

L-THEANINE SHOWING THE NEUROPROTECTIVE ACTIVITY AGAINST LIPOPOLYSACCHARIDE INDUCED NEUROINFLAMMATION IN MICE MODEL

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Article Received on
15 April 2019,

Revised on 05 May 2019,
Accepted on 26 May 2019

DOI: 10.20959/wjpr20197-15044

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ABSTRACT

Neuroinflammation is associated with a number of neurodegenerative diseases such as Alzheimer's disease. It is reported that lipopolysaccharide (LPS) can induce neuroinflammation and cognitive impairment. L-Theanine is non-protein amino acid obtained from tea leaves *Camellia sinensis* and it is identified to possess neuroprotective activity. The aim of this study was to explore the possibility of L-Theanine in preventing LPS induced cognitive impairment. The study was carried out on Swiss albino mice in different groups by giving saline, L-Theanine (2mg/kg, 4mg/kg), LPS (0.25mg/kg) and Rivastigmine respectively. Various behavioral parameters and antioxidant assays were carried out. L-Theanine demonstrated antioxidant activity and significant increase in memory as evidenced

by Y-maze spontaneous alteration test and Morris water maze test.

KEYWORDS: Alzheimer's disease, Cognitive impairment, Neuroprotective, Lipopolysaccharide, L- Theanine, Antioxidant.

1. INTRODUCTION

Alzheimer's disease (AD) is a progressive, neurodegenerative disease of the brain and is the most common form of dementia among the elderly population. The two core hallmarks of

AD are amyloid plaques (APs) and neurofibrillary tangles (NFTs) in addition to a dramatic decrease in the hippocampal and cortical levels of the neurotransmitter acetylcholine (ACh).^[1]

Plaques are dense, mostly insoluble deposits of beta amyloid peptide along with cellular material outside and around neurons. Tangles (neurofibrillary tangles) are aggregates of the microtubule-associated protein tau which gets hyperphosphorylated and accumulates inside the cells.^[2]

The accumulation of the amyloid protein aggregates in the brain of AD patients leads to oxidative stress and inflammation. Neuroinflammation has been known to play a critical role in the pathogenesis of chronic neurodegenerative disease in general, and in AD specifically. Numerous studies show the presence of a number of markers of inflammation in the brain of patients affected by AD. This includes elevated inflammatory cytokines, chemokines along with accumulation of activated microglia in the damaged regions.^[3]

Oxidative stress is a key component of the pathology of the chronic neurodegenerative conditions. It is now established that oxidative/nitrosative stress (OS/NS) has a pivotal role in the pathophysiology of neurodegenerative diseases and many other types of human maladies. Oxidative damage to neuronal molecules, accumulation of iron species in the brain, and decreased cellular reserve antioxidant pool are major pathological aspects of neurodegenerative disorders, like Alzheimer's disease (AD), Parkinson's disease (PD) or Amyotrophic lateral sclerosis.^[4] Hence drugs with potential anti-inflammatory, antioxidant and neuroprotective effects could provide a major breakthrough in the treatment of AD.^[2]

Worldwide, nearly 44 million people have Alzheimer's or related dementia. However, only 1-in-4 people with Alzheimer's disease have been diagnosed so far. Alzheimer's and dementia are most common in Western Europe (North America is close behind). Alzheimer's is least prevalent in Sub-Saharan Africa. Alzheimer's and other dementias are the foremost reasons of disabilities in later life. Medications currently used to treat the cognitive problems of AD are Tacrine, Rivastigmine, Galantamine, Donepezil and Memantine.^[5]

The present drugs have a number of drawbacks such as poor bioavailability, adverse effects, narrow therapeutic ranges and limited efficacy. Herbs may play a promising role in the early treatment of Alzheimer's and other conditions involving poor memory and dementia. One of the chief benefits is that they have a low toxicity compared to pharmaceutical agents.^[6]

Dementia is a progressive neurological disease of the brain. It is chronic and progressive syndrome characterized by a decline in mental functioning in which memory thinking, judgmental behavior and ability to concentrate are impaired.^[7] One of the most common causes of dementia in the elderly include Alzheimer's disease, cerebrovascular disease and dementia with Lewy bodies. Of these, Alzheimer's disease is the leading cause followed by cerebrovascular disease and their mixed dementia.^[5]

The World Health Organization (WHO) predicts that by 2025, about 75% of the estimated 1.2 billion people aged 60 years and older will reside in developing countries.^[8] It is estimated that the number of people living with dementia will almost double every 20 years to 42.3 million in 2020 and 81.1 million in 2040. The rate of growth will be the highest (around 33.6%) in India, China, South Asia, and Western Pacific regions, 23-39% in Latin America and Africa, and the lowest (10%) in developed regions. Based on 2001 global population, about 24.3 million have dementia and 4.6 million incident or new cases are added yearly.^[9] According to global burden of disease study by WHO and World Bank, dementia contributes 4.1% of all disability-adjusted life years (DALYs).^[8]

Neuroinflammation, the inflammation associated with the brain, differs from that found in the periphery. Although edema and neutrophil invasion, the typical features of inflammation, are not seen in the AD brain, tissue levels of inflammatory mediators including cytokines, chemokines, oxygen free radicals and reactive nitrogen species are altered. Numerous reports have indicated that neuroinflammatory process contributes to the pathogenesis of AD.^[10]

Microglia, the brain-resident macrophages, serve as the first line of defense when injury occurs and play a critical role in the immune surveillance of the CNS. Upon activation, microglia produce various proinflammatory mediators, such as tumor necrosis factor- α (TNF- α), interleukin-1 (IL-1) β , IL-6, nitric oxide, and reactive oxygen species, which are associated with a number of neurodegenerative diseases.^[11]

Astrocytes, the most abundant glial cells in the brain, play an important role in maintaining CNS homeostasis. Like microglia, they readily become activated in response to injury and regulate neuroinflammatory events. Over-activation of glial cells can cause neuronal damage, which may result in neuropathological changes in a number of CNS. Therefore, inhibition of the activation of microglia and astrocytes and the consequent release of proinflammatory mediators is regarded as an important therapeutic strategy for neuroinflammation mediated diseases.^[11]

Hence, considering the aforementioned scenario, the aim of the present study was to explore the possibility of L-Theanine in preventing LPS induced cognitive impairment.

