Research Article

L-Theanine, an Aminoacid in Tea Augments Methylmercury (Global Pollutant) Induced Biochemical, Behavioural and Histological Changes in Rat Cerebellum

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ABSTRACT
Methylmercury (MeHg) is an environmental toxicant that leads to long-lasting neurological and developmental deficits in animals and humans. L-Theanine is a relatively uncommon amino acid found almost exclusively in green tea leaves. This study was aimed to evaluate the effect of L-Theanine (LT) on MeHg induced oxidative stress in cerebellum of rats. Male Wistar rats were administered with MeHg orally at a dose of 5 mg/kg b.w. for 21 days. Experimental rats were given MeHg and also administered with LT (100 mg/kg, orally) 1 hour prior to the administration of MeHg for 21 days. After treatment period, MeHg exposure significantly decreased the level of glutathione and the activities of superoxide dismutase, catalase and, glutathione peroxidase. Whereas, the thiobarbituric acid reactive substances and the activity of glutathione reductase were found to be increased. Behavioural changes like decreased motor coordination, increased immobility, decreased tail flick responses and decreased locomotor activities were also observed along with histopathological alterations. All the above behavioural, biochemical and histopathological changes were found to be reversed by pretreatment with LT.

Keywords: Methylmercury; L-theanine; Oxidative stress; Behavioural changes; Histology

INTRODUCTION
Methylmercury (MeHg) is recognized as a global environmental pollutant. Its primary target organ is the central nervous system (CNS). MeHg disrupts multiple biochemical and physiological processes1. In humans, MeHg damages the visual cortex and interferes with somatosensory processing. Signs of intoxication include the constriction of the visual field, hearing loss, sensory impairment of the extremities, muscle weakness, tremors and mental deterioration2. Several mechanisms have been proposed for MeHg-induced neurotoxicity, such as the induction of oxidative stress3, disruption of neurotrophic signaling4, disruption of intracellular calcium homeostasis5, and alterations in neurotransmitter systems6. Nevertheless, the precise nature of MeHg-induced neurotoxicity remains to be fully elucidated, likely reflecting upon its multifaceted disruptive cellular mechanisms7-9. The accumulation of MeHg in fish tissue (10–40 ppm) and subsequent consumption of this contaminated fish tissue by humans pose the greatest health risk. Faroe Islands cohort study reported MeHg-induced deficits in some neurological and cognitive functions in school-age children10. The principal sources of exposure to mercury in the general population are ingestion and inhalation of mercury compounds from dental amalgams, and ingestion of fish (fresh water and marine) and seafood, which contain mercury, primarily as methyl mercury. Due to its ubiquitous presence in the environment health concerns are increasing11. Some studies have focused their efforts on the protective effects of plants or natural compounds in various neuropathological conditions. Of particular importance, the beneficial effects of plants/ natural compounds against metal-induced neurotoxicity have been reported under both in vitro and in vivo conditions12, 13, 14. Recently, our research group15 had published for the first time the protective effect of alcoholic extract of Bacopa monniera against MeHg induced neurotoxicity. In this present study we have selected a natural compound L-Theanine to determine for first time the protective effect against Methylmercury induced biochemical and behavioural changes. L-theanine (γ-glutamylethylamide) is a unique amino acid present almost exclusively in the tea plant (Camellia sinensis). It appears to only occur in three other species; one mushroom species and two other species of the Camellia genus. Theanine is synthesized in the root of the plant and concentrates in the leaves, where sunlight converts theanine to polyphenols. Because of this, some tea cultivators grow their plants out of direct sunlight to preserve the theanine content and thus the flavour16. Theanine crosses the blood-brain barrier via the large neutral amino acid (leucine-prefering) transport system. After oral administration, L-theanine is easily absorbed
into the bloodstream and transported to major organs, including the brain. Reports revealed that L-theanine is an effective antihypertensive drug that significantly reduced the systolic, diastolic and mean blood pressure in spontaneously hypertensive rats without any influence on normal rats. Consistently, theanine was found to reduce blood pressure and to antagonize the effect of caffeine-induced blood pressure increases in humans. L-Theanine is hydrolyzed in the kidney to glutamic acid and ethylamine by the enzyme glutaminase. In the brain L-theanine increases dopamine and serotonin production although one study showed a decrease in serotonin in rats administered theanine. Regardless of the mechanism, theanine increases alpha-brain wave activity, a sign of induced relaxation. L-Theanine has been reported to promote relaxation and have neuroprotective effects. It is also shown to have an antagonistic effect on the excitement induced by caffeine.

