Evaluation of neuroprotective effect of Ellagic acid against lead induced neurotoxicity

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Running title: Neuroprotective effect of Ellagic acid

ABSTRACT

Aim of the study: The widespread use of lead causes rise in the amount of free lead in the different organs as well as in the environment. The nervous system is affected most for being major target of number of metals. Ellagic acid (EA), a natural polyphenolic compound, is having numerous potential medicinal properties. Materials and methods: The lead induced neurotoxicity was carried out in adult albino mice of both sex weighing 25-30 g. The doses of Ellagic acid were selected as 50mg/kg and 25mg/kg through oral route. After acclimatization, the animals were randomly divided into 4 groups of 8 animals and treated with ellagic acid high

and low doses orally and simultaneously treated with lead acetate for three months. The extent to which EA treatment provides neuroprotective effect was measured by locomotor and cognitive function test, antioxidants like SOD, Catalase, MDH activity were measured in brain tissue homogenate. Other than that, estimation of MAO and acetyl cholinesterase were also performed. Remaining brains were subjected for histological examination. Results and conclusions: Observed results showed dose dependent beneficial effects for EA against lead acetate induced neurotoxicity and it was concluded that EA exhibited dose-dependent protection against lead induced neurotoxicity.

Keywords: Ellagic acid, neurotoxicity, lead toxicity, heavy metal toxicity.

INTRODUCTION

Heavy metals induced toxicity is responsible for major damage to the nervous system apart from other organ toxicites. Some metals like lead, tin and mercury and their alkyl derivatives are neuro-toxic[1]. Acute lead exposure symptoms usually are cognitive changes, nausea, headaches, as well as emotional disruptions. The symptoms of chronic exposure are fatigue, reduced processing speed, motor deficits, reduction in cognitive functioning, neuro-degeneration as well as psychiatric symptoms such as anxiety, depression, and irritability are observed more[2].

The immature organisms are found to be more susceptible to the neurological effects caused due to detrimental effect of chronic exposure to lead. Lead is absorbed and retained more perinatally compared to adults, the developing nervous system seems to be more sensitive to the harmful adverse effects of low-level lead exposure, it is responsible for intellectual and behavioral deficits in children, including hyperactivity; deficits in fine motor function, hand-eye coordination, reaction time; and lowered performance on intelligence tests[3][4].

Continued research on toxic effect of lead on nervous system revealed that multiple mechanisms are involved in lead toxicity. Mainly the mechanisms are of two types; the first is neuro-developmental toxicity, perhaps due to involvement with cell adhesion molecules, which caused miswiring of the central nervous system during early development and permanent dysfunction; the other one is neuropharmacological toxicity, probably involving interactions between lead and

calcium; and lead and zinc, responsible for interference with neurotransmission at the synapse and this may be reversible[5].

Several fascinating theories about the mechanism of lead-induced neuropharmacological toxicity have been proposed. Lead has strong effect on calcium metabolism and transport responsible for inhibiting the acetylcholine release. Lead is also responsible for disrupting heme synthesis. Reduction of heme containing enzymes is responsible for change in mitochondrial function interfering energy metabolism. Lead may hamper neurotransmission by interfering with neurotransmitters. In fact, aminolevulinic acid, produced as a result of lead acting on synthesis of heme, is structurally similar to γ-amino-isobutyric acid. Lead acting on GABA system may be responsible to produce neurotoxicity resembling in case of severe lead poisoning [6].

Besides, lead exposure is responsible for various pathological conditions such as impaired renal and thyroid function, vitamin D deficiency, hypertension and preterm birth which have very strong impact on nervous system[5].

Natural phytochemicals have been found having similar potency compared to various active ingredients having more safety profile as well as efficacy when chcked in various disease models. Polyphenols are usually isolated from various natural sources such as different plants, fruits and vegetables. The protective effect of polyphenols in various heavy metal toxicity is found in many disease models[7,8]. The possible mechanism of polyphenolic compounds is by free radicals scavenging activity. It is also evident that they are responsible for removal of accumulated heavy metals from major organs and detoxification. Reactive oxygen species (ROS) and other free radicals are responsible for development of heavy metal toxicity in different organs[9,10].