2. METHODS ANIMALS

In the present study, we used the adult male swiss albino mice of 7 weeks old age (20-25g) from Bombay Veterinary College, Parel. The animals were procured one week before the initiation of study. The mice were housed in opaque polypropylene cages and maintained under standard laboratory conditions of temperature ($22 \pm 2^{\circ}\text{C}$) and humidity ($50 \pm 10\%$) following 12 hour light/dark cycle. All mice were given the Standard pellet diet (Amrut brand, Sangli, India) and purified water *ad libitum*. In the experiment, 5 groups of 8 animals each were randomized. The animals were acclimatized for 7 days before the initiation of experiments. The animal study was approved by the Institutional Animal Ethics Committee (IAEC) (Reg. No.87/1999/CPCSEA dated 28th April 1999), constituted for the purpose of control and supervision of experimental animals by Ministry of Environmental and Forest, Government of India, New Delhi. All behavioral experiments were carried out between 09:00 a.m. and 05:00 p.m.

Materials

L-theanine was purchased from SRL Mumbai. Rivastigmine was purchased from S. D. Fine Chemicals, Mumbai and Lipopolysaccharide was obtained from Sigma Chemicals, USA. All other chemicals and biologicals, obtained were of analytical grade with the highest purity. LPS was dissolved in saline (0.9% NaCl) and all drug solutions were prepared freshly before every experiment for producing total injection volume of 1.0 ml/kg (*i.p.*) and 3.0 ml/kg (*p.o.*). All drugs were administered between 8.30 am to 10.00 am, LPS was administered (*i.p.*) daily one hour before L-Theanine administration for seven days with 14 days prior treatment of L-Theanine. The dose of L-Theanine was selected based on the previous studies performed.

Experimental Design

In this study we used forty male swiss albino mice which were divided into five groups containing eight animals each (5 groups \times 8 animals = 40). Group 1: Normal control receive vehicle (Normal saline); Group 2: Disease control receive LPS along with vehicle; Group 3 and Group 4: L- Theanine (2 mg/kg and 4 mg/kg body weight; *p.o.*) treated group along with LPS for seven days one hour before L-Theanine; Group 5: Positive control group receiving Rivastigmine (250 $\mu\text{g/ml}$) along with LPS for seven days.

Evaluation of *in vitro* antioxidant activity

The assay was performed in triplicates and percentage inhibition was calculated using the following formula:

$$\text{Percentage Inhibition} = [(\text{Control} - \text{Test}) / \text{Control}] \times 100.$$

Thiobarbituric Acid Reactive Substances (TBARS) assay***Procedure***

Lipid peroxidation induced by Sodium nitroprusside and Fe^{2+} ions in brain homogenate was estimated by TBARS method of Ohkawa et al.^[12] The reaction mixture contained brain homogenate 0.1 ml in phosphate buffer (pH 7.4) FeSO_4 (10 mM) or Sodium nitroprusside (5mM) and various concentrations of L-Theanine in a final volume of 0.5ml. The reaction mixture was incubated at 37°C for 1 h. After the incubation period, 0.4ml was removed and treated with 0.2 ml sodium dodecyl sulphate (SDS) (8.1%); 1.5 ml thiobarbituric acid (TBA) (0.8%); and 1.5 ml acetic acid (20%, pH 3.5). The total volume was made up to 4.0 ml with distilled water and then kept in a water bath at 95 to 100°C for 1 h. After cooling, 1.0 ml of distilled water and 5.0 ml of n- butanol and pyridine mixture (15:1 v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The butanol-pyridine layer was removed and its absorbance was measured at 532 nm to quantify TBARS. Inhibition of lipid peroxidation was determined by comparing the treatments with that of the standard. L-ascorbic acid was used as a standard.

Nitric oxide assay***Procedure***

One ml of sodium nitroprusside (10 mM) in Phosphate-buffered saline (pH 7.4) was mixed with 1 ml of the test substance at various concentrations (1-800 $\mu\text{g/ml}$) dissolved in methanol. The mixture was incubated at 25°C for 30 min. After 30 min, 1 ml of incubated solution was withdrawn and mixed with 1 ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthyl ethylenediamine dihydrochloride). The absorbance of the pink chromophore formed during the diazotization of the nitrite with sulphanilamide and the subsequent coupling with naphthyl ethylenediamine dihydrochloride was measured at 546 nm. All the tests were performed in duplicate.^[13]

Percentage inhibition was calculated using following equation:

$$\text{Percentage inhibition} = ((A \text{ control} - A \text{ test}) / (A \text{ control})) \times 100$$

Drug administration

The animals were divided into five groups containing eight animals in each. LPS (250 µg/kg, *i.p.*) was administered after 14 days prior treatment with L-Theanine (2 and 4 mg/kg, *p.o.*) and revastigmine (250 µg/kg, *p.o.*) except normal control group which received normal saline. L- Theanine was given with normal saline and administered orally in respective groups. The dose of L-Theanine was selected based on literature survey reports.^[14]

Behavioral studies***Behavioral studies of mice were done as follows***

On the day of behavioral evaluation, home cages were placed in the testing room 30 minutes before testing to allow habituation. All behavioral observations were made between 9:00 and 17:00 hours. The order of observation during this period was randomized across animals.

