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CARDIOPROTECTIVE EFFECT OF ELLAGIC ACID AGAINST LEAD INDUCED CARDIOTOXICITY

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ABSTRACT

The present study was undertaken to evaluate the cardioprotective effect of Ellagic acid (EA) against Lead (Pb) induced cardiotoxicity.

In this model, Albino rats of either sex were divided in to four groups of six animals. Cardiotoxicity were induced in these rats (180-200 g) by administering lead (100mg/L) (as lead acetate, $(Pb(CH_3COO)_2)$) through drinking water from the first day of experimental method. Ellagic acid will be administered orally in the respective groups along with lead daily for 8 weeks. The influence of the treatment was analysed by quantification of antioxidants. Superoxide Dismutase (SOD), Reduced Glutathione (GSH) and Catalase activities were significantly increased in heart tissue homogenate in all treated groups compared to Lead(Pb) group. Thus, investigational finding conclude that Ellagic acid possess potential benefits against cardiotoxicity induced by the most toxic heavy metal lead.

Key words: Cardiotoxicity, Ellagic acid, Lead, Antioxidants

INTRODUCTION

The heart is a hollow muscular organ which functions as pump in the cardiovascular system toprovide a continuous circulation of blood throughout the body via the circulatory system,supplying oxygen and nutrients to the tissues and removing carbon dioxide and other wastes.[1] Cardiovascular disease is a significant and ever-growing problem in the United Kingdom, commonly defined as coronary heart disease, cerebrovascular disease,peripheral arterial disease, rheumatic and congenital heart diseases and venousthromboembolism.[2]

Heavy metals are the metallic elements found naturally in the earth's crust. These elements have relatively high atomic weight and density than water. Heavy metals are considered as trace elements duo to their trace concentration in environment. Theses metals are very essential to maintain and control various chemical and physiological functions in all living organisms at low concentrations.[3] Lead (Pb) is the most important toxic heavy metal present naturally in the earth's crust and it is considered as one of the most harmful environmental pollutant that effect all biological systems through exposure to air, water and food sources.[5] Lead toxicity is particularly insidious hazard causing irreversible health effects. It interfere with a number of body functions and primarily effecting the central nervous, cardiovascular , hematopoietic, hepatic and renal system, resulting in serious disorders.[6] Lead can interfere with autonomic nervous control of the heart and decrease heart rate variability. Decreased heart rate variability is associated with increased coronary heart disease. [7]

The toxicity of heavy metal is mainly due to their cumulative deleterious effect that can cause chronic degenerative changes. Oxidative stress, impairment of antioxidants metabolism and enzymatic inhibition are the major mechanisms of heavy metal toxicity. Many of the heavymetals can cause DNA damage and lipid peroxidation by the generation of free radicals.[8] Long-term exposure to lead can produce lead toxicity in body. Lead toxicity is particularly insidious hazard causing irreversible health effects. It interferes with a number of body functions and produces adverse effects on central nervous, cardiovascular, hematopoietic, hepatic and renal system, and results in serious disorders.[9]

Cardiovascular effects[10]

Chronic and acute lead poisoning causes severe cardiac and vascular damage with hypertension and other cardiovascular disorders like ischemic coronary heart disease, cerebrovascular accidents and peripheral disease. Exposure to lead may cause alterations in force of contraction, ECG and hypertension in both humans and animals.

Lead Toxicokinetics

Inhalation and ingestion are the main two routes of exposure for Pb. Inhalation exposure is more efficient route of absorption than ingestion. Passive and facilitated diffusion mediates the absorption of Pb^{2+} from intestine. Passive diffusion plays a crucial role in total absorption oflead. In blood Pb^{2+} ions are seen by bounding to proteins. Once absorbed 99% of lead binds tored blood cells and 1% remains in serum. Pb^{2+} cross blood brain barrier (BBB) as free ion ($PbOH^{+}$). Absorbed lead is excreted from the body via urine, sweat, hair and nails.[11]

Mechanisms of lead toxicity

Oxidative stress[12]

Oxidative stress and ionic mechanism are the two major ways for lead metal toxicity. The exposure to lead from the polluted environment causes this heavy metal to attack on cells and initiate the production of free radicals and the generation of antioxidants to detoxify the reactive intermediates. The imbalance between production of ROS and antioxidants is the main reason forthe development of oxidative stress in living cells.