**MATERIALS AND METHODS**

**Chemicals**

Methyl mercuric chloride was purchased from Sigma Chemicals. Glutathione reductase (GR), glutathione (GSH)-reduced form, glutathione oxidized form (GSSG), tert-butyl hydroperoxide, 5, 50-dithiobis-(2-nitrobenzoic acid (DTNB) were purchased from SRL, b-Nicotinamide adenine dinucleotide phosphate reduced (NADPH) was purchased from CDH. All the other chemicals used were of the analytical grade. L-Theanine was purchased from SRL. All the other chemicals used were of the analytical grade.

**Animals**

Male Wistar Albino rats weighing 250-300g were obtained from Central Animal House, Dr. ALMPGIBMS, University of Madras, Taramani campus, Chennai-113, TamilNadu, India. Rats were housed separately in polypropylene cages and fed standard pellet diet (purchased from Hindustan Lever) kept under hygienic conditions. Rats were kept on a 12hr light and dark cycles with free access to water (RO water) ad libitum. All experiments and protocols described in the present study were approved by the Institutional Animal Ethics Committee (IEAC) of Dr. ALMPGIBMS, University of Madras, Taramani campus, Chennai-113, TamilNadu, India. Rats were divided into four experimental groups of 6 animals each. Group I: control (vehicle orally), Group II: MeHg(5mg/kg, b.w.) orally for 21 days, Group III: MeHg+LT (100mg/kg b.w) orally 1 h prior to the administration of MeHg for 21 days, Group IV: LT alone (100mg/kg b.w) orally for 21 days. Experimental animals were handled according to the University and Institutional legislation, regulated by the Committee for the Purpose of Control and Supervision of Experimental on Animals (CPCSEA).

**Tissue preparation**

After treatment period, experimental animals and control animals were killed by cervical dislocation. Brains were immediately taken out and washed with ice cold saline to remove blood and kept at -80°C. The cerebellum was rapidly dissected from the intact brain carefully on ice plate according to the stereotaxic atlas of Paxinos and Watson.

**Behavioral Parameters**

All the behavioral parameters were performed at room temperature in a calm room without any outside interference. All of the behavioral parameters were performed between 10.00 A.M and 5.00 P.M.

**Rotarod Task**

The rats were subjected to rotarod task, which was based on the study of Duham and Miyaw. Briefly, the apparatus consisted of a bar with a diameter of 2.5 cm, subdivided into four compartments by disks, 25 cm in diameter. After treatments, the rats were subjected to the rotarod task, and the time of permanence on the apparatus was recorded. The maximum time allowed on the rotarod apparatus was 60 sec. Each rat was subjected to three different trials with 3 minutes of interval between each trial, and the mean of their falling latency values was used in the statistical analysis as actual value.

**Tail Suspension Test (TST)**

We used the test as described by. A short piece of paper adhesive tape (about 6 cm) was attached along half the length of the tail (3 cm). The free end of the tape was attached to a 30 cm long rigid tape which hung from a horizontal bar clamped to a heavy laboratory support stand. Suspected animals were surrounded by a white wooden enclosure (45 cm high, 40 cm wide and 40 cm deep) such that the rat’s head was about 20 cm above the floor. For testing each rat was suspended by its tail and observed for 6 min. an observer scored the total duration of a passive, “dead weight” hanging (immobility), between the periods of wriggling of the animal to avoid aversive situation.