Polyphenols are also reported to reduce ROS-mediated inflammatory cytokines secretion through ERK/JNK/p38 pathways causing protection against lead induced inflammatory reactions[10,11].

Ellagic acid (EA) is a polyphenolic phytochemical constituent and an important component of fruits, nuts and vegetables, is well known for many medicinal properties[12]. Pomegranates, raspberries, blackberries, strawberries, red guava, black raspberries, white guava, beefsteak fungus, Cranberries, walnuts and almonds are rich in EA [13,14]. It is proved to be potential antioxidant, with hepato-protective, Neuro protective, Antiulcer, Anti inflammatory,

Cardioprotective, Antineoplastic, Anti-cataract and Antiviral activities [15–17]. Hence the present study has been designed for evaluation of hepatoprotective effect of Ellagic acid against lead induced hepatotoxicity.

METHODOLOGY

Chemicals

All the analytical grade chemicals were obtained from standard companies. Lead acetate was procured from Loba Chemicals, Mumbai. Biochemical kits were purchased from Crest Biosystems (Goa, India).

Phyto-chemicals

Ellagic acid samples were procured from Yucca Enterprises (Mumbai, India).

Experimental Animals

Healthy adult albino mice of both sex weighing 25-30 g were kept in polypropylene cages, maintained in specified condition with12 hours light and day cycles, and a temperature of 25°±5°C) with paddy husk bedding in Central Animal House. They received standard pellet food and had free access to purified drinking water. The CPCSEA guidelines (Committee for the Purpose of Control and Supervision of Experiments on Animals), guidelines from Ministry of Social Justice and Empowerment, Government of India were followed and prior permission was sought from the Institutional Animal Ethics Committee for conducting the study.

Dose selection

Mice doses of Ellagic acid were taken as 50 mg/kg and 25 mg/kg through oral route based on earlier literature as high and low dose respectively[18].

Experimental design

After one week acclimatization, the animals were divided randomly into 4 groups having 8 animals in each. Group I, normal control: received normal saline 2 ml/kg, *p.o.* through oral route,

group II, toxic control: received lead acetate (20 mg/kg, orally) for three months[19]. Group III and IV animals treated by Ellagic acid 50 and 25 mg/kg *p.o.* respectively for three months, along with lead acetate administration as in group II.

Twenty four hours after the last treatments, motor and Locomotor functions were tested by rota rod & actophotometer. Cognitive functions were estimated by active avoidance test. Thereafter, the animals were sacrificed without anesthesia to avoid animals stress, brain of each animal were weighed. Then part of the brain was used for estimation of antioxidants like super oxide dismutase (SOD), catalase, MDA. Apart from that, estimation of MAO and acetyl cholinesterase was performed. Remaining brain samples were collected in 10% formaline for histopathological studies.

Estimation of cognitive functions by active avoidance test

In this experimental protocol acrylic chamber was divided into two equal compartments by a wall with a hole. Active avoidance was performed by the following technique. The training session was designed with 10-s of an acoustic conditioned stimulus followed by electric footshock (0.5–0.7 mA) through the electrified floor of the chamber.

If mice did not enter the safe compartment within 10s, both the stimuli were turned off. The stimuli were applied again after a 30s interval. If the mice entered the adjacent compartment during the sound presentation, it avoided the footshock, and the sound was turned off. If the mice entered the adjacent compartment during the footshock, both stimuli were turned off. Each training session was designed with application of stimulus for 25 times. Avoidance responses were observed for the mice of all the experimental groups for the last five days of treatment protocol[20,21].

Preparation of brain Tissue Homogenate:

Brain samples were rapidly removed, and rinsed in ice cold saline [0.9% NaCl). Then, one lobe of brain was homogenized in a twice volume of ice cold TBS (50 mM TRIS, 150 mM NaCl, pH 7.4), the homogenates were centrifuged at 10000 g for 15 min at 4^{o}C, and the resultant supernatant were used for oxidative parameters analysis[22].