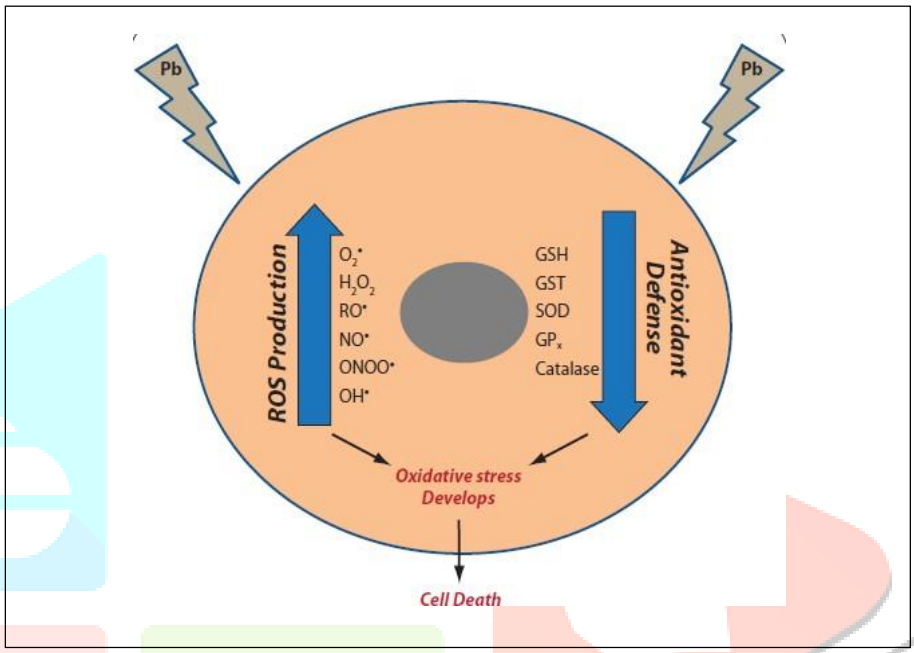

Figure 1: Mechanism underlying the development of oxidative stress in a cell on lead exposure

Glutathione is an antioxidant present in the cell to protect it from free radicals. Exposure to lead causes the increased level of ROS and decreased level of antioxidants. Glutathione exists mainly in reduced (GSH) and oxidized (GSSG) state. Under normal conditions the reduced form (GSH) consists 90% and oxidized form (GSSH) consists 10% of total glutathione. Oxidative stress results in an increased concentration of GSSG than GSH. Increased production of ROS causes structural damage to cells, nucleic acid, proteins and membranes.

Ionic Mechanism[13]

The lead metal ion possess an ability to replace other bivalent cations like Ca^{2+}, Mg^{2}, Fe^{2+} and monovalent cations like Na^{+}, which disturbs the biological metabolism of the cell. This ability of lead metal causes the ionic mechanism of lead toxicity. Lead inhibits the actions of calcium and can interact with many proteins. The ionic mechanism of lead toxicity can interfere with many biological processes like protein folding, ionic transportation, cell adhesion, apoptosis and release of neurotransmitters.

Lead toxicity is associated with many cardiovascular diseases like coronary heart disease, hypertension, left ventricular hypertrophy, stroke and heart rate variability. It causes alterations in cardiac rhythm also. Heart rate variability is the measurement of fluctuation of heart rate around the mean heart rate. Lead can interfere with autonomic nervous control of the heart and decrease heart rate variability. Decreased heart rate variability is associated with increased coronary heart disease. [14]

Lead is established as a major risk factor for hypertension. Long term exposure can cause an increase in the blood pressure.[15] Once absorbed most of the lead is bind to red blood cells. A low level of blood lead level can increase the blood pressure. Changes in the blood lead level concentration can results substantial changes in blood pressure.[16]

Antioxidants

Antioxidants are the molecules which are capable of slowing or preventing the oxidation reactions which can produce free radicals that damage cells. Fruits, vegetables, meats and fish are the main sources of antioxidants.[17]

Superoxide dismutase (SOD) is an enzyme which plays an important role in protecting cells from oxidative stress. It catalyzes the conversion of anion into oxygen and hydrogen peroxide.[18]

Catalase is found in peroxisomes in eukaryotic cells. It promotes the breakdown of H_2O_2 to water and molecular oxygen.[19]

Glutathione peroxidase is group of enzyme which plays a major role in protecting the organisms from oxidative damage. Like catalase, these enzymes also promoting the breakdownof hydrogen peroxide.[20]

Ellagic acid is a natural polyphenol compound associated with numerous health benefits. It is found mainly in some of the nuts, seeds and fruits especially in berries. Strawberries are considered as one of the main source of ellagic acid.[21]

Ellagic acid is a natural polyphenol compound obtained mainly from strawberry belonging to family rosaceae.[22] strawberry achenes and leaves are rich in ellagic acid. Ellagic acid rich foods and formulations are exerts beneficial health effects in human health. Ellagic acid is one of the secondary metabolite produced by the plants.[23]

Mechanism of action[24]

Ellagic acid is a natural phenolic compound having well anti-oxidant potential, free radical scavenging property and metal ion chelating capacity. Phenolic compounds quench ROS, chelate metal, and regenerate membrane-bound antioxidants. Ellagic acid quench ROS andprotecting the cells from damage due to oxidative stress and forming complex with metal ion andcan results in detoxification.