**The nociceptive testing (tail flick reflex)**

Pain thresholds were assessed using a modification of tail flick (TF) test. All TF testings occurred in a dimly lit room maintained at 27°C. Rats were loosely held in plexiglas cylinders, constructed so that each rat’s tail protruded from the rear of cylinder. This allowed TF testing to occur without disturbing the animals. Following a 30-minutes habituation period, baseline and TF latencies were recorded. The tail was placed over a radiant heat source (50W projector lamp) adjusted to provide baseline latencies in native animals of approximately 6 ses. The change in thermal withdrawal latencies of tail to noxious heat was measured to the nearest tenth of a second as the time from onset of heating of tail using a tail-flick (TF) device. A cut-off latency of 20 s was used to avoid tissue damage to the tail.

**Open Field Test**

The floor of the open field measured 60 cm×90 cm and was divided into six equal squares. The floor was surrounded by a 30-cm-high opaque wall. The rats were tested individually, and after each session, the open field was cleaned with 70% alcohol followed by wet cotton. These assays were always performed at a fixed hour of the day with controlled light and sound. At the beginning of each session the animals were placed at the centre of
the open field, and the behaviour of each rat was observed for 5 min.

**Forced Swim Test**

The forced swim test was performed according to the method of Porsolt et al. A vertical glass cylinder (25 cm high, 14 cm in diameter) was filled with water (30°C) to a depth of 20 cm. The water depth was adjusted so that the animals must swim or float without their hind limbs or tail touching the bottom. For testing, each animal was placed in the cylinder for 6 min, and the latency to float, and the duration of floating (i.e. the time during which rat made only the small movements necessary to keep their heads above water) was scored. As suggested by Porsolt, only the data scored during the last 4 min were analyzed and presented.

In the forced swim procedure, rats were forced to swim in un-escapable situation. After a period of vigorous struggling, the animal becomes immobile, or makes only those movements necessary to keep its head above the water. The immobility observed in this test is considered to reflect a state of despair. The forced swim test has a high degree of pharmacological validity as reflected by its sensitivity to major classes of antidepressants, including tricyclic antidepressant and Selective Serotonin Reuptake Inhibitors (SSRIs).

**Biochemical Determinations**

Thiobarbituric acid-reactive substances (TBARS), an index of lipid peroxidation, were estimated by the method of Okhawa et al. The amount of TBARS was determined spectrophotometrically at 532 nm and expressed as 1 moles of TBARS/mg. Protein carbonyl levels were measured by the method of Levine et al. and expressed as nmol/mg protein. The level of reduced glutathione (GSH) was measured by the method of Moron et al. on the basis of the reaction of 5,5′-dithiobis-2-nitrobenzoic acid which is readily reduced by sulphydryls forming a yellow substance which was measured at 412 nm and expressed as 1 moles/mg protein. The enzyme glutathione peroxidase (GPx) was assayed according to the method of Rotruck et al. The assay takes advantage of concomitant oxidation of NADPH by GR, which was measured at 340 nm. Enzyme activity was expressed as 1 g/min/mg protein. GR activity was assayed by the method of Carlberg and Mannervik. The enzyme activity was quantitated at room temperature by measuring the disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidized/min/mg protein. Superoxide dismutase (SOD) activity, expressed as units/mg of protein, was based on the inhibition of superoxide radical reaction with pyrogallol. Catalase (CAT) activity was determined by following the decrease in 240 nm absorption of hydrogen peroxide (H₂O₂). It was expressed as nanomoles of H₂O₂ reduced/min/mg of protein. The protein content was measured by Lowry et al.

**Histopathology**

After sacrifice of all animals, the cerebellum was fixed in 10% neutral formalin. The tissues were processed in the usual way for paraffin embedding and sections were mainly stained with Hematoxylin and Eosin (H&E) for histopathology (400x).

