ROS was measured after incubation with DCFH-DA for 30 min with astrocytes by excitation and emission wavelengths fixed at 485 and 530 nm, respectively. Each data point represents the mean of six animals. $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$ with respect to the value in the corresponding control.

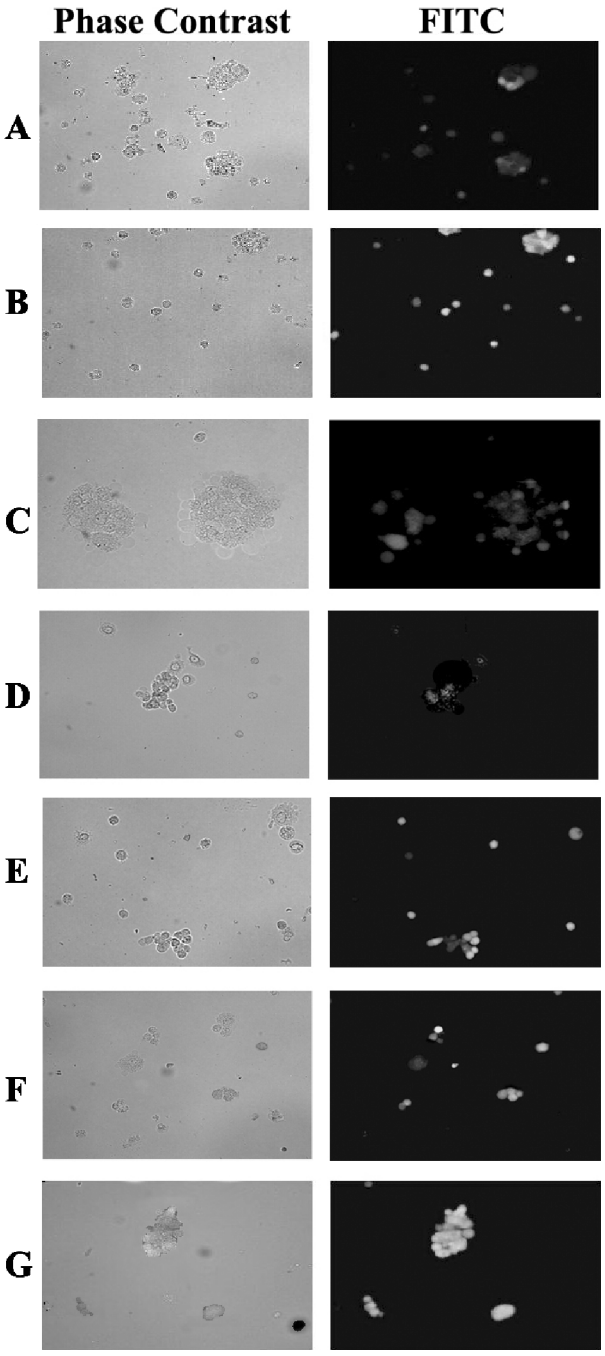

Figure 4. Observation of reactive oxygen species in the astrocytes isolated from PBS, silver nitrate solution, adjuvant, $A\beta_{35-25}$, protofibrillar form of $A\beta_{25-35}$, $A\beta_{1-42}$ and silver nitrate along with $A\beta_{25-35}$ protofibril treated group of rats using DCFH-DA as a fluorogenic probe. Cells glowing green is a positive signal for ROS production in FITC filter.

difference in ROS production between $A\beta_{1-42}$ and $A\beta_{25-35}$ injected groups. Our data also highlights that there were no significant difference in the production of ROS among control, complete Freund's adjuvant and $A\beta_{35-25}$ injected groups. Figure 4 gives the comparison between astrocytes with, without and partial ROS production. Cells glowing green showed positive signal for ROS, which is an index for free radical level in the

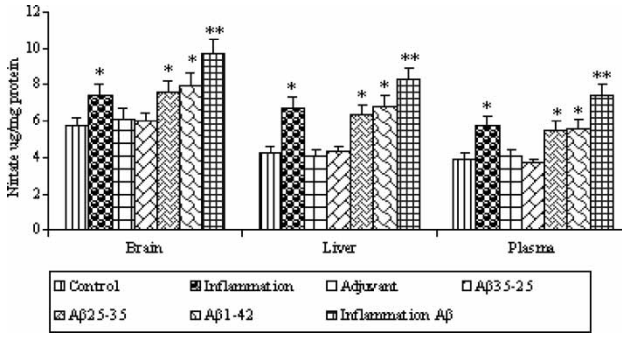

Figure 5. Variation of NO levels in the brain, liver and plasma of PBS, silver nitrate, adjuvant, $A\beta_{35-25}$, protofibrillar form of $A\beta_{25-35}$, $A\beta_{1-42}$ and silver nitrate along with $A\beta_{25-35}$ protofibril treated group of rats. Each data point represents the mean of six animals. $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$ with respect to the value in the corresponding control.

cell. The number of green signals increased in the case of inflammation, Aβ and inflammation with Aβ treated groups (Figure 4).

NO is a short-lived cytotoxic mediator that has been contributing to several neurological diseases. Our result shows that NO concentration was significantly higher ($P > 0.05$) in the inflammation with $A\beta_{25-35}$ injected rat when compared to other groups (Figure 5). There was no significant difference between controls, complete Freund's adjuvant and $A\beta_{35-25}$ injected rats.

Analysis of postmortem AD brain tissue using biochemical and immunohistochemical approaches has clearly documented an increase in membrane lipid peroxidation [35]. LPO levels were significantly higher in plasma, liver and brain of the inflammation, $A\beta_{25-35}$ and $A\beta_{1-42}$ administered groups when compared with complete Freund's adjuvant, $A\beta_{35-25}$ injected rat. Our data indicates that significant increase in the level of LPO was observed in the inflammation together with $A\beta_{25-35}$ treated group when compared with other experimental groups (Figure 6). No significant

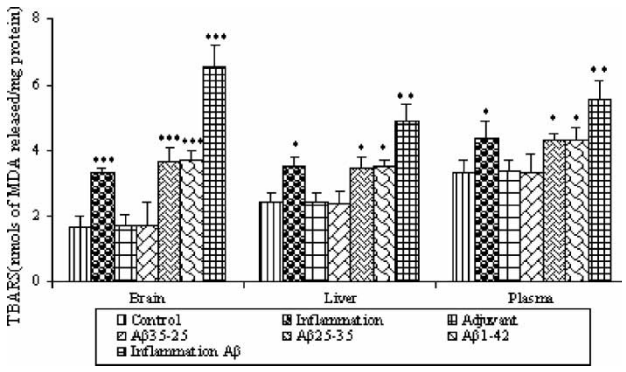

Figure 6. Variation of LPO levels in the brain, liver and plasma of PBS, silver nitrate, adjuvant, $A\beta_{35-25}$, protofibrillar form of $A\beta_{25-35}$, $A\beta_{1-42}$ and silver nitrate along with $A\beta_{25-35}$ protofibril treated group of rats. Each data point represents the mean of six animals. $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$ with respect to the value in the corresponding control.

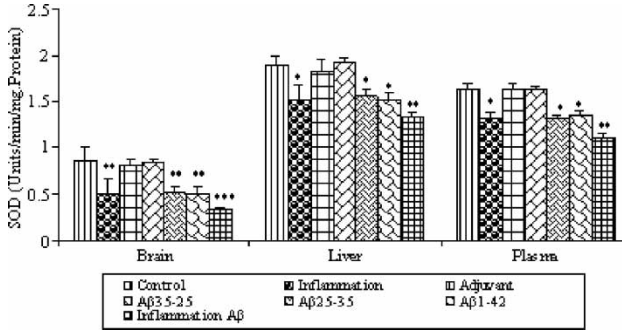

Figure 7. Variation of SOD activity in the brain, liver and plasma of PBS, silver nitrate, adjuvant, $A\beta_{35-25}$, protofibrillar form of $A\beta_{25-35}$, $A\beta_{1-42}$ and silver nitrate along with $A\beta_{25-35}$ protofibril treated group of rats. Each data point represents the mean of six animals. $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$ with respect to the value in the corresponding control.

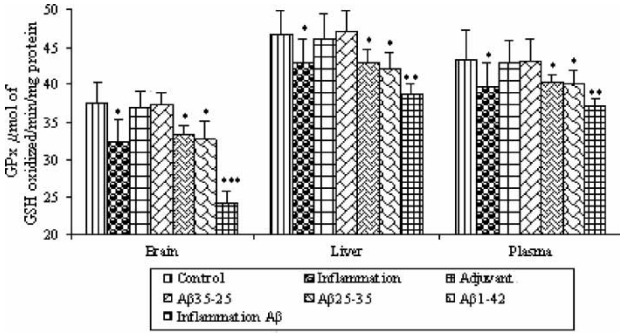

Figure 9. Variation of GPx activity in the brain, liver and plasma of PBS, silver nitrate, adjuvant, $A\beta_{35-25}$, protofibrillar form of $A\beta_{25-35}$, $A\beta_{1-42}$ and silver nitrate along with $A\beta_{25-35}$ protofibril treated group of rats. Each data point represents the mean of six animals. $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$ with respect to the value in the corresponding control.

difference in LPO production was found between $A\beta_{1-42}$ and $A\beta_{25-35}$ injected groups. It should be noted that LPO level in the brain was highly elevated when compared to plasma and liver.

There is increasing experimental evidence indicating the impairment in cellular total antioxidant capacity in AD. The activities of the antioxidant enzymes SOD, catalase and GPx are significantly reduced in the frontal and temporal cortex of AD patients [36]. Our result shows that free radical scavenging enzyme activities were significantly decreased in plasma, liver and brain of inflammation, $A\beta_{25-35}$ and $A\beta_{1-42}$ treated groups when compared with control. The significant changes were similar between inflammation, $A\beta_{25-35}$ and $A\beta_{1-42}$ injected groups. The free radical scavenging enzymes were found to decrease drastically in the inflammation with Aβ treated animal group when compared to other groups (Figures 7–9). There was no significant difference observed between control, complete Freund's adjuvant and scramble $A\beta_{35-25}$ injected rat.

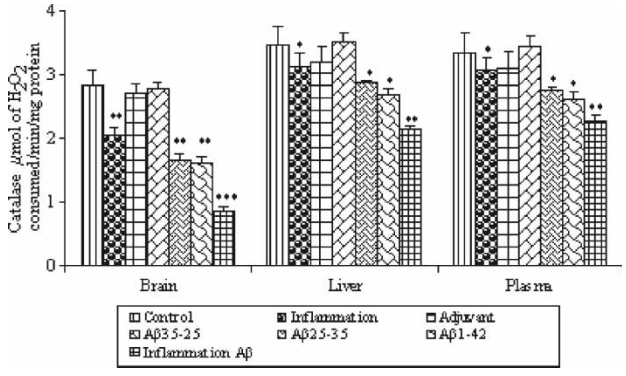

Figure 8. Variation of catalase activity in the brain, liver and plasma of PBS, silver nitrate, adjuvant, $A\beta_{35-25}$, protofibrillar form of $A\beta_{25-35}$, $A\beta_{1-42}$ and silver nitrate along with $A\beta_{25-35}$ protofibril treated group of rats. Each data point represents the mean of six animals. $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$ with respect to the value in the corresponding control.

GSH and ascorbic acid are important intracellular antioxidants that protects cells against a variety of different oxidant species/radicals. The disturbance of GSH and ascorbic acid homeostasis has been implicated in the pathogenesis of several neurodegenerative diseases including AD [37]. The present result shows that GSH and ascorbic acid levels were significantly decreased in the inflammation with Aβ treated group when compared to inflammation induced, complete Freund's adjuvant, Aβ alone treated groups. There was no significant difference observed between control, complete Freund's adjuvant and scramble $A\beta_{35-25}$ injected rat. Our result also highlights that brain was more susceptible than plasma and liver (Figures 10 and 11).

Discussion

Our data demonstrate that, systemic inflammation induced by silver nitrate significantly increases the proinflammatory cytokines such as IL1α and TNFα in the plasma, liver and brain (Figures 1 and 2).