Pharmacological activities

Anti-inflammatory activity[25]

The enzymes like nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2) enzymes and cytokines possess a key role in many inflammatory conditions. Paw edema is induced in mouse through intraplantar injection of carrageenan led to development of peripheral inflammation, which resulted in a significant increase in the levels of tumor necrosis factor α (TNF-α) and interleukin 1 (IL-1) β, nitric oxide (NO) and prostaglandin E2 (PGE2) and also iNOS and COX-2 protein expression in the paw. The intraperitoneal administration of EA (1-30 mg/kg) could reduce the inflammation in the paw.

Anti-oxidant activity[26]

Anti-oxidant activity is defined as the ability to inhibit oxidative degeneration. Ellagic acid at a dose of (50 mg/kg) shows antioxidant activity and exhibit a protective effect against cyclosporin induced liver injury in male Wistar rats.

Anti-proliferative activity[27]

Ellagic acid inhibits the proliferation of cancer cells. EA at 1-100 micromol/L inhibited human umbilical vein endothelial cell (HUVEC) tube formation. EA shows strong anti-proliferative activity against the colon, breast, and prostatic cancer cell lines. The mechanism of apoptosis in ellagic acid-treated cancer cells is associated with decreased ATP production, which is essential for the viability of the cancer cells.

Anti-carcinogenic activity[28]

Ellagic acid consumed in the form of nuts and fruits can protect from carcinogenesis by blockingthe binding of carcinogen to DNA and acts as a natural anti-initiator. It can also inhibit the growth of tumor cells by inhibiting two enzymes (topo I and II), which are essential for DNA replication and cell proliferation.

Anti-genotoxicity[29]

Ellagic acid at a dose of 50 and 100 mg/kg body weight reported antigenotoxic activity against cyclophosphamide induced genotoxicity.

Anti-diabetic activity[30]

EA present in the *Emblica officinalis* extract shows anti-diabetic activity. Action of EA on β- cells of pancreas causes an increase in β-cell size and number, increasing antioxidant status, increase in serum insulin, and decrease in blood glucose.

MATERIALS AND METHODS

Albino rats of either sex weighing 180-200g were housed at 25° ± 5°C in a well-ventilatedanimal house under 12:12 h light dark cycle. All the rats were provided with commercially available standard pellet diet, water ad libitum. Institutional Animal Ethics Committee approved the experimental protocol. The animals were maintained under standard conditions in an animal house as per the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA). The Institutional ethical committee approved the experimental protocol (SDCP/IAEC-01/2017-18).

Dose Selection

Based on earlier literature review, dose of lead in rat was found to be 100 mg/L administered through drinking water[29] and dose of ellagic acid in rat was found to be 100 mg/kg and 200 mg/kg[30]. The same dose was selected for the present study.

Table 1: Apparatus and Chemicals used

SI.NO	APPARATUS	COMPANY
1	Autoanalyser	Robonik , Mumbai
2	Centrifuge	Remi, India
3	Analytical balance	Schimadzu, Japan

4	Colorimeter	Systronics, Baroda
5	Spectrophotometer	Schimadzu, Japan
6	Micropipettors	Unitron Bio-medicals, Bangalore

SI.NO	**CHEMICAL**	**COMPANY**
1	Ellagic acid	Yucca enterprises, Mumbai
2	Lead acetate	SD scientifics, mangalore
3	Calcium chloride	S D Fine Chemicals, Mumbai, India
4	CK-MB kits	kits Lab-Care diagnostic, Pvt Ltd, Mumbai
5	CK-NAC kits	kits Lab-Care diagnostic, Pvt Ltd, Mumbai
6	LDH kits	Accurex, India
7	ALT kits	Robonik India Pvt Ltd, Mumbai
8	AST kits	Robonik India Pvt Ltd, Mumbai
9	D Glucose	Loba Chemicals, Mumbai, India
10	EDTA	Nice Chemicals Pvