INTRACEREBROVENTRICULAR INJECTION OF OLIGOMERISED β-AMYLOID ($A\beta_{1-42}$) PEPTIDE INDUCES NEUROINFLAMMATION, NEURODEGENERATION AND CHANGES IN PERIPHERAL IMMUNE RESPONSES IN RATS

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ABSTRACT

Alzheimer's Disease (AD) is a progressive neurodegenerative disorder. Intracerebroventricular (icv) administration of $A\beta_{1-42}$ to develop rat model of AD since β-amyloid can induce dementia, neurodegeneration and neuroinflammation which may alter peripheral immune responses due to leakiness blood brain barrier in that condition. But the effect of $A\beta_{1-42}$ induced neuroinflammation on peripheral immune responses is unclear. The present study designed to investigate neuroinflamation, neurodegeneration and changes in peripheral immune responses in rats of all test groups (control, sham operated and Aβ injected rats). After 28 days rats from all test groups were anesthetized and histopathology of hippocampul tissue, levels of inflammatory markers (ROS, nitrite, TNF-α and IL-1β) of hippocampus and serum and some peripheral immune responses [phagocytic activity of blood WBC and polymormhonuclear cells of spleen, Leucocyte adhesive inhibition index(LAI) and cytotoxicity of splenic mononuclear cells (MNC)] were studied. In the Aβ injected AD rats results neurodegeneration (Presence of hippocampal plaques and chromatolysis of hippocampal neurons were observed) and indicating the prevailing neuroinflammatory condition [Hippocampal levels of inflammatory markers (ROS, nitrite, TNF-α and IL-1β) were significantly higher] compared to control and sham operated rats. Present study showed serum levels of inflammatory markers were also elevated in the AD rats along with alterations in peripheral immune responses. The study supports the fact that the peripheral immunological changes seen in the AD rats may be associated with neuroinflammation (mediated through leaky blood brain barrier). Thus, it can be concluded that neuroinflammation in Aβ-AD rats can induce alterations in peripheral immunological responses.

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and is the potential factor responsible for occurrence of dementia in the elderly population worldwide (Pillai et al, 2013). It is characterized by two major neuropathological features- extracellular senile or neuritic plaques composed chiefly of β- amyloid peptide and intracellular neurofibrillary tangles composed of hyperphosphorylated tau protein (Alzheimer et al, 1907; Hardy

et al, 1986; Cuello et al, 2007; Blennow, 2006; Selkoe et al, 1991) in different brain areas such as cortex, hippocampus, basal forebrain and amygdala (Mattson et al, 2004).

Origin of AD is often categorized as familial or sporadic. The incidence of familial cases is low (0.1%) (Blennow., 2006) and is associated with mutations in the β - amyloid Precursor Protein (β-APP) gene, presenilin-1(PSEN1) and presenilin-2 (PSEN 2) genes (Sherrington et al, 1995; Levy-Lahad et al, 1995) whereas sporadic AD represents 90–95% (Meraz-Rios et al.,2013) of total cases, Though the etiology of AD is multi-factorial but researchers have mostly focused on the role of β- amyloid peptide in the development of AD. The 'amyloid cascade hypothesis' attributes the aberrant production, aggregation, and deposition of Aβ to be the chief causative agent in the initiation of the pathogenesis of AD (Hardyet al, 1991). APP is a transmembrane protein whose sequential cleavage by β and γ secretases leads to the production of of $A\beta_{40}$ and $A\beta_{42}$(LaFerla et al, 2007).The Aβ cascade is thought to be initiated by an elevatedAβ concentration, in particular $A\beta_{42}$, which aggregates toform soluble dimers, trimers, and the low-ordered oligomers.Further aggregation forms insoluble and proteolysis-resistantfibrils, which accumulate as beta-amyloid deposits (Selkoe, 2011).Increasing studies have demonstrated the toxicity of diverse Aβ species invitro and in vivo, confirming the importance of age-dependent Aβaccumulation in AD pathogenesis (Troy et al, 2000; Cleary et al, 2005;Gouras et al, 2005; Chong et al, 2006).This accumulated Aβis said to be neurotoxic and has the potential to induce oxidative stress, nitrosative stress, inflammatory response , synaptic dysfunction and eventually these factors lead to progressive neurodegeneration (Cetin et al, 2007; Sastre et al, 2006;Goodwin et al, 1997; Wallace et al, 1997; Akama et al, 1998). Hence Aβ, the major constituent of senile plaques is cytotoxic to neurons and has a central role in the pathogenesis of the disease (Cetin and Dincer, 2007).

Evidences suggest chronic inflammation is a characteristic feature of the AD brain (Eikelenboom et al, 2006; Akiyama et al, 2000). Aβ promotes an inflammatory response mediated by microglia and astrocytes (Meraz-Ríos et al, 2013)which are located in the vicinity of the senile plaques and neurofibrillary tangles (Craft et al , 2006)accounting for the synthesis of different cytokines, and pro-inflammatory mediators (Mrak and Griffin, 2001; Tuppo and Arias, 2005). Astrocytes, and microglia, are the cellular component of the resident innate immune system in the CNS. (Ransohoff and Brown, 2012). They get activated in response to any type of brain injury. Once activated they start producing a wide variety of proinflammatory cytokines tumor necrosis factor(TNF-α) and interleukin-1β (IL-1β), free radicals such as nitricoxide (NO) and superoxides activation of COX-2, production of PGE2 which could eventually promote neuronal death (Akiyama et al, 2000; Kitazawa et al, 2004).This inflammatory process once initiated contributes to neural dysfunction, and cell death, thereby establishing a self-perpetuating cycle, by which the inflammation becomes a cause of further neurodegeneration (Block and Hong, 2005; Glass et al, 2010).Several in vitro studies suggest that these factors act concomitantly

rather than individually to exert their effect for example combined action of IL-1β and TNF-α resulted in neurodegeneration of cortical neurons (Chao et al,1995).

Nitric oxide (NO) has been implicated in neurodegenerative diseases because of its free radical properties (Cetin and Dincer, 2007).NO is a cell signaling molecule thatis synthesisedfrom L-arginine by the action of the enzyme nitric oxide synthase (NOS). High levels of NO are generated in inflammatory conditions, which is responsible for synaptic dysfunction, protein and lipid oxidative damage, excitotoxicity, and neuronal death (Liu et al, 2002; Bishop and Anderson, 2005; Calabrese et al, 2007).

Oxidative stress is also responsible for the progression of the AD. Studies have reported that Aβ accumulation induces an elevation in levels of reactive oxygen species (ROS) in neurons, leading to apoptotic neuronal death in rats and mice (Allanbutterfield et al, 2002).Oxidative stress is also associated with mitochondrial dysfunction. Aberrant energy metabolism results in low ATP generation and excessive ROS generation due to an imbalance in the redox status of the cell which accelerates progression of the disease (Butterfield et al, 2006; Aliev et al, 2010; Massad et al, 2009).

The neuroinflammatory statepersisting in the AD brain may cause alterations in the systemic immune responses. Various inflammatory meditors generated during neuroinflammation can pass out easilyinto the periphery due to leakiness of blood brain barrieras there is extensive communication between the central and systemic immune responses in neurodegeneration(Britschgi and Wyss-Coray, 2007). Such changes have also been reported in AD patients (Shalit et al, 1994).

Many researchers have administered β-Amyloid peptide intracerebroventricularly in rats/mice to study the neurodegeneration and neuroinflammatory state characteristic of the AD brain (Frautschy et al, 1992; Cetin et al, 2013; Li et al, 2015; Cetin et al, 2007). In β-amyloid peptide induced AD (Aβ-AD) rat's neuroinflammation may cause alterations in the peripheral immunological responses due to efflux of inflammatory mediators through leaky blood brain barrier but no investigation has been carried out in this regard. Hence the current study aims to assess the inflammatory markers (TNF-α, IL-1β, nitrite and ROS) of brain and serum, and certain peripheral immunological parameters (phagocytic activity of blood WBC and splenic PMN, leucocyte adhesion inhibition index and cytotoxicity of splenic MNC) to investigate the influence of Aβ induced neuroinflammation on peripheral immunological responses.

AIM AND OBJECTIVES

The aim of the study is to investigate the effect of i.c.v. injection of oligomerised β-amyloid protein on neurodegeneration, neuroinflammatory markers of brain and some select peripheral immunological parameters in rats. The objectives of the study are as follows:

1. To study the inflammatory markers of hippocampus (TNF-α, IL- 1β, ROS, nitrite) in icv β- amyloid protein injected experimental AD rats (Aβ-AD rats), control and sham operated rats (receiving PBS).
2. To study levels of TNF-α, IL- 1β, ROS, nitrite in the serum of Aβ-AD rats, control and sham operated rats.
3. To study the systemic immunological parameters [phagocytic activity of blood WBC and polymormhonuclear (PMN)cells of spleen, Leucocyte adhesive inhibition index(LAI) and cytotoxicity of splenic mononuclear cells (MNC)] in icv β - amyloid protein injected experimental AD rats(Aβ-AD rats) , control and sham operated rats (receiving PBS).
4. To study the histopathology of the hippocampus (Congo red staining for plaque and Cresyl Violet staining for Nissl granules) in icv β - amyloid protein injected experimental AD rats (Aβ-AD rats), control and sham operated rats (receiving PBS).

MATERIALS AND METHODS

Animals

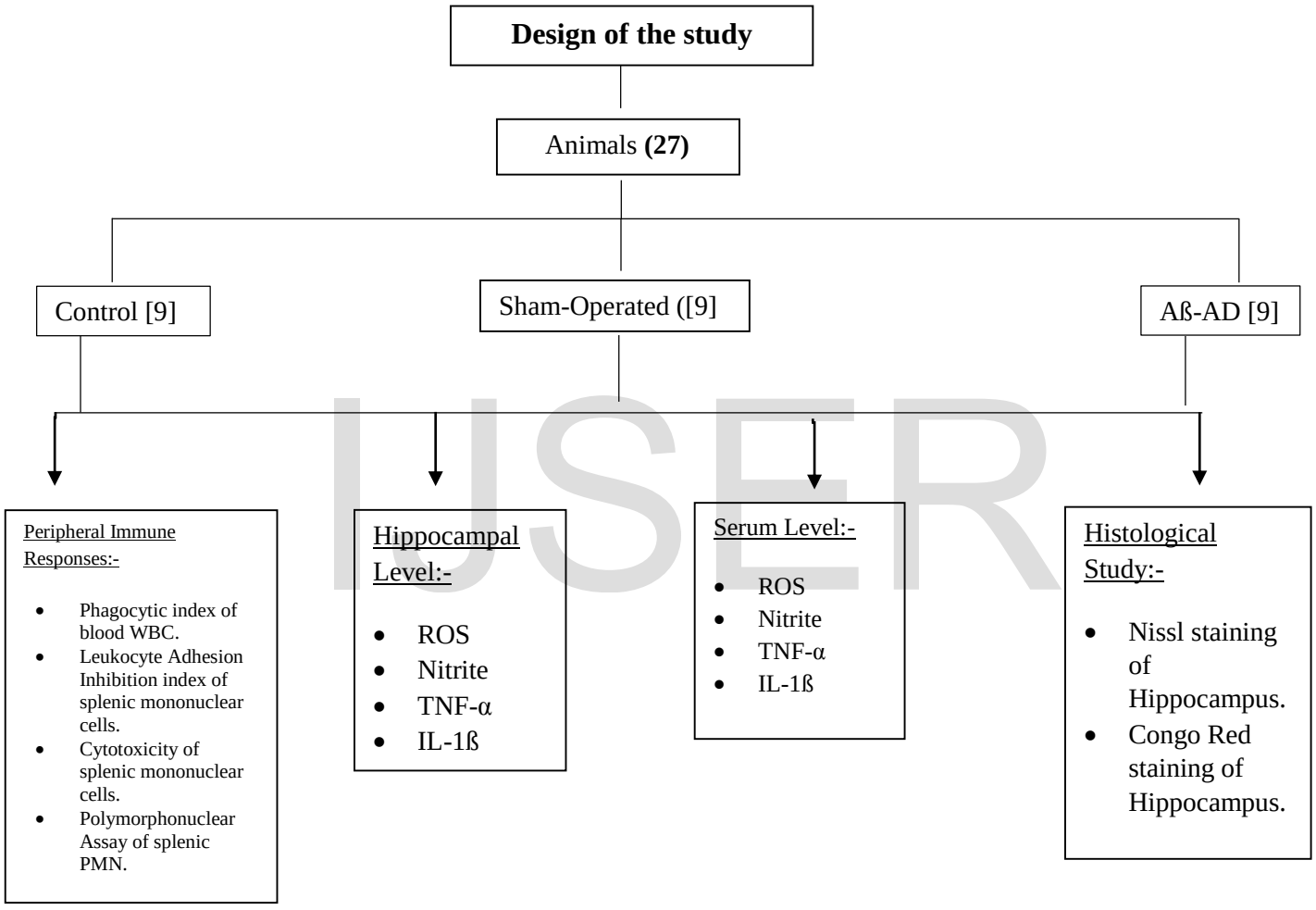
Adult male albino rats weighing 200–250 g were used in this study. Animals were housed individually in polypropylene animal cages with food pellet and water ad libitum. The animal room was maintained at a temperature of 25 ± 1 ° C with a 12-hour light/dark cycle (lights on from 7 a.m. to 7 p.m.). According to the regulations set by the institutional animal ethical committee, all adequate measures were taken to minimise the pain and discomfort in the rat.

Design of study

27 rats were randomly divided into three groups: oligomerised β- amyloid protein injected (icv) experimental AD rats (Aβ AD rats), control and sham operated group (received PBS). The Aβ - AD rats and sham operated rats were sacrificed after 4 weeks of surgery along with control rats, and blood was collected to measure phagocytic index of blood WBC, and spleens were collected to measure leukocyte adhesion inhibition index (LAI) of splenic MNC, phagocytic activity of splenic PMN and cytotoxicity of splenic MNC. The IL 1β, TNFα, ROS, nitrite were measured in the hippocampus of the three groups of rats. Histopathology of the hippocampus in the Aβ AD rats, control and sham operated rats were studied.

Preparation of Aβ- AD rats

The rats were anaesthetized with intra peritoneal thiopentone injection (50mg/kg body wt). The anaesthetized animals were placed on stereotaxic instrument (INCO, India Ltd.) equipped with a custom-made ear bar, which prevents the damage of the tympanic membrane.



Head was fixed in such a position that bregma and lambda sutures were in same horizontal plane by introducing the incisor bar properly attached to the mouth. An incision was given on the scalp in the midline and the pericranial muscles and fascia were retracted laterally. After retracting the nuchal musculature the overlying bone was drilled at the specific loci for approaching the lateral ventricle following the coordinates of the stereotaxic atlas (Paxinos and Watson, 1980). The coordinates for the lateral ventricles were: 0.6mm posterior to bregma, 1.5 mm lateral to the midline and 2.8mm below the cortical surface. The β-amyloid protein or PBS (5µl vol) was infused through

a 10µl Hamilton syringe in to each lateral. Thus, 2.5µl of PBS was infused bilaterally. The composition of PBS: KCl 0.05g/100ml, NaCl 2g/100ml, KH_2PO_4 0.05g/100ml, Na_2HPO_4.

Preparation of oligomerised Aβ amyloid protein for icv injection

Animal model of AD was prepared by intracerebroventricular injection of $A\beta_{1-42}$ (Sigma Aldrich, St.Louis, MO, USA). $A\beta_{1-42}$ was dissolved in phosphate buffer saline at a concentration of 1mg/ml and incubated at 37° C for 7 days prior to use for oligomerisation or to obtain fibrillary aggregation (Liu et al, 2008) 10 µl of this solution (containing 5µg of $A\beta_{1-42}$) was injected into lateral ventricles (5 µl/ lateral ventricle) of each rat.

Immunological Study

Blood Collection

Blood was collected (0.5 ml) for FACS analysis from the heart of deeply anaesthetised rats (diethyl ether; Kabra Drugs, India) by a syringe containing 100 µl of Na citrate (3.8%; Sigma, USA). Blood (1.5 ml) was also sampled for serum collection without any anticoagulant.

Leukocyte Adhesion Inhibition (LAI)

The spleens of rats were collected aseptically after anaesthesia (diethyl ether; Kabra Drugs) and LAI was measured according to the method of Maluish and Halliday, 1979. The spleens of 3 rats of a subgroup were combined (considered as a sub group and there were 3 subgroups) in phosphate-buffered saline (PBS) containing 3.8% Na citrate (Sigma) in a ratio of 10: 1 (v/v). Single-cell suspension of those spleens was made and WBCs were separated by a Percoll density gradient of 1.092. MNC and PMN were separated then with a Percoll density gradient of 1.077. MNC (98% purity of separation) in isolated suspension were counted using a Neubauer haemocytometer; the haemocytometer was incubated for 30 min at 37 ° C in a moist environment. After 30 min of incubation, the counted field was washed gently with PBS using a Pasteur pipette and adherent cells were counted: LAI (%) = (number of adherent cells after incubation × 100)/ (number of the total cell count before incubation). A smear of the isolated MNC suspension was made and stained with Leishman stain (Merck, India). The percentage of MNC in this smear was determined to verify the purity of separation.

Phagocytic Activity of WBC

According the method of Oben and Foreman, 1998. The fluorescein isothiocyanate (FITC; Sigma)-tagged bacterial cell membrane was prepared. A 100-µl blood sample was taken from the collected blood and transferred to four microcentrifuge tubes; 20 µl of FITC-tagged bacterial cell membrane and 380 µl of Roswell Park Memorial Institute (RPMI) medium (AT1640; HiMedia, India) were mixed in each of the four microcentrifuge tubes, and

tubes were incubated for different durations (0, 15, 60 and 90 min) at 37 ° C. After incubation, the tubes were dipped into ice for 15 min to stop the reaction. Then, 1 ml of red blood cell lysing solution (10 times dilution with distilled water; BD) was added to each tube and the tube was left for 5 min in the dark. The tube was centrifuged at 800 *g* for 3 min and washed with PBS (pH 7.4). Mean fluorescence values were analysed by BD FACS Verse using BD FACSuite software after 10,000 events inlight scatter leukocyte gate were acquired. Mean fluorescence values of samples for different time duration studies were taken from FITC-positive cell population from histogram. A regression line was drawn from the mean fluorescence values of different time intervals (MINITAB statistical software) for each group of rats.

PMN Assay

PMN were separated from the single-cell suspension of spleens following the method described in the previous section for LAI (Hudson and Hay,1980) Effectors cells (PMN) as well as target cells [Ehrlich's ascites carcinoma (EAC)] were washed separately in 15- ml centrifuge tubes with PBS, and their pellets were dissolved separately in phenol red-free RPMI 1640 (HiMedia) medium. PMN (98% purity of separation) and target cells in isolated suspension were counted using a Neubauer haemocytometer. Effector:target cells were mixed in a 10: 1 ratio in a 15-ml centrifuge tube, and 200 µl of 1% nitro blue tetrazolium (NBT) were added, and, to reach a volume of 3 ml, RPMI was added, followed by incubation at 37 ° C for 18 h. After 18 h, chilled 0.1 N HCl was added to stop the reaction. The centrifuge tube was kept for 5–10 min at room temperature and then centrifuged at 2,000 *g* for 30 min. The supernatant was discarded from the tube and 3 ml of pyridine solution were added to the tube. The mixture was kept in boiling water for 10 min, cooled at room temperature and centrifuged at 2,000 *g* for 10 min. In the resulting blue-coloured soup, absorbance was measured spectrophotometrically at 480 nm.

Calculation for PMN Assay:

Two sample tubes named blank (B) and test (T) were taken. Then the percentages of PMN were calculated using the following formula from the spectrophotometer reading:

PMN (%) = T – B/B × 100.