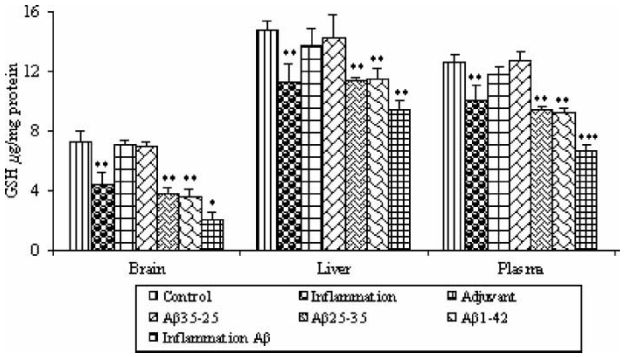

Figure 10. Variation of GSH levels in the brain, liver and plasma of PBS, silver nitrate, adjuvant, $A\beta_{35-25}$, protofibrillar form of $A\beta_{25-35}$, $A\beta_{1-42}$ and silver nitrate along with $A\beta_{25-35}$ protofibril treated group of rats. $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$ with respect to the value in the corresponding control.

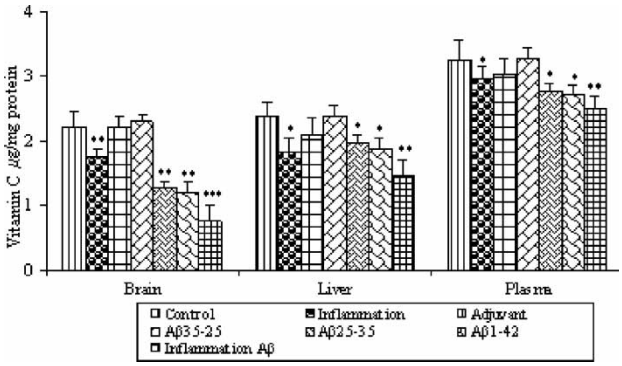

Figure 11. Variation of ascorbic acid levels in the brain, liver and plasma of PBS, silver nitrate, adjuvant, $A\beta_{35-25}$, protofibrillar form of $A\beta_{25-35}$, $A\beta_{1-42}$ and silver nitrate along with $A\beta_{25-35}$ protofibril treated group of rats. Each data point represents the mean of six animals. $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$ with respect to the value in the corresponding control.

Induction of silver nitrate to mice produces 1000-fold increase in serum amyloid A (SAA), a classic acute phase protein [18]. Such an increase in SAA concentration is primarily caused by a transcriptional induction event mediated by several cytokines, such as IL1, IL6, TNFα and NFkB released during inflammation [38]. In addition to this, SAA also induces the release of proinflammatory cytokines such as TNFα, IL1β and chemokine IL8 [39]. This proinflammatory cytokines are transported to various tissues through the blood stream [40]. Hopkins and Rothwell [41] reported that the proinflammatory agents in the circulatory system enter the brain at circumventricular sites, the area which lack functional blood–brain barrier (BBB). Guo et al. [15] reported that systemic inflammation could activate astrocytes and increase IL-1β, IL-6 and TNFα expression in the transgenic mice. Intracerebroventricular injection of the proinflammatory cytokine IL1β induces the formation of ROS in the rat hippocampus [42]. This may be the possible reason for the significant production of ROS in the astrocytes of the inflammation-induced rat. Johnstone et al. [43] reported that Aβ activates astrocytes to release TNFα, IL-1β and ROS. Our results are in accordance with the above findings. Present study provides evidence on the additive effect of Aβ in the inflammation animals in triggering cytokines and ROS production.

Activated astrocytes secrete a variety of factors, including cytokines, free radicals and NO [43,44]. Our results demonstrate that inflammation-induced rat causes a significant increase in NO level in the plasma, liver and brain (Figure 5). Induction of silver nitrate induces the activation of transcription factor NFkB [38], which regulates the genes for proinflammatory cytokines and iNOS. The expression of iNOS would thereby induce the production of NO. Aβ activates iNOS expression and NO production in cultured rat astrocytes through NFkB dependent mechanism [44]. This might be the reason for higher production of NO in our study.

Oxidative damage in the central nervous system predominantly manifests as LPO because of the high content of PUFA that are highly susceptible to oxidation. We have also observed a significant increase of LPO in the inflammation-induced group when compared to control (Figure 6). It should be noted that LPO are the markers of free radical generation and oxidative stress in AD [45]. The oxidative destruction of polyunsaturated fatty acids (PUFA) in membrane lipids is well manifested in AD [46]. Significant increase of LPO recorded in the brain when compared to plasma and liver of the inflammation induced rats (Figure 6) supports that brain is more exposed to oxidative damage than other organs.

Furthermore, Aβ is also known to produce inflammatory/toxic protofibls that can cross the BBB [47–49]. The present result shows that LPO level in Aβ treated group was significantly higher in plasma, liver and brain when compared to control (Figure 6). And also, Aβ peptide produces H_2O_2 through metal ion reduction, with concomitant release of LPO products, measured as thiobarbituric acid reactive substance (TBARS), a process probably mediated by formation of hydroxyl radicals [50]. In support to this, Markesbery [51] showed that LPO is a major cause for the depletion of membrane phospholipids in AD. Our result shows that LPO level in the inflammation together with Aβ treated group was significantly higher when compared to all other groups. Misonou et al. [52] reported that oxidative stress induced by H_2O_2 caused an increase in the level of intracellular Aβ in human neuroblastoma cells. Dyrks et al. [53] reported that Aβ and oxidative stress may be related to oxidation-mediated increase in Aβ aggregation. In a mouse model, during inflammation, evidence of cell stress and expression of RAGE were closely linked [54]. RAGE contributes to the transport of circulating Aβ across the BBB [55]. This may be the possible reason for higher LPO level in the inflammation with Aβ treated group.

SOD, catalase and GPx are antioxidant enzymes involved in the prevention of free radical induced damages. These enzymes contribute to a mutually supportive group of defense against ROS [56]. It has been observed that protofibril Aβ ($A\beta_{25-35}$) fragment at low concentration plays a role as antioxidant whereas in elevated concentration its antioxidant action is abolished [57]. SOD is the first enzyme involved in antioxidant defense, which dismutase superoxide anion to H_2O_2. The biosynthesis of SOD is mainly controlled by its substrate and the superoxide anion [56]. It has also been reported that SOD activity is decreased in different region in AD patients [58]. Catalase and GPx catalyze the elimination of H_2O_2 from the system [58]. Since GSH acts as the substrate for GPx, the decreased availability of GSH for the

activity of GPx results in the accumulation of H_2O_2. The excess H_2O_2 is toxic to SOD [59] and catalase [60]. The ability of Aβ to inhibit catalase activity [61] could be the cause for the two-fold rise in H_2O_2 [13]. This may be responsible for the observed decline in the activities of SOD, catalase and GPx in the inflammation, Aβ, inflammation with Aβ treated rats when compared to control (Figures 7–9).

Concentrations of ascorbic acid were significantly decreased in the present study in line with the decrease in GSH levels (Figures 10 and 11). The decreased availability of GSH in inflammation condition and Aβ treated rats are responsible for the decreased concentration of ascorbic acid. Ascorbic acid is a water-soluble antioxidant, which scavenge ROS, during which ascorbic acid is converted to dehydroascorbic acid. For the reconversion of dehydroascarbic acid to ascorbic acid, GSH is essential [62]. Martensson and Meister [63] have clearly demonstrated the decline of tissue ascorbic acid level along with decreased GSH level. Thus, the level of ascorbic acid is dependent on the level of GSH. Hence in the present study, the decline in the ascorbic acid can be attributed to inflammatory and Aβ -induced GSH deficiency. Further, Aβ neurotoxicity can be inhibited by various free radical scavengers or antioxidants such as SOD, catalase and GSH and other molecules [13].

Our findings suggest that oxidative vulnerability was significantly higher in the Aβ treated inflammation-induced rat. These results will be particularly relevant in the Aβ immunization method of AD treatment as people with inflammatory disease should be treated with caution because Aβ will trigger the brain inflammation in uncontrollable level.

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