Histological analysis:

Brain was dissected and immediately fixed in formalin solution for 24 hr, and then it was embedded in paraffin. Thin sections was cut (5 μm) into slices and was stained with hematoxylin and eosin (H&E) following standard staining technique and the sections were then viewed observing changes in histology [22](Figure no. 1).

Determination of MAO:

The assay mixture containing 1.0 ml of semicarbazide hydrochloride (0.05 M, pH 7.4), 1.6 ml of phosphate buffer (0.2 M, pH 7.4) and 0.4 ml of mitochondrial fraction were incubated at 37°C for a duration of 20 min in a water bath with a shaking device. The reaction began by the addition of 0.5 ml of tyramine hydrochloride (0.1 M, pH 7.4), after incubating for 30 min it was stopped by the addition of 1.0 ml of 0.5 N acetic acid, then it was placed in a water bath and kept for a period of 30 min. then it was subjected for centrifugation for about 10 min at 1000× g and, to 2.0 ml of the supernatant, 2.0 ml of 2,4-dinitriphenylhydrazine (0.5 mg/ml in 2N HCl) were added. It was kept at room temperature for 15 min, then 5 ml of benzene was added. The tubes were vortexed, later aqueous layer was discarded. The benzene layer was washed with 4 ml of distilled water, then 4 ml of 0.1N NaOH solution was added and the contents of the tubes were mixed thoroughly. The benzene layer was discarded, and then the NaOH layer was kept at room temperature for 1 h. The absorbance of the samples was measured at 425 nm[23].

Determination of acetyl cholinesterase:

After sacrificing, mice brains were removed quickly and placed in ice-cold saline. Frontal cortex, hippocampus and septum were quickly dissected out on a Petri dish covered with crushed ice. The weight of the tissues were taken, then homogenized in 0.1M Phosphate buffer (pH 8). 0.4ml aliquot of the homogenate was added to a cuvette containing 2.6 ml phosphate buffer (0.1M, pH 8) and 100μl of DTNB. The contents of the cuvette were mixed, the absorbance was measured at 412 nm in a spectrophotometer. When absorbance becomes stable, it was recorded as the basal reading. 20μl of acetylthiocholine was added and change in absorbance was recorded. Change in the absorbance per minute was determined[24].

Statistical analysis:

Results were expressed as mean +/- SEM. Statistical significance was evaluated by One-way Analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison tests. $P<0.05$ is considered significant.

RESULTS

Estimation of motor function and Locomotor functions by rotarod and actophotometer (Table no. 1)

One hour after the last treatment, each mouse were subjected to rotarod test for a period of 5 min to measure changes in their balancing behavior and fall off time was measured [25]

Similarly, one hour after the last treatment, each mice were placed individually in actophotometer for 10 minutes and locomotor activity was measured in scores[24,26].

Marked reduction in muscular strength and locomotor activity was observed in lead treatment group compared to normal control group after 3months of exposure to lead 20 mg/kg, orally. The both treatment groups significantly restored the muscular strength as well as locomotor score compared to toxic control group.

Table no. 1: Estimation of motor function and Locomotor functions by rotarod, actophotometer & active avoidance test.

Treatment	Locomotor Scores	Muscular strength (seconds)
Normal control	170.72 ± 10.91	120.65±1.15
Toxic control	141.72 ± 8.72***	40.32±2.13***
EA 50	160.76±9.94##	98.11±1.04##
EA 25	159.52±8.31##	85.87±1..91##

*All values are mean ± SEM, n=8, ***P <0.001 when compared to normal control; ##P <0.01 compared to Toxic control group. EA 50=Ellagic acid 50 mg/kg, EA 25=Ellagic acid 25 mg/kg.*

Estimation of cognitive functions by active avoidance test (Table no. 2)

Lead acetate treatment showed significant decrease in avoidance response compared to normal control. Both the treatment groups showed significant improvement in avoidance response compared to toxic control group.

Table no. 2: Estimation of active avoidance test.