**Statistical analysis**

Data represents mean ± SD. Statistical comparisons were performed by one way analysis of variance (ANOVA) followed by student’s test using SPSS 10 version. If ANOVA analysis indicated significant differences, Tukey’s post-hoc test was performed to compare mean values between treatment groups and control. A value of p<0.05 was considered as statistically significant.

**RESULTS**

Effect of LT on MeHg induced Rota rod task in control and experimental rats. The effect of LT on MeHg induced motor performance by rotarod task in control and experimental rats were shown in figure.1. The motor performance was found to be significantly reduced (p<0.01) in MeHg induced group when compared with control. LT pretreated rats showed increased performance in rotarod task (p<0.05). The motor performance was found to be equal to control in LT alone treated group.

**Protective effect of LT on MeHg induced Tail Suspension Test**

The total duration of passive, “dead weight” hanging (immobility) was scored as a measure of depression like behaviour. MeHg induced rats showed significant (p<0.01) increase in dead weight (immobility) than control animals. LT pretreatment was significantly reduced the immobility (p<0.05). LT alone treated rats resembled to that of control group.

**Protective effect of LT on MeHg induced Tail Flicking Test in control and experimental rats**

MeHg administration caused increase tail flick response (p<0.01) when compared to control rats. Pretreatment with LT significantly (p<0.05) reduced the response. LT alone treated group showed a response which was similar to control.

**Effect of LT on MeHg induced Open Field Test in control and experimental rats**

The locomotor activity was found to be significantly decreased (p<0.01) in MeHg induced animals compared to control groups. LT pretreated rats showed significantly (p<0.05) increased locomotor activity. LT alone treated group did not show any effect on the locomotor activity.

**Protective effect of LT on MeHg induced Forced Swim Test in control and experimental rats**

Response to inescapable aversive situation interpreted as a measure of depression like behaviour. MeHg exposed rats showed (p<0.01) longer immobility time than control animals. LT pretreatment was significantly (p<0.05) reduced the immobility time when compared to MeHg induced group. LT alone treated resembled to that of control group.

**Effect of LT on MeHg induced changes on the level of TBARS and GSH in control and experimental rats**

Level of LPO and TBARS and GSH were estimated by the method of Okhawa et al. GSH was significantly decreased in MeHg induced group compared with control. Administration of LT
significantly decreased the levels of the TBARS and reduced GSH was completely restored significantly (p<0.05) increased in pretreatment with LT. LT alone treated groups resembles control.

**Protective effect of LT on MeHg induced GR and GPX activities in control and experimental rats**
MeHg administration caused a significant decrease in GPx activity (p<0.01), whereas the activity of GR was found to be significantly increased (p<0.01) when compared to control rats. These alterations in the activity of GPx and GR activity were observed to be maintained at near normally when the rats were pretreated with LT (p<0.05). Rats treated with LT alone did not show any alterations and was similar to that of control.

**Effect of LT on MeHg induced SOD and CAT activities in control and experimental rats**
The activities of SOD and CAT were found to be significantly (p<0.01) reduced in MeHg intoxicated groups when compared to control rats. LT pretreatment significantly (p<0.05) increased the activities of both the antioxidant enzymes. LT alone pretreated rats resembled control rats.

**Protective effect of LT on MeHg induced Histopathological changes in control and experimental rats**
Effect of LT on MeHg induced histopathological changes were shown in Figure.9. Figure.9a (Control) shows the normal histo-architecture of cerebellum. Compared with the control rats, the numbers of Purkinje cells were found to be reduced in MeHg induced animals (Figure.9b). LT pretreated rats (Figure.9c) exhibited the restoration of Purkinje cells. LT alone treated rats (Figure.9d) showed normal architecture of cerebellum.

**DISCUSSION**
Methylmercury (MeHg) is well known for causing Minamata disease. Oxidative stress has been known to contribute to MeHg induced central nervous system (CNS) damage. Many studies have reported that the activation of antioxidant enzyme system plays a significant role in the reduction of MeHg toxicity. A previous study from our group reported the significant beneficial effects of the alcoholic extract of Bacopa monniera against MeHg induced oxidative stress and behavior.