Cytotoxicity Assay

Cytotoxicity of splenic MNC (the effector cells collected from the spleen using the procedure described for LAI) against target cells (EAC cells) was tested in a lactate dehydrogenase (LDH) release assay using the LDH-FS non-radioactive cytotoxicity assay kit (DiaSys Diagnostic Systems GmbH, Germany) following the procedure of Weidmann et al,1995. Effector as well as target cells were washed separately in 15-ml centrifuge tubes with PBS and their pellets were dissolved separately in phenol red-free RPMI 1640 (HiMedia) medium. The volume of the

dissolved pellet was made up to 3 ml in each centrifuge tube. The number of effector or target cells in 1 ml of dissolved pellet solution was determined with the help of a counting chamber. 1×10^8 effectors cells were transferred from the dissolved pellet solution to two 1.5-ml microcentrifuge tubes (tubes 1 and 2). 1×10^7 target cells from the dissolved target cells were transferred to tube 1 (containing 1 × 10 8 effectors cells) and two other microcentrifuge tubes (tubes 3 and 4). Thus, the ratio of effector:target cells in tube 1 was 10: 1. 1% Triton X-100 (Merck) was added to tube 4 for lysis of the target cells. The volume of each of the four microcentrifuge tubes was made up to 500 µl with phenol red-free RPMI 1640. All the four microcentrifuge tubes were incubated at 37 ° C for 3 h. The microcentrifuge tubes were centrifuged at 200 *g* for 2 min. The supernatant was collected and LDH release into the supernatant was quantified following the kit manufacturer's instructions and recording the absorbance at 340 nm. Microcentrifuge tube 1 indicates the release of LDH from effector/target co-culture (C). The spontaneous release of LDH from the effector (E) and target cells (T) was determined from microcentrifuge tubes 2 and 3. The total target cell LDH release was obtained from microcentrifuge tube 4 (M). All tests were performed in triplicate and the amount of LDH released was calculated according to the following formula:

Cytotoxicity (%) = {[(C – E) – T]/ (M – T)} × 100

Cytokine Assay

TNF-α Assay

TNF-α levels were measured in the hippocampus and serum using the rat TNF flex set and BD cytometric bead array rat soluble protein master buffer kit (BD Biosciences,USA) in BD FACS Verse.FCAP Array software was used for data analysis. Samples were prepared according to the method of Csolle and Sperlagh, 2010; Csolle and Sperlagh 2011. Briefly the method was as follows: the hippocampus was homogenised in a solution containing 10mM Tris-HCl buffer, 1mM EDTA, 0.2 mM PMSF and 4 M urea per 0.1g of tissue. The samples were centrifuged at 15,000 g (4 for 20 mins). The supernatant was collected and mixed in a solution containing 10mM Tris-HCl buffer, 1% bovine serum albumin (BSA) and and0.2% Tween 20; 50µl of this solution were used to assess TNF-α in the kit method. The cytokine levels are expressed as pictograms per 100 mg protein in the sample.

IL-1β Assay

IL-1_ levels in different areas of the brain were assayed using a commercial rat IL-1_ ELISA Kit (Ray Bio, Norcross, GA). For these analyses, 100 ml of the solutions prepared above were used to assay IL-1β levels, according to manufacturer protocols. In this case the sample content was based on measures of absorbance at

450nm/well in a 96 well plate reader (Ray Bio). Cytokine levels were expressed as pg IL-1β/ mg protein in the sample. The sensitivity of the kit was 80 pg IL-1β /ml.

ROS Estimation

ROS was estimated by spectroflurometry using a modified method of Socci et al, 1995. In brief, the hippocampus was homogenised in 500 µl ice-cold (40mM) Tris-HCl buffer (pH 7.4) for ROS, nitrite and protein estimation.The brain homogenate (10µl) was incubated with 5µM DCF-DA (Loba-Chemie,India) for 30 min at 37^{o} C in a water bath. The formation of fluorescent product DCF was measured using a spectrofluorometer (JASCO FP 6200) at excitation and emission wavelengths of 495 and 529 nm, respectively. ROS is expressed as percent of control.

Nitrite Estimation

Nitrite levels were estimated by the method of Green et al, 1982. In brief, 10µl of brain homogenate (as described for the ROS assay) was mixed with equal volume of Griess reagent [0.1%N-(1-napthyl) ethylenediamine dihydrochloride, 1% sulphanilamide and 2.5% phosphoric acid] and incubated at 37^{o} C for 10 min followed by absorbance read spectrophotometrically at a wavelength of 550nm. Sodium nitrite was used as standard (Rai et al, 2013) and nitrite values are expressed in micrograms per milligram protein.

Protein Estimation

Protein was estimated in all the brain samples and serum by the method of Lowry et al, 1951. BSA (1 mg/ml) was used as standard. (Rai et al, 2013).

Histological Study

Cresyl Violet Staining for Nissl Granules

Nissl granules in the neurons of paraffin-embedded brain sections were stained with cresyl violet acetate (Sigma Aldrich, Germany) (Cox, 1977).The sections were deparaffinised, dehydrated and then brought to water medium. Cresyl fast violet (1 g/100 ml) was allowed to stain the section for 20–30 min. After rinsing the section with distilled water, it was treated with 96% alcohol and absolute alcohol. The section was mounted in DPX after dehydration (with absolute alcohol) and clearing with xylene.

Congo Red Staining

Plaques in the neurons of paraffin-embedded brain sections were stained with Congo red (Central Drug House, India). The sections were deparaffinised, dehydrated and then brought to water medium. The sections were stained with Gill's haematoxylin and rinsed in tap water for 2 min. Then the sections were immersed in alkaline sodium chloride solution for 20 min. Then Congo red working solution (1 g/100 ml sodium hydroxide, Congo red stock solution) was allowed to stain the section for 20 min. After rinsing the section with distilled water, it was differentiated with alkaline alcohol and again rinsed in distilled water. The sections were then dehydrated and mounted in DPX.

Statistical Analysis

All data are expressed as mean ± SEM. A two-way ANOVA was employed to compare the data of different groups control, sham-operated, ICIR followed by the Tukey-Kramer multiple comparison test using the statistical package for social science (20.00, SPSS, USA) software.

RESULTS

Nitrite level of Hippocampus

There was a significant increase in hippocampal nitrite level of AD rats compared to control and sham operated rats ($p < 0.001$). There was no significant difference in hippocampal nitrite level between control and sham operated rats.

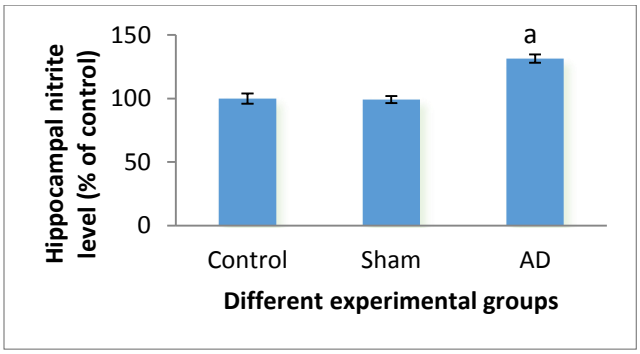


Fig. 1: The hippocampal nitrite level of different experimental groups of rats. [a]Significant difference in AD rats compared to control/ sham operated rats ($p < 0.001$). Abbreviations: Sham = Sham operated group; AD = intracerebroventricular Aβ injected group Values are expressed in mean ± SEM (n = 6).

ROS level of Hippocampus

There was a significant increase in hippocampal ROS levelof AD rats compared to control and sham operated rats ($p < 0.001$). There was no significant difference in hippocampal nitrite level between control and sham operated rats.

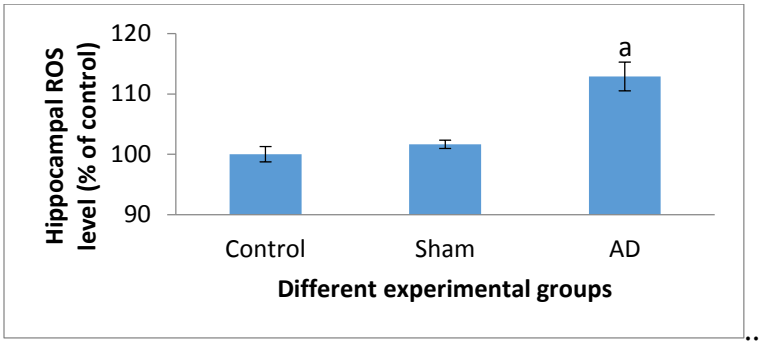


Fig. 2 The hippocampal ROS of different experimental groups of rats. [a]Significant difference in AD rats compared to control/ sham operated rats ($p < 0.001$). Abbreviations: Sham = Sham operated group; AD = intracerebroventricular Aβ injected group Values are expressed in mean ± SEM (n = 6).

IL-1β level of hippocampus*

There was a significant increase in IL-1β level of hippocampus of AD rats compared to control and sham operated rats ($p < 0.001$). There was no significant difference in hippocampal nitrite level between control and sham operated rats.

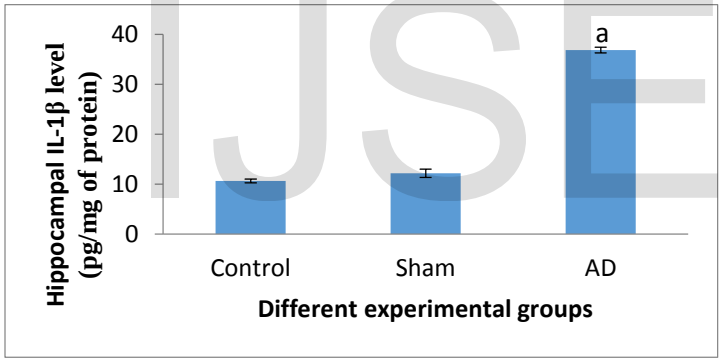


Fig. 3. The Hippocampal IL-1β level of different experimental groups of rats in 21-day study. [a]Significant difference in AD rats compared to control/ sham operated rats ($p < 0.001$). Abbreviations: Sham = Sham operated group; AD = intracerebroventricular Aβ injected group Values are expressed in mean ± SEM (n = 6).

TNF-α level of hippocampus

There was a significant increase in hippocampal level of TNF-α level of AD rats compared to control and sham operated rats ($p < 0.001$). There was no significant difference in hippocampal nitrite level between control and sham operated rats.

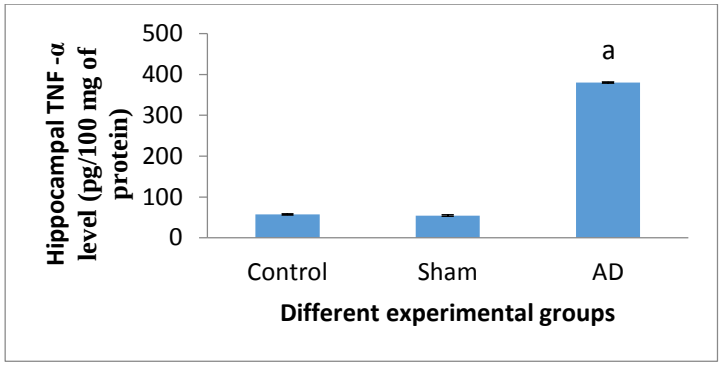


Fig. 4. The TNF-α level of hippocampus of different experimental groups of rats. [a]Significant difference in AD rats compared to control/ sham operated rats ($p < 0.001$). Abbreviations: Sham = Sham operated group; AD = intracerebroventricular Aβ injected group Values are expressed in mean ± SEM (n = 6).

Nitrite level of Serum

There was a significant increase in serum nitrite level of AD rats compared to control and sham operated rats ($p < 0.001$). There was no significant difference in serum nitrite level between control and sham operated rats.

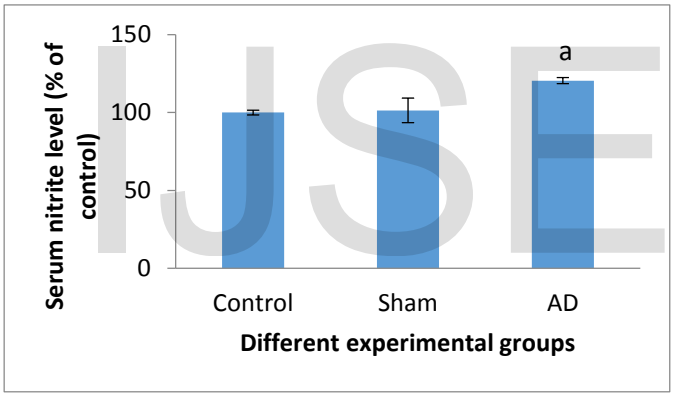


Fig. 5: The serum nitrite level of different experimental groups of rats. [a]Significant difference in AD rats compared to control/ sham operated rats ($p < 0.001$). Abbreviations: Sham = Sham operated group; AD = intracerebroventricular Aβ injected group Values are expressed in mean ± SEM (n = 6).

ROS level of Serum

There was a significant increase in ROS level of serum in AD rats compared to control and sham operated rats ($p < 0.001$). There was no significant difference in serum nitrite level between control and sham operated rats.

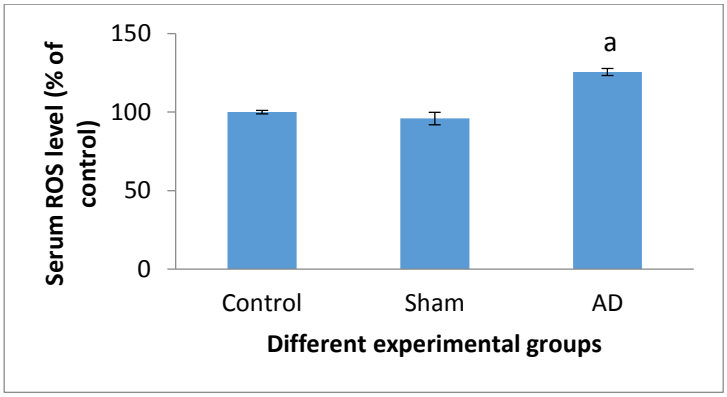


Fig. 6: The serum ROS level of different experimental groups of rats. [a]Significant difference in AD rats compared to control/ sham operated rats ($p < 0.001$). Abbreviations: Sham = Sham operated group; AD = intracerebroventricular Aβ injected group Values are expressed in mean ± SEM (n = 6).

IL-1β level of Serum

There was a significant increase in IL-1β level of serum in AD rats compared to control and sham operated rats ($p < 0.001$). There was no significant difference in serum nitrite level between control and sham operated rats.

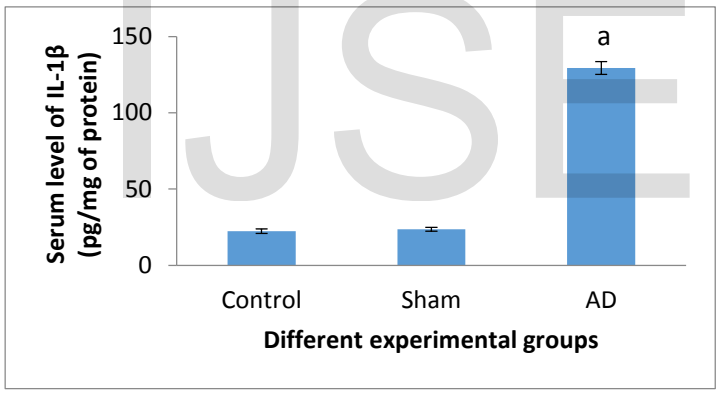


Fig. 7: The serum IL-1β level of different experimental groups of rats. [a]Significant difference in AD rats compared to control/ sham operated rats ($p < 0.001$). Abbreviations: Sham = Sham operated group; AD = intracerebroventricular Aβ injected group. Values are expressed in mean ± SEM (n = 6).

TNF-α level of Serum

There was a significant increase in TNF-α level of serum in AD rats compared to control and sham operated rats ($p < 0.001$). There was no significant difference in serum nitrite level between control and sham operated rats.

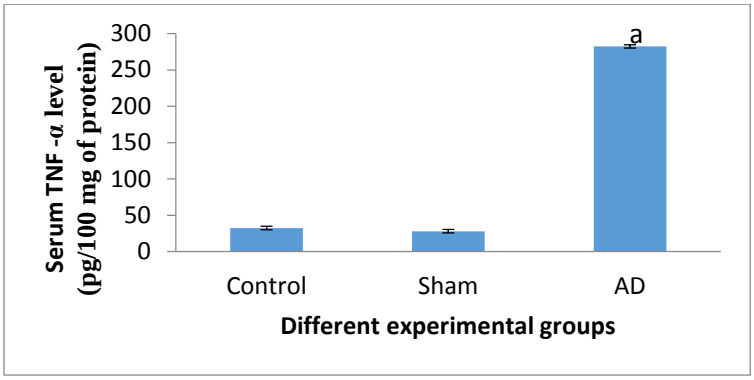


Fig. 8: The serum TNF-α level of different experimental groups of rats. [a]Significant difference in AD rats compared to control/ sham operated rats ($p < 0.001$). Abbreviations: Sham = Sham operated group; AD = intracerebroventricular Aβ injected group Values are expressed in mean ± SEM (n = 6).

Phagocytic activity of splenic PMN

There was a significant increase in phagocytic activity of splenic PMN of AD rats compared to control and sham operated rats ($p < 0.001$). There was no significant difference in phagocytic activity of splenic PMN between control and sham operated rats.

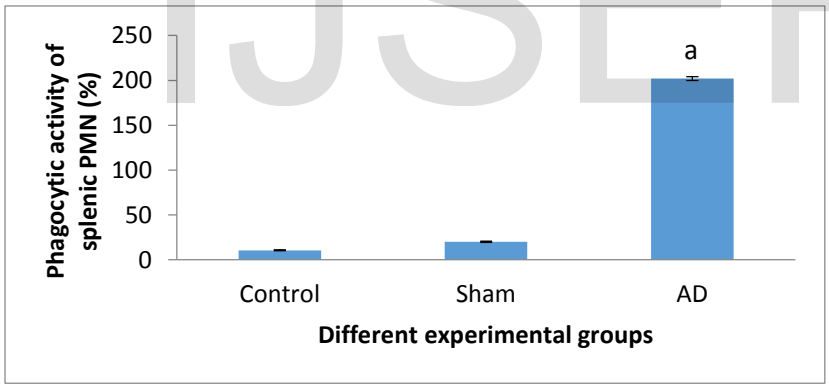


Fig. 9 The phagocytic activity of splenic PMN of different experimental groups of rats. [a]Significant difference in AD rats compared to control/ sham operated rats ($p < 0.001$). Abbreviations: Sham = Sham operated group; AD = intracerebroventricular Aβ injected group Values are expressed in mean ± SEM (n = 6).

Cytotoxicity of Splenic MNC

There was a significant increase in cytotoxicity of splenic MNC of AD rats compared to control and sham operated rats ($p < 0.001$). There was no significant difference in cytotoxicity of splenic MNC between control and sham operated rats.

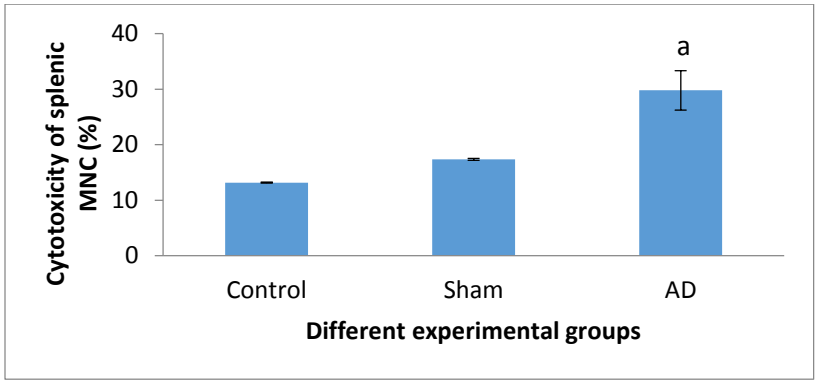


Fig. 10: The cytotoxicity of splenic MNC of different experimental groups of rats. [a]Significant difference in AD rats compared to control/ sham operated rats ($p < 0.001$). Abbreviations: Sham = Sham operated group; AD = intracerebroventricular Aβ injected group Values are expressed in mean ± SEM (n = 6).

LAI

There was a significant decrease in LAI of AD rats compared to control and sham operated rats ($p < 0.001$). There was no significant difference in cytotoxicity of splenic MNC between control and sham operated rats.

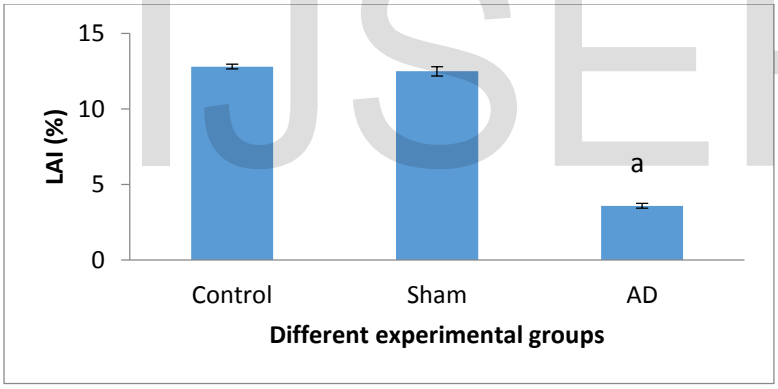


Fig. 11. LAI of different experimental groups of rats. [a]Significant difference in AD rats compared to control/ sham operated rats ($p < 0.001$). Abbreviations: Sham = Sham operated group; AD = intracerebroventricular Aβ injected group Values are expressed in mean ± SEM (n = 6).

Phagocytic activity of WBC

There was a significant increase in phagocytic activity of splenic PMN of AD rats compared to control and sham operated rats ($p < 0.001$). There was no significant difference in cytotoxicity of splenic MNC between control and sham operated rats.

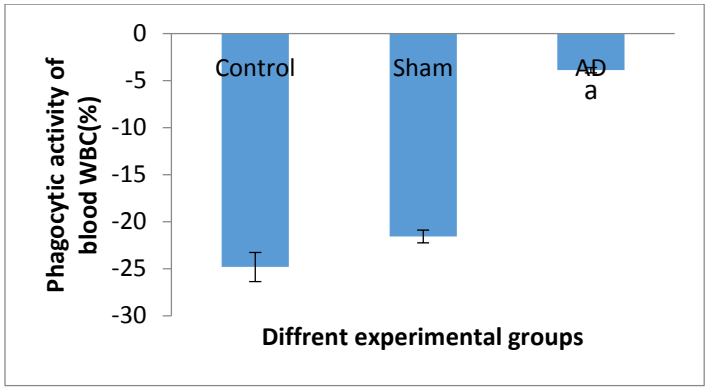


Fig. 12: The PI of WBC of different experimental groups of rats. [a]Significant difference in AD rats compared to control/ sham operated rats ($p < 0.001$). Abbreviations: Sham = Sham operated group; AD = intracerebroventricular Aβ injected group Values are expressed in mean ± SEM (n = 6).

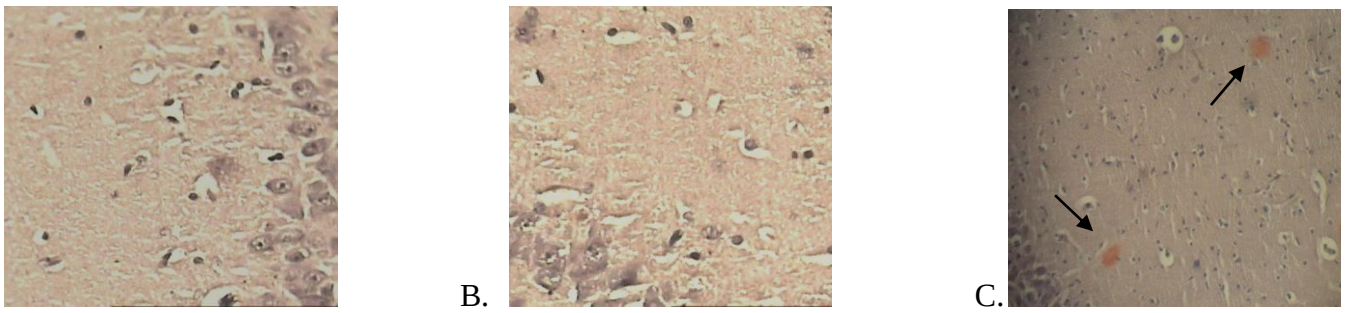
Histopathological observations

Congo Red staining of hippocampal plaques:-

Plaques were present in the hippocampus of the Aβ-AD rats compared to control and sham operated rats.

CONTROL **SHAM** **AD**

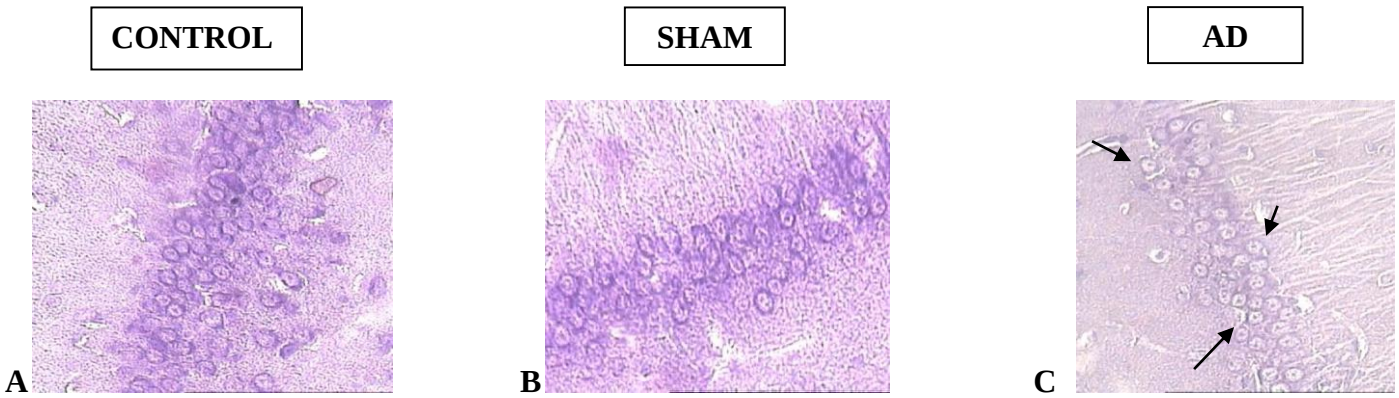
A. B. C.



Figures A , B and C represents congo – red stained hippocampal sections of control, sham operated and Aβ-AD rats. Arrows in Fig.C indicate hippocampal plaques.

Nissl Staining of Hippocampal Neurons

Nissl granules of neurons of the hippocampus are stained less intensely in Aβ-AD rats bycresyl fast violet staining method compared to that of control and sham operated rats.



Figures A, B and C represents cresyl – violet stained hippocampal sections of control, sham operated and Aβ-AD rats. Arrows in Fig.C indicate chromatolysis of nissl granules.

DISCUSSIONS

In the present study AD model was established by intracerebroventricular (icv) administration of $A\beta_{1-42}$due to the role of β – amyloid peptide in the pathogenesis of AD. Histological examination was carried out using Cresyl Violet and Congo Red staining techniques. Brain sections stained with cresyl violet showed considerable chromatolysis of hippocampal neurones of the Aβ – AD rats. Whereas no such changes were observed in the hippocampi of the control and PBS treated (sham operated) rats. Such changes have also been previously reported in other rats models of AD (Sil et al, 2014).

Congo red stained brain sections of Aβ – AD rats showed the presence of distinct amyloid plaques. But no such plaques were observed in the hippocampi of the control and sham operated rats. The occurrence of amyloid deposits in the form of plaque is an important neuropathological marker of Aβ induced AD rats. Previous investigations by Maurice have also reported similar histological alterations in β-amyloid injected AD (Maurice et al, 1996). Immunohistochemical studies have also reported the presence of amyloid plaques in the hippocampus of $A\beta_{1-42}$injected rats (Liu et al, 2008). So the histological changes observed in this AD model corroborates with the observations made by previous investigators.

In the current study there was a significant increase in the hippocampal levels of IL-1β and TNF-α in the Aβ – AD rats compared to control and sham operated rats. Elevated levels of these cytokines clearly indicate the ongoing chronic inflammatory cycle in the AD rats. In our study the presence of amyloid plaques in the hippocampus accompanied by high levels of proinflammatory cytokines (TNF-α and IL-1β) can be explained by the concept that the inflammatory process is a plaque induced event as previously described by researchers (Dandrea et al., 2001; Mehlhom et al,2000) . The continuous accumulation of of Aβ leads to activation of migroglia and astrocytes (Kurt et al, 1999; Yu et al, 2005) leading to secretion of a wide range of inflammatory

factors which then damages nearby neurones (Lue et al, 2001; Meraz-Rios et al, 2013; Gunderson et al, 2015; Mrak and Griffin, 2001; Pekny et al, 2014) enhancing the degree of neurodegeneration (Hirschet al, 2009; Rai et al, 2013).

Presence of high levels of TNF- α and IL -1β in the Aβ-AD rats corroborates the idea that they are two major cytokines participating in the pathogenesis of AD (Shaftel et al, 2008; Perry et al, 2001). Reports suggests that IL -1β is overexpressed in the initial phase of AD (Shaftel et al.,2008).Moreover the immunoreactivity of IL -1β is also heightened in the hippocampus following $A\beta_{42}$ injection (Nitta et al, 1997; Nitta et al, 1994). TNF-α supposedly augments the the expression of β and γ secretases (Blasko et al, 2000; Liao et al, 2004). In vitro studies suggest that TNF- α directly stimulates BACE 1 expression favouring APP processing (Yamamoto et al, 2007). These reports can help us to explain our findings that high level of TNF- α and IL – 1β in the hippocampus play an important role in $A\beta_{1-42}$induced neuroinflammation and neurodegeneration. In our present study elevated levels of ROS and nitrite (NO) were observed in the hippocampus of Aβ – AD rats compared to control and sham operated rats.Our findings are in conformity with studies that suggest that Aβ insult can lead to oxidative and nitrosative stress (Frank-Cannon et al ,2009). It has been reported that accumulated Aβ in the plaques can stimulate the release of reactive oxygen and reactive nitrogen species, other inflammatory mediators which can exert neurotoxic effect (Li et al, 2003; Esposito et al, 2006; Solenski et al, 2003). Neuronal analyses from AD patients have supported the idea that Aβ promotes NOS expression and NO production in the microglia and astrocytes (Goodwin et al, 1997; Wallace et al, 1997; Akama et al, 1998).So it can be said that an inflammatory response is often accompanied by a high nitrite level, similar to our finding .

Elevated levels of TNF- α and IL -1β were also observed in the serum of Aβ – AD rats in comparison to control and sham operated rats. These findings are in conformity with the fact that in case of AD a link between the cytokine profile in the blood stream and that in the brain exist because there is an active and highly regulated communication between the brain and the immune system (Huberman et al, 1994. Human studies have also reported high TNF –α levels in the blood plasma of AD patients (Tarkowski et al, 1999; Alvarez et al, 2007; Zuliani et al, 2007). The levels of ROS and nitrite were also significantly higher in serum of AD rats compared to control and sham operated rats.

The role of the immune system is indispensable in development of AD since there is bidirectional communication between brain and periphery through the blood brain barrier (Britschgi et al*, 2007). Hence certain peripheral immunological parameters were assessed in the Aβ – AD rats , control and sham operated rats (PBS treated).Results showed that there was an increase in the cytotoxicity of splenic mononuclear cells and phagocytic activity of splenic polymorphonuclear cells.The increased cytotoxicity of splenic mononuclear cells and

phagocytic activity of splenic polymorphonuclear cells in the Aβ – AD rats can be due to the presence of inflammatory mediators in the periphery , as reported by Sil et al .,2014 in colchicine induced AD rats.

There was a decrease in the phagocytic activity of blood WBC and leukocyte adhesion inhibition index in the Aβ – AD rats compared to control and sham operated rats. The decrease in the phagocytic activity of blood WBC has also been previously reported in AD patients (Davydova et al, 2003). However, the decrease of LAI in the AD rats cannot be explained on the basis of increased inflammatory mediators in the periphery rather it may be due to a compensatory response to other inflammatory responses (Dutta et al, 2011) .These changes in the peripheral immune responses can be justified by the explanation that the blood brain barrier may be become leaky during and hence there could be passage of inflammatory mediators through the blood brain barrier during neuroinflammation which in turn influences the peripheral immune responses.

SUMMARY AND CONCLUSION

Alzheimer's Disease (AD) is an age related progressive neurodegenerative disorder. Researchers have opted for intracerebroventricular (i.c.v.) administration of $A\beta_{1-42}$ to develop rat model of AD since β- amyloid can induce dementia, neurodegeneration and neuroinflammation. This neuroinflammation may alter peripheral immune responses due to leakiness of blood brain barrier in that condition. But the effect of $A\beta_{1-42}$ induced neuroinflammation on peripheral immune responses has not been investigated. In the present study AD model was prepared by (icv) administration of $A\beta_{1-42}$ (Aβ-AD rats). Histopathology of hippocampus , levels of inflammatory markers (ROS, nitrite, TNF-α and IL-1β) of hippocampus and serum and some peripheral immune responses (phagocytic activity of blood WBC and polymormhonuclear cells of spleen, Leucocyte adhesive inhibition index(LAI) and cytotoxicity of splenic mononuclear cells MNC) were studied in rats Aβ-AD. These parameters were also studied in control and sham operated rats (receiving PBS). Presence of hippocampal plaques and chromatolysis of hippocampal neurons were observed in Aβ-AD rats indicating neurodegeneration. Hippocampal levels of inflammatory markers (ROS, nitrite, TNF-α and IL-1β) were significantly higher in the Aβ-AD rats compared to control and sham operated rats indicating the prevailing neuroinflammatory condition. Hence these results are indicative of neuroinflammation linked neurodegeneration.

Serum levels of inflammatory markers (ROS, nitrite, TNF-α and IL-1β) were also elevated in the AD rats alongwith alterations in peripheral immune responses .It was observed that the immunological parameters like cytotoxicity of splenic mononuclear cells and phagocytic activity of splenic polymorphonuclear cells showed significant increase in the Aβ-AD rats compared to control and sham operated rats. The LAI of splenic MNC was decreased in the Aβ-AD ras in comparison to control and sham operated animals. The phagocytic activity of blood WBC was also decreased in the Aβ-AD rats.

The peripheral immunological changes seen in the AD rats may be associated with neuroinflammation (mediated by passage of neuroinflammatory markers through leaky blood brain barrier). Thus, it can be concluded that neuroinflammation in Aβ-AD rats can induce alterations in peripheral immunological responses.

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