Y-maze spontaneous alternation test

Each animal was placed in turn in arm A of the Y-maze and allowed to explore for 8 minutes and the arm entries made by each animal were recorded. Arm entry is defined as having all 4 paws in the arm. Spontaneous alternation is defined as a successive entry into 3 different arms, on overlapping triplet sets. The percentage number of alternations was calculated as the number of actual alternations divided by the maximum number of alternations (the total number of arm entries minus 2). The total number of moves were also recorded as an index of ambulatory activity.^[15]

Morris Water Maze test

During the MWM test, mice were given 2 days of visual platform training followed by 8 days of hidden platform training and a 1-day probe trial. Briefly, for the acquisition of the visual platform training, mice were placed into the maze without spatial cues and were allowed to locate a visual flagged platform. If the platform was not found within 2 minutes, the mouse was gently guided to it. Mice were given 4 trials each day for 2 days with a different start position and flagged platform location each trial. For the acquisition of the hidden platform test, 4 trials per day were conducted for 8 days. The sequence of start positions was different on each training day and visual spatial cues were located outside the tank. The latency to find the platform was recorded with a maximum of 2 minutes allowable. To test memory retention of the platform location, mice underwent a probe trial 24 hours after the final hidden platform training trial. During the probe trial, the platform was removed, and the mouse was placed in the pool and allowed to swim for 30 seconds. Time spent in each quadrant was recorded. The

percentage time spent in the target quadrant during the probe trial was calculated.^[14]

Biochemical Parameters Study

Assessment of protein concentration

Protein content in the brain homogenate was measured using the standard total protein kit by the method of Biuret test; wherein bovine serum albumin (BSA) was used as a standard. 5µl of tissue homogenate was added to 200µl of Bradford reagent incubated at 37 °C for 10 min and absorbance was measured at 540 nm with the help of a microplate spectrophotometer.^[16,17]

Assessment of reduced glutathione

Reduced GSH was estimated according to Smith and Johnson 1988 with small modifications. Each 3 ml reaction mixture consisted of 2.9 ml of 5,5-dithiobis(2-nitrobenzoic acid) prepared in potassium phosphate buffer (0.1 M, pH 7.4) and 0.1 ml of tissue homogenate. The reaction mixture was incubated at 37 °C for 15 min and absorbance was measured at 412 nm and results were expressed as GSH/mg protein.¹⁷

Assessment superoxide dismutase

The activity of SOD in the brain homogenate was assayed by monitoring its ability to scavenge superoxide radicals generated by auto-oxidation of pyrogallol in the alkaline medium. SOD was determined with the method of Zhang et al. 2012. Each 200 µl reaction mixture contained 180 µl of potassium phosphate buffer (0.1 M, pH 7.4), 10 µl tissue homogenate and 10 µl pyrogallol solution (2.6 mM in 10 mM HCl). The rate of increase in the absorbance at 325 nm was recorded for a period 5 min with 30s interval. One unit of SOD is described as the amount of enzyme required to cause 50% inhibition of pyrogallol autoxidation per 200 µl of the assay mixture.^[18]

Assessment of catalase

The activity of catalase was determined by using the mixture consisting of 1.95 ml of phosphate buffer (50 mM, pH 7.0), 1 ml of H₂O₂ (10 mM) and 0.05 ml aliquots of homogenates (10% w/v) in a final volume of 3 ml. The control contained all the components except substrate. The change in absorbance was recorded at 240 nm for 10 min, at an interval of 1 min. The catalase activity was expressed in U/mg of protein.^[19]

Assessment of lipid peroxidation

The LPO content in the brain homogenate was determined by the spectrophotometric method as described by Ohkawa et al.^[20] Briefly, the method describes, 0.2 ml tissue homogenate was added to a mixture of 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid solution adjusted to pH 3.5 with NaOH, and 1.5 ml of 0.8% aqueous solution of TBA. The final mixture volume was adjusted to 4.0 ml with distilled water and then heated at 95 °C for 60 min in a water bath. After cooling, 1 ml of distilled water and 5.0 ml of the mixture of n-butanol and pyridine (15:1, v/v) were added to the above reaction mixture and shaken vigorously. After centrifugation at 4000 rpm for 10 min, the absorbance of the organic layer was measured at 532 nm. LPO was expressed in terms of nmol of MDA/mg of protein.

Assessment of whole brain acetylcholinesterase activity

The estimation of whole brain acetylcholinesterase activity was carried out based on Ellman's method with slight modifications. The animals were decapitated and brains were dissected out immediately and placed in ice-cold saline. The tissue was weighed and homogenized in 0.1 M phosphate buffer pH 8 (10% w/v), homogenized tissue was centrifuged at 4,000 xg for 15 min. 0.4 ml aliquot of the supernatant was added to a cuvette containing 2.6 ml phosphate buffer (0.1 M, pH 8) and 100 µl of 5,5-dithiobis (2-nitro benzoic acid). The contents of the cuvette were mixed thoroughly by bubbling air and absorbance was measured at 412 nm in a spectrophotometer. When absorbance reached a stable value, it was recorded as the basal reading. 20 µl of substrate i.e., acetylthiocholine was added and the change in absorbance was recorded for a period of 10 min at intervals of 1 min. Change in the absorbance per min was thus determined.^[20,21]

Statistical analysis

Data of all the results were presented as mean \pm SEM. The analysis of all the studies was done with the help of an analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test by using GraphPad Prism Version 5, San Diego California USA. *P<0.05, **P<0.01, ***P<0.001 were considered to be statistically significant when compared with the LPS treated (disease control) group.

Histopathological examination

At the end of experiments, all the animals were sacrificed with an overdose of carbon dioxide and their brains were removed and perfused with cold phosphate-buffered saline followed by 4% paraformaldehyde. From every group, two animals were used for histopathology.^[22]

Tissue specimens were collected from animals belonging to different treatment groups. After collection, the tissues were immediately preserved in the 10% neutral buffered formalin. Processed tissue were sectioned (at 5µm) and taken on the clean glass slides and stained with hematoxylin and eosin and observed under microscopes at different magnifications. Sections were examined histologically to check for any microscopic alterations of pathological significance.

3. RESULTS

Effect of L-Theanine on *In vitro* antioxidant activity

L-Theanine scavenged NO free radicals in a dose dependent manner with an IC₅₀ value of 776.44 µg/ml. This reveals the good antioxidant potential of L-Theanine. Also L-Theanine was able to inhibit the lipid peroxidation in the brain homogenate induced by Fe²⁺ and sodium nitropruside in the TBARS assay. The IC values were found to be 11.17 µg/ ml and 24.77µg/ml respectively (Figure 1 and 2).