In this current study, we observed the protective effect of L-Theanine against MeHg induced neurotoxicity. There is some evidence that MeHg affects the motor system due to cerebellar damage. In this regard, we have also observed motor deficit in MeHg induced rats. It is interesting to note that LT increased the motor performance. In case of the open field test a reduced locomotor activity was noted in MeHg intoxicated rats. Previous studies also reported that that MeHg inhibits the locomotor in rodents and in humans. LT pretreatment increased the locomotor activity. Furthermore in earlier studies also LT has shown effect in open field test. Tail suspension test and Forced swim test are the good predictive experiments in evaluation of antidepressive activity. In forced swim test and tail suspension test methylmercury intoxicated rats showed longer immobility. It represented as depression like
Depression syndromes have been reported in humans later in life after occupational exposure to inorganic mercury. The results obtained by this study are in agreement with Nabi et al., who reported that alterations in depression like behaviour induced by MeHg. LT pretreatment decrease the immobility. Antinociception was assessed by Tail flick reflex. In our study tail flick latency was reduced in MeHg induced rats. The tail flick reflex was subjected to a complex regulatory system including peripheral neuromuscular transmission, the excitation–contraction coupling system, as well as the central regulatory system of brainstem and cerebellum. Further earlier studies also reported that MeHg caused antinociceptive effect. Preatreatment with LT increase the tail flick response. Mechanistically, it is well known that MeHg-induced neurotoxicity is related to oxidative stress, which is closely associated with glutamate and calcium dyshomeostasis. Notably, the glutathione antioxidant system has been reported to represent a molecular target for the deleterious effects of MeHg in the central nervous system. Gpx activity was significantly reduced in MeHg exposed rats when compared to control. Since GPx catalyzes the reduction of hydrogen peroxide, phospholipid-hydroperoxide and other organic hydroperoxides at the expense of GSH, LT significantly increased the activity. Glutathione reductase is an important enzyme involved in the reduction of glutathione disulfide (GSSG also known as oxidised glutathione) to glutathione (GSH), using NADPH as a reducing cofactor. In our study GR activity was increased in MeHg induced rats. In agreement with these result, previous studies were also reported the same. GR activity was found to be reduced by LT pretreatment. TBARS level was elevated in MeHg induced rats in our study. This indicates lipid peroxidation. It was previously reported by Yamashita et al. that the elevation of TBARS and protein carbonyls were due to oxidative injury by free radicals. LT decrease the level of TBARS. Previous study also reported that LT decreases the oxidation levels in the brain. GSH system is an important target in mediating MeHg neurotoxicity. In accordance with mori et al. and Farina et al., we found from our study that MeHg induction caused reduction of GSH. It was interestingly found that LT increased the GSH level. The level of GSH increased by L-Theanine was already reported by Kurihara et al. in the study of L-Theanine enhances antigen-specific IgG production partly through augmentation of GSH levels. SOD and CAT are the antioxidants play a vital role in preventing both free radical damage and generating oxidative stress like conditions. From our studies the SOD and CAT activities were found to be decreased in MeHg intoxicated rats. These results obtained by this study are in agreement with mori et al. LT pretreatment increased the activites of...
these antioxidants which might be due to enhancing antioxidant abilities. Histopathological changes were also observed in rat cerebellar tissues. In our studies purkinje cells were decreased in MeHg intoxicated rats. In agreement with our findings, it has been evidenced from Sanfelii et al\(^6\) that cerebellar cells are selectively targeted by mercurials in vivo. LT pretreated rats showed retaining of the purkinje cells in cerebellum, which could be due to the antioxidant properties of LT reported earlier by Nishida et al\(^8\). From the above results; we conclude that L-Theanine has the capacity to protect the cerebellum from chronic exposure of MeHg.

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