Treatment	Experimental Days				
	Day 1	Day 2	Day 3	Day 4	Day 5
Normal control	12.7±3.2	27.4±3.8	45.3±4.7	68.4±5.4	87.9±4.2
Toxic control	11.5±4.9	24.7±3.7***	29.4±5.3***	31.2±3.7***	36.8±5.4***
EA 50	12.6±5.1	26.6±5.4##	39.0±2.8##	59.6±2.2##	62.7±3.7##
EA 25	12.5±4.2	25.4±2.2##	37.7±3.6##	52.4±4.6##	58.4±5.3##

*All values are mean ± SEM, n=8, ***P <0.001 when compared to normal control; ##P <0.01 compared to Toxic control group. EA 50=Ellagic acid 50 mg/kg, EA 25=Ellagic acid 25 mg/kg.*

Determination of antioxidants like SOD, Catalase, MDA in brain tissues homogenate (BTH). (Table no. 3)

Superoxide dismutase and catalase activity assay

Toxic control group reported extremely significant (P <0.001) decrease in SOD and Catalase activity compared to normal control. Experimental groups EA 50 and EA 25 demonstrated moderately significant increase (P <0.01) in SOD and Catalase values compared to toxic control group.

Malondialdehyde assay

MDA levels in brain samples were measured by the formation of MDA as an indicator of lipid peroxidation, which reacts with thiobarbituric acid producing thiobarbituric acid reactive substances (TBARS), measured spectrophotometrically at 532 nm[27,28]. Toxic control group demonstrated extremely significant (P <0.001) increase in MDA activity compared to normal control.

Both the treatment groups EA 50 and EA 25 showed moderately significant (P <0.01) decrease in TBARS activity compared to toxic control group.

Table no. 3: Determination of antioxidants like SOD, Catalase, MDA in brain tissues homogenate (BTH).

Treatment	SOD	Catalase	MDA
Normal control	1.12 ± 0.014	0.25±0.015	12.23±0.54
Toxic control	0.88 ± 0.022***	0.16±0.013***	19.65±0.43***
EA 50	1.00±0.046##	0.22±0.024##	14±0.52##
EA 25	0.96±0.031##	0.20±0.019##	15±0.28##

*All values are mean ± SEM, n=8, ***P <0.001 when compared to normal control; ##P <0.01 compared to Toxic control group. SOD= Super oxide dismutase, MDA= Malondialdehyde, EA 50=Ellagic acid 50 mg/kg, EA 25=Ellagic acid 25 mg/kg.*

Determination of MAO & acetylcholineesterase using spectrophotometer. (Table no. 4)

The absorbances of the samples for the estimation of MAO were measured at 425 nm. Similarly, the absorbance of the samples for the estimation of acetylcholinesterase was measured at 412 nm in a spectrophotometer[29]. Toxic control (only lead acetate treated) group demonstrated extremely significant (P <0.001) decrease in brain monoamine oxidase and acetylcholinesterase activity compared to normal control. Treatment groups EA 50 and EA 25 showed moderately significant (P <0.01) increase in brain monoamine oxidase and acetylcholinesterase activity compared to toxic control.

Table no. 4: Determination of MAO & acetylcholineesterase using spectrophotometer.

Treatment	Brain monoamine oxidase activity (nmol/min/mg protein)	Brain Acetylcholineesterase (nmol/min/mg protein)
Normal control	7.5±0.03	0.68±0.05
Toxic control	3.5±0.04***	0.23±0.06***
EA 50	6.5±0.05##	0.59±0.07##
EA 25	6.0±0.03##	0.47±0.04##

All values are mean ± SEM, n=8, $^{***}P$ <0.001 when compared to normal control; $^{\#\#}P$ <0.01 compared to Toxic control group. EA 50=Ellagic acid 50 mg/kg, EA 25=Ellagic acid 25 mg/kg.