Effect of L-Theanine on reduced glutathione, superoxide dismutase, catalase and MDA activity

Our data reveals that chronic LPS treatment elevated the *in vivo* oxidative stress in the brain as evidenced by lower values of GSH, SOD, CAT and an increase in MDA levels as compared to vehicle control. However, chronic L-Theanine (2mg/kg and 4mg/kg) and Rivastigmine administration significantly increased the GSH, SOD, CAT values and reverse the increased MDA activity in the hippocampus and cortex thus confirming their antioxidant potential (Table 1).

Effect of L-Theanine on acetylcholinesterase activity

Acetylcholinesterase activity was found to be significantly higher in the brain of LPS treated mice as compared to vehicle treated group. L-Theanine administration significantly reversed the increase in acetylcholinesterase activity in the brain in a dose dependent manner. Rivastigmine being a known acetylcholinesterase inhibitor also reversed the increase in acetylcholinesterase activity (Table 1).

Effect of L-Theanine on behavioral parameters

Effect of L-Theanine on Y-maze spontaneous alteration test

The alteration of spatial and learning memory was significantly decreased in lipopolysaccharide induced memory impairment group, while administration of L-Theanine at dose 4 mg/kg and Rivastigmine (0.25 mg/kg) caused a significant increase in memory but L-Theanine 2 mg/kg does not significantly improve the memory (Figure 3).

Effect of L-Theanine on Morris water maze test

The alteration of learning and memory were significantly decreased in LPS induced memory impairment group, while administration of L-Theanine at dose 4 mg/kg and Rivastigmine (0.25 mg/kg) caused a significant increase in memory but L-Theanine 2 mg/kg not significantly improve the memory (Figure 4).

Histopathological examination

Histology of brain in experimental mice was observed after 28 days of treatment (H&E staining, 400X) showed no abnormalities with respect to neuronal dendrites in the hippocampus, Vacuolation, Hippocampus and its degeneration and blood capillaries in normal control group. LPS induced severe neuron degeneration was observed consistently in the hippocampus in LPS treated group, whereas mild damage to the hippocampus area was observed in rivastigmine treated group. L-Theanine treated groups had shown moderate to mild damage in dose dependent manner to the hippocampus area indicated by the presence of a medium number of degenerative cells compared to disease control group (Figure 5).

Table 1: Effect of L-Theanine on Antioxidant and Acetylcholinesterase enzyme levels in Brain tissue homogenate.

Treatment	GSH (μ mole/mg protein)	SOD (U/mg protein)	CAT (nmoles H_2O_2 /min/mg protein)	LPO (nmoles MDA/mg protein)	AchE activity (nmoles/min/g m tissue)
Saline control	63.00 \pm 2.44	63.00 \pm 2.44	64.44 \pm 4.657	2.453 \pm 0.2113	42.01 \pm 3.388
Disease control	42.30 \pm 1.819	42.30 \pm 1.819	26.56 \pm 2.443	4.420 \pm 0.03710	18.15 \pm 2.566
L-Theanine (2mg/kg)	58.49 \pm 1.250	58.49 \pm 1.250	43.67 \pm 3.728	3.057 \pm 0.2311	21.78 \pm 2.628
L-Theanine (4mg/kg)	58.55 \pm 1.077	58.55 \pm 1.077	43.89 \pm 4.124	3.527 \pm 0.527	31.56 \pm 1.149
Positive control	59.39 \pm 1.317	59.39 \pm 1.317	56.73 \pm 5.619	3.057 \pm 0.3241	35.38 \pm 2.681

The results are expressed as mean \pm SEM (n = 6) and were evaluated by one-way ANOVA followed by Tukey's Multiple Comparison Test. *P< 0.05, **P< 0.001, ***P< 0.0001 vs. Disease control.

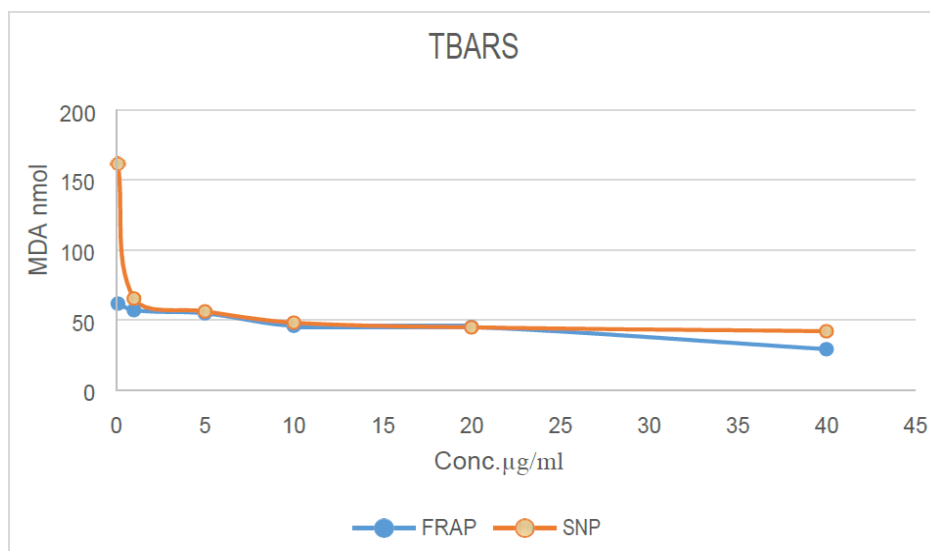


Figure 1: In-vitro antioxidant activity and free radical scavenging activity of L-Theanine using Malondialdehyde assay.

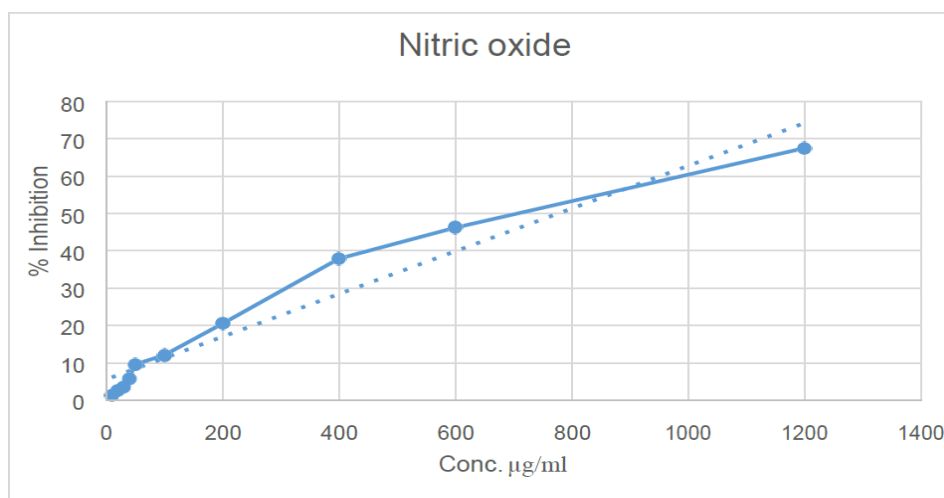


Fig. 2: Effect of L-Theanine on nitric oxide radical scavenging activity.

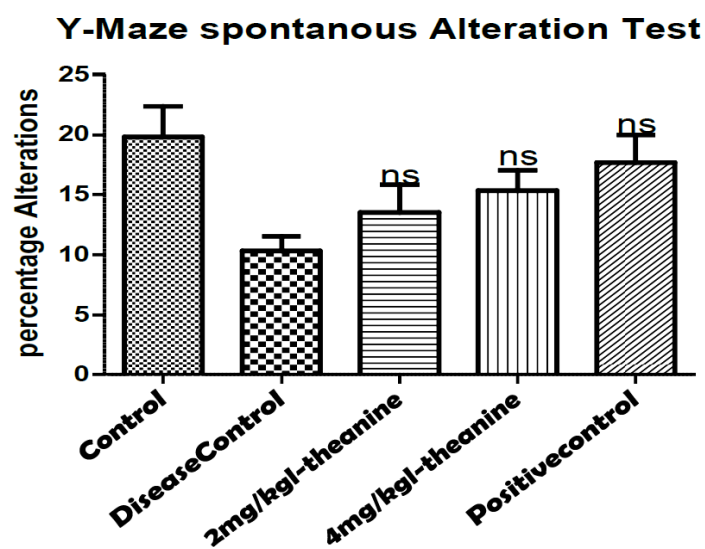


Figure 3: Effect of L-Theanine on Y-maze spontaneous alteration test.

The results are expressed as mean \pm SEM ($n = 8$) and were evaluated by one-way ANOVA followed by Tukey's Multiple Comparison Test. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$ vs. Disease control.

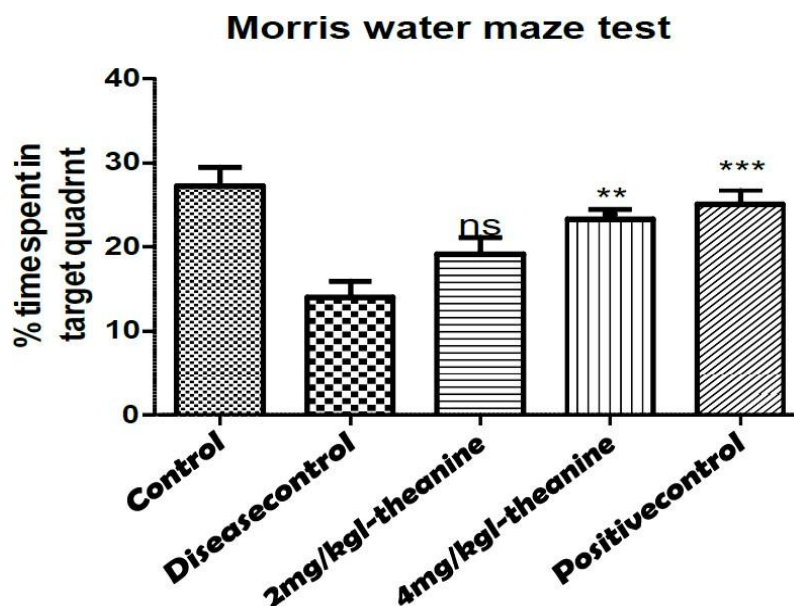
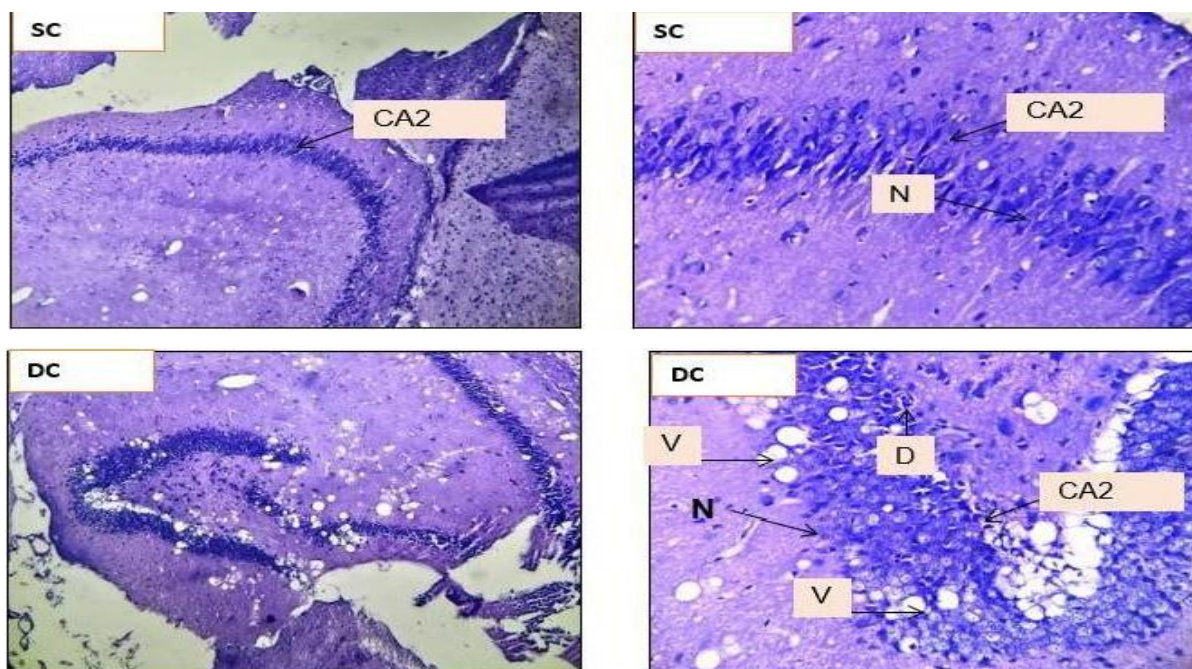


Figure 4: Effect of L-Theanine on morris water maze test.

The results are expressed as mean \pm SEM ($n = 8$) and were evaluated by one-way ANOVA followed by Tukey's Multiple Comparison Test. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$ vs. Disease control. Time spent in target quadrant in probe trial.



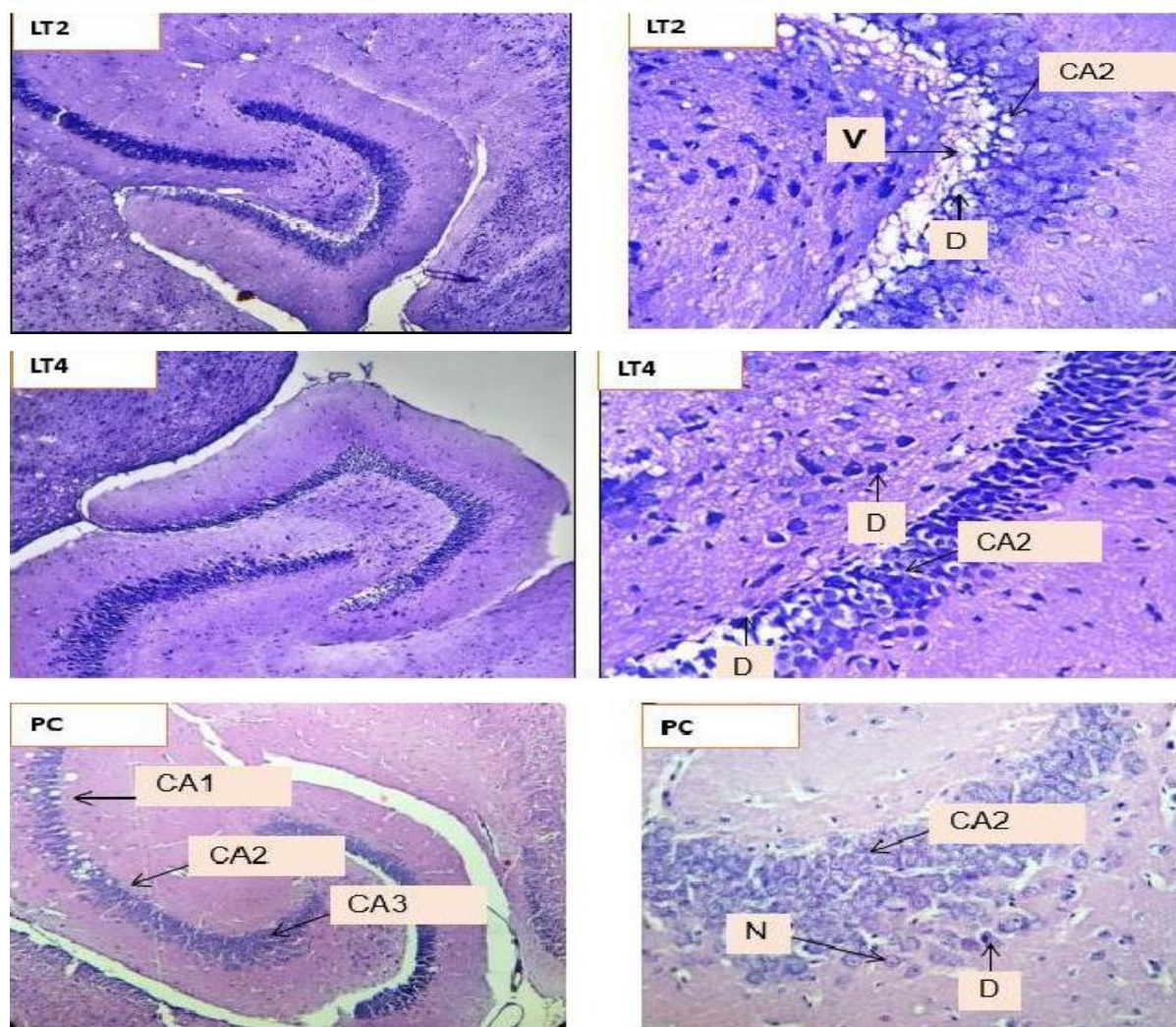


Figure 5: Histopathology of brain hippocampus H & E staining.

Representing SC- Saline control, DC-Disease control, LT2- L-Theanine 2 mg/kg, LT4- L-Theanine 4 mg/kg, PC – positive control; **D**: degeneration; **V**: Vacuolation (Degeneration of parenchymatous neurons in hippocampus); **CA1**, **CA2** and **CA3**: regions of the neuronal dendrites in hippocampus; **H**: hippocampus; **C**: Blood capillary and **N**: Neuron myelinated.

4. DISCUSSION

Alzheimer's disease is a progressive neurodegenerative disorder and the most common cause of dementia worldwide. It mainly affects older individuals. It is characterized by the presence of beta-amyloid plaques and neurofibrillary tangles within the brain of patients presenting with the memory defects including impairment in learning and memory. As the exact mechanism about the genesis of disease is unknown, various hypothesis have been given for the cause of Alzheimer's. These are genetics, cholinergic hypothesis, amyloid hypothesis and tau hypothesis. L-theanine crosses the blood-brain barrier and is transported in a dose-

dependent manner into the brain. Transport into the brain occurs via the leucine-preferring transport system of the blood- brain barrier. The plasma concentrations of L-theanine and ethylamine reach their highest levels about 0.5 and 2 hours, respectively and L-theanine (administered intraperitoneally) is taken up by brain tissue within 30 minutes after its administration without any metabolic changes in mice.^[23]

The present study demonstrated the neuroprotective effect of L-Theanine on LPS induced memory impairment model of Alzheimer's disease in mice. In the study, various parameters were studied. These include the various antioxidant assays, behavioral studies of animals, biochemical parameters and also the histopathological changes.

An antioxidant is defined as any substance that when present at low concentrations compared with those of an oxidisable substrate, significantly delays or inhibits oxidation of that substrate. Antioxidant compounds are able to donate electrons to reactive radicals converting them into more stable and unreactive species. This is called the reducing power of the antioxidant.

There are many reports that support the use of antioxidants from natural sources as alternative to synthetic antioxidants in reducing the level of oxidative stress by counteracting the free radicals and in delaying or preventing the development of complications associated with diseases. Many synthetic antioxidant compounds have shown toxic and /or mutagenic effects. Hence attention needs to be given to antioxidant occurring from natural sources. A great number of naturally occurring substances have been recognized to have free radical scavenging and antioxidant abilities. Oxidative stress is one of the main mechanisms involved in the induction of neurodegenerative disorders. This oxidative stress is due to an imbalance between free radical generation and disturbance in the natural antioxidant protective system in the body. Free radicals are the molecular species that contain unpaired electrons in atomic orbital's. These include hydroxyl (OH.), superoxide (O₂.), nitric oxide (NO.), nitrogen dioxide (NO₂.), radicals; are highly unstable molecules that have an unpaired electron available to react with various organic substances such as lipids, proteins, DNA, peroxy (ROO.) and lipid peroxy (LOO.). When these free radicals are produced in excess, natural enzymatic antioxidant system present in the cell of the body like superoxide dismutase, catalase, glutathione helps to neutralize them thereby shielding down the oxidative damage. In the present study, different methods of *in vitro* assays were performed to evaluate the free radical scavenging and antioxidant activity of L-Theanine.

Nitric oxide (NO) is a potent pleiotropic mediator generated from amino acid L-arginine by vascular endothelial cells, phagocytes and certain cells of brain. The toxicity of NO is increased when it reacts with superoxide radical, forming a highly reactive peroxynitrite anion (ONOO⁻).

The nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. L-Theanine inhibited nitrite formation by competing with oxygen to react with nitric oxide directly and also to inhibit its synthesis. L-Theanine showed a significant increase in percent inhibition in the nitric oxide radical scavenging activity in a concentration dependent manner. The IC₅₀ value of L-Theanine was found to be 776.44 µg/ml. Thiobarbituric Acid Reactive Substance (TBARS) are different types of aldehydes and lipid peroxidation is considered to occur due to the presence of reactive oxygen species (ROS) in the cells. Intermediates of lipid peroxidation ultimately convert to different types of aldehydes eg. formaldehyde, acetaldehydes etc. Malondialdehyde (MDA) is the end product. So MDA is the commonly accepted biomarker of lipid peroxidation and its reaction with TBA is used to determine a number of aldehydes or the products of lipid peroxidation. It is a test for estimation of lipid peroxidation with its end product (MDA). L-Theanine was able to reduce the MDA by competing with the formation of aldehydes. The MDA inhibition study showed that L-Theanine possesses good reduction potential with a 50% inhibition (IC₅₀) at a concentration of 11.17 µg/ml and 24.77 µg/ml respectively with ferrous sulfate and sodium nitroprusside reagents. Spontaneous alternation is a behavioral test which tests spatial learning and memory. The purpose of the test is for the rat or mouse to remember which maze arm was last visited and try to enter as many different arms as possible. The task is testing hippocampal memory and can be weakened by lesions to the hippocampus. The sequence of arm entries and total amount of arm entries are scored and a percentage is calculated. The cognition of the animal can be assessed based on the score where a lower score is considered cognitively impaired. Y-maze spontaneous alternation test showed dose dependent increase in percentage alternations in L-Theanine treated group. There was increase in percentage alteration in L-Theanine (2mg/kg) group but not significantly. Significant ($P^* < 0.05$) increase in percentage alteration was observed in L-Theanine 4mg/kg ($15.33 \pm 1.667^*$) as compared to disease control group (10.33 ± 1.202). The present study showed that the memory was less affected as good scores were obtained in L-Theanine treated groups.

The Morris water maze (MWM) is a test of spatial learning for rodents that relies on distal cues to navigate from start locations around the perimeter of an open swimming arena to locate a submerged escape platform. Spatial learning is assessed across repeated trials and reference memory is determined by a preference for the platform area when the platform is absent. The MWM has proven to be a robust and reliable test that is strongly correlated with hippocampal synaptic plasticity and NMDA receptor function. During visual platform training of MWM test, there was no significant difference in escape latency between any groups. During hidden platform training, a decrease in escape latency in rivastigmine and L-Theanine treated groups were observed as compared to LPS treated group. Rivastigmine and L-Theanine treated groups got the platform quicker as compared to LPS treated group, which shows that the LPS group had less learning ability. During probe trial, the time spent in targeted quadrant was significantly ($P^{**} < 0.01$) more in L-Theanine (4mg/kg) as compared to LPS treated group.

To further confirm antioxidant activity as the mode of neuroprotective, *in vivo* antioxidant properties were investigated in the animal model. However, there are many factors which are responsible for the brain or nerve damage or injuries. LPS is the bacterial endotoxin which causes inflammation in neurons. The mechanism thought to be involved is impairment of antioxidant defenses as well as reactive oxygen species (ROS). The latter formed *in vivo*, such as superoxide anion, hydroxyl radical and hydrogen peroxide which is highly reactive and potentially damaging transient chemical species. These are continuously produced in the human body, as they are essential for energy supply, detoxification, chemical signaling and immune function. ROS are regulated by endogenous superoxide dismutase, glutathione peroxidase, and catalase; As a result of over-production of ROS, due to exposure to external oxidant substances or a failure of enzyme regulatory mechanisms leading to damage of cell structure, DNA, lipids and proteins. LPS is known to have a profound effect on lipids and proteins metabolism. It has been demonstrated that oxidative stress plays a major role in neuroinflammation induced by LPS. The present study shows a significant decrease in the levels of antioxidants such as superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), while the level of malondialdehyde, an end product of lipid peroxidation, was increased in the LPS-treated group. The decreased levels of antioxidant viz., SOD, GSH, CAT activities may be due to an overwhelming oxidative modification of enzymatic proteins by excessive ROS generation. More so reduction in the activities of these enzymes may stem from a decrease in their rate of synthesis.

Superoxide dismutase (SOD) is an enzymatic antioxidant that protects the tissue from highly reactive superoxide radicals by catalyzing and converting it into hydrogen peroxide and water. Hydrogen peroxide is further acted upon catalase and gets converted to water and oxygen. Decreased activity of SOD (42.30 ± 1.819 U/mg of protein) may be due to excessive accumulation of the end product of LPS metabolism or accumulation as a result of which there could be increased oxidative stress. Increased activity of SOD in mice administered with L-Theanine 2 mg/kg (58.49 ± 1.250 U/mg of protein) and 4 mg/kg (58.55 ± 1.077 U/mg of protein) shows the protective role of L-Theanine in the accumulation of excessive free radical in brain and thereby protection of brain LPS induced inflammation.

Catalase converted hydrogen peroxide to water and oxygen, which is the intermediate of SOD cleavage. Decreased activity of CAT (26.56 ± 2.443 U/mg of protein) may be due to excessive accumulation of the end product of LPS metabolism or accumulation as a result of which there could be increased oxidative stress. Increased activity of CAT in mice administered with L-Theanine 2 mg/kg (43.67 ± 3.728 U/mg of protein) and 4 mg/kg (43.89 ± 4.124 U/mg of protein) shows the protective role of L-Theanine in the accumulation of excessive free radical in brain and thereby protection of brain LPS induced inflammation.

Glutathione (GSH) is a critical determinant of tissue susceptibility to oxidative damage and the depletion of brain GSH has shown to be associated with an enhanced toxicity to LPS. This is attributed to conjugation of GSH to other endogenous molecules. In the present study, the GSH levels were significantly depleted in LPS treated group (42.30 ± 1.819 U/mg of protein), however L-Theanine treated groups at a dose of 2 mg/kg (58.49 ± 1.250 U/mg of protein) and 4 mg/kg (58.55 ± 1.077 U/mg of protein) have shown restorability in the GSH levels which may be due to its ability to scavenge free radical and reduce the oxidative stress.

The level of lipid peroxide (LPO) is a measure of membrane damage and alterations in structure and function of cellular membranes. Lipids undergo peroxidation in the presence of reactive oxygen species, which interferes with the structural appearance of these lipids and make them deleterious in the process. One of the main end products of the lipid peroxidation is malondialdehyde (MDA), the levels of which are hampered by brain injury. The increase in MDA levels in the brain on LPS administration (4.420 ± 0.03710 nmol/mg of protein) suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defence mechanism to prevent the formation of excessive free radicals. Treatment with L-Theanine at a dose of 2 mg/kg and 4 mg/kg (3.057 ± 0.2311 , 3.527 ± 0.527 nmol/mg of protein

respectively) significantly reversed these changes. Hence it is possible that the mechanism of neuroprotection of L-Theanine may be due to its antioxidant action. From the results it is clear that the L- Theanine shows protective activity compared with disease control. These results indicate that L- Theanine protects the structural integrity of the hippocampus membrane and brain cell architecture damaged by LPS which was further confirmed by histopathological examination.

Acetylcholine (ACh) is a key neurotransmitter in learning and memory, and its activity is terminated by a hydrolytic enzyme acetylcholinesterase (AChE) into acetate and choline. The AChE activity has been shown to be increased within and around amnesic brain. LPS induced amnesia leads to increased calcium influx followed by oxidative stress which in turn increases activity of AChE. The increase in AChE levels in brain on LPS administration (18.15 ± 2.566 nmol/min/gm of tissue) suggests enhanced neurodegradation leading to nerve damage and reduce neurotransmitter levels in neuron and failure of neurotransmitter signaling mechanism. Treatment with L-Theanine at a dose of 2 mg/kg and 4 mg/kg (21.78 ± 2.628 , 31.56 ± 1.149 nmol/min/gm) of tissue respectively reversed these changes. Hence it is possible that the mechanism of neuroprotection of L-Theanine may be due to its neurotransmitter action.

Histopathological evaluation is in good agreement with results of biochemical parameters. The histopathological observations of the brain tissue of mice challenged with LPS showed severe neuron degeneration and vacuolation in comparison with normal control. On the other hand, mice treated with L-Theanine at a dose of 2 mg/kg and 4 mg/kg showed mild to moderate neurodegeneration which was incomparable with rivastigmine treated group.

5. CONCLUSION

L-Theanine protected against these cellular changes, thereby supporting their memory impairment activity in Alzheimer's disease.

Conflict of interest

None.

ACKNOWLEDGEMENTS

This work was supported by All India Council for Technical Education (AICTE), New Delhi, India. Authors would like to thank Ms. Sarayu Pai, Ms. Malvika Gursahani, Mr. Nitin

Gawali, Ms. Snehal Mestry, and Ms. Amrita Chowdhury for their assistance during the experiment. Authors also thank the help of Dr. Sanjay Pawar (Pathologist) (Regd No: MSVC 7238) for histopathological examination.

Abbreviations

AD – Alzheimer's disease

LPS – Lipopolysaccharide

A β – Amyloid Beta

NFT – Neurofibrillary tangle

ROS – Reactive Oxygen Species

FDA – Food and Drug Administrations

NMDA – Nicotinamide Adenine dinucleotide

CMC – Carboxyl Methyl Cellulose

MWM – Morris Water maze

BSA – Bovine Albumin Serum

GSH - Glutathione

LPO – Lipid Peroxidation

MDA – Malondialdehyde

CAT - Catalase

AchE - Acetylcholinesterase

ANOVA – Analysis of Variance

AICTE – All India Council for Technical Education

SDS – Sodium dodecylsulphate

SOD – Superoxide dismutase

TBA – Thiobarbituric acid

TBARS- Thiobarbituric Acid Reactive Substance

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