Figure no. 1: Haematoxylin and eosin (H&E) stained section of cortex in lead acetate induced damage. Photographed at magnification 100X

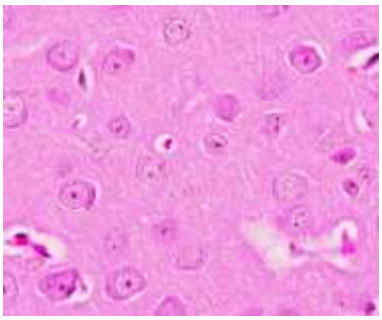

Figure- 1a: (H&E) stained cortex section (100X) of normal control mice

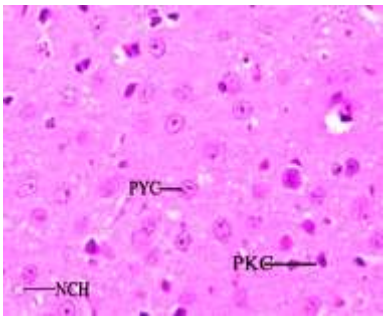

Figure- 1b: (H&E) stained cortex section (100X) of toxic control mice, PYC= Pyramidal neurons, PKC= degenerated pyramidal cells, NCH= neurocytechromatolysis

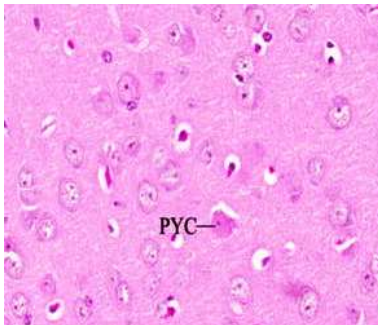

Figure- 1c: (H&E) stained cortex section (100X) of EA50 treated mice, PYC= Pyramidal neurons

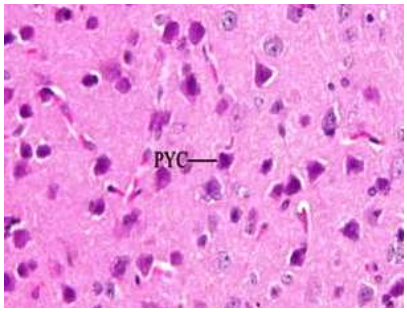

Figure- 1d: (H&E) stained cortex section (100X) of EA25 treated mice, PYC= Pyramidal neurons

DISCUSSION

The aim of the present study was to investigate the protective effect of Ellagic acid (EA) against lead acetate induced neurotoxicity. Observed results demonstrated dose dependent beneficial effects for EA against lead acetate induced neurotoxicity.

As lead is widely distributed in the environment, it alters several organ systems, such as nervous, renal, reproductive and haematological systems are important among them. Behavior can be

regarded as the indication of the sensory, motor and cognitive functions occurring in the nervous system and it can serve as potentially sensitive indicators of chemically-induced neurotoxicity. Lead exposure has been reported to decrease the locomotor activity. Long term lead exposure can be responsible to cause cognitive and motor impairment, with behavioral alterations as long-term effects. These were indicated by a decrease in muscle strength and locomotor activities in lead exposed rats compared with normal control. Significant improvement in muscle grip strength and locomotor activities along with number of squares crossed were observed in the treatment groups. Improvement in neurotransmitter enzymes such as AChE could be responsible for the improvement in learning, memory, cognition and locomotor functions [25,29].

It has been observed chronic exposure of lead acetate was responsible for significant inhibition of avoidance response which is an indication of deterioration of cognitive functions[20,21]. EA reported significant improvement in avoidance response which is a clear demonstration of protection against lead acetate induced memory impairment.

The antioxidant enzymes are the first line of antioxidant defense. SOD and CAT effects are sufficient to remove superoxide anions and hydrogen peroxide and protect cells against reactive hydroxyl radicals. In the present study, the activity of SOD and CAT has been significantly decreased after lead exposure. Both treatment groups of Ellagic acid was found to significantly increase the activity of SOD and catalase in brain when compared to mice treated with lead alone[30].

Lead exposure to the cell or tissue may cause damage to the nervous system by various mechanisms. It can cross the blood-brain barrier and damage the structural components of brain by producing injury to the glial cells, primarily in the cerebral cortex, cerebellum and hippocampus. It gets deposited in these regions of the brain and is associated with behavioral abnormalities, learning impairment, decreased hearing, neuromuscular weakness and impaired cognitive functions in humans and in experimental animals. It may also cause in many biochemical alterations and a variety of neurological disorders, such as mental retardation, behavioral problems, nerve damage, Alzheimer's disease, schizophrenia and Parkinson's disease. From the studies it has been found that there is association of lead exposure to CNS damage which can cause forgetfulness, irritability, poor attention span, headache, fatigue, impotence, dizziness and depression[31–33].

The free radicals generation linked with the enhanced oxidative stress after lead exposure has been reported. Experimental studies have shown an increase in lipid peroxidation and impaired antioxidant defence enzymes in brain following lead exposure, suggesting enhanced oxidative stress.

Studies revealed that lead-exposed animals showed reduced antioxidant defense mechanisms, such as catalase and superoxide dismutase, which shows an increase in oxidative stress. In vitro and in vivo studies have reported lead exposure may have enhanced the generation of ROS as well as increased oxidative stress by changing the antioxidant defense systems in animals. Lead has high affinity for free-SH groups in enzymes and proteins, its binding with these enzymes can alter their efficacy and function. Oxidative stress, induced by disrupting the antioxidant balance in the cells, deregulation of cell signalling and neurotransmission alteration have been regarded as potential mechanisms involved in lead-induced neurotoxicity. In this study, an increase in the level of lipid peroxidation and protein carbonyl contents, decreased levels of SOD and catalase clearly indicate the enhanced oxidative stress following lead exposure in mice[31,34,35].

Free radicals have been found to be significant in lead neurotoxicity, as lead causes its neurotoxicity through oxidative stress. Lead induced disruption of the antioxidant balance in the brain can induce oxidative damage to critical biomolecules. Lead increases the rate of lipid oxidation in brain membranes. Increased oxidative stress reflected as a higher MDA level in lead-treated mice which was prevented by different treatment groups dose dependently and decreases the MDA content[28].

Lead exposure causes dysfunction of cholinergic innervations. Early lead exposure causes a significant reduction in high-affinity choline uptake in mouse forebrain synaptosomes and a depressed acetylcholine turnover in rat brain. These changes in the cholinergic ways are associated with the peroxydative damage caused on the neuronal membrane[23].

It was observed that administration of lead acetate significantly decreased the MAO activity in various cerebral areas. Lead administration modifies the aminergic system by reducing the activity of mitochondrial MAO and tyrosin hydroxylase. The effects during high lead exposure on MAO and catecholamines at the cerebral level are not a direct result of the intoxication by lead but a resultant of the inhibiting effect of the cholinergic system. The reduction in the activity of MAOs in the various cerebral areas, during the exposure to lead, can be due to the cellular damage and with the high affinity of lead to sulfhydryl group of these enzymes[23,28,36].

Sections of the brain sample were stained with eosin and haematoxylin to study the neurodegeneration. In the normal control group it was found to be intact and no neuronal loss was observed. Gross histopathology changes, including neurodegeneration and vacuolated cytoplasm was observed in lead control group. These changes were not prominent in the different treatment groups, and remarkable improvements were found dose dependently.

EA, phytoconstituent widely available in fruits and vegetable, showed potential medicinal properties majorly because of potential antioxidant property. In this present study also protective activity associated with EA may be due to potent antioxidant activity, apart from that polyphenols are having capacity to make chelated with heavy metals like lead which may cause significant removal of lead[10,37,38].

CONCLUSION

It can be concluded from the present study that EA exhibited dose-dependent protection against lead induced neurotoxicity through antioxidant mechanisms from the oxygen-derived free radicals. Findings of the present study can be important for those who are chronically exposed to high level lead. Ellagic acid in the dietary source or in the form of formulation can keep the nervous system healthy and safe. Future studies can be carried out to establish the fact clinically.

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SOURCE OF SUPPORT

Nil

CONFLICT OF INTEREST

The authors declare no conflicts of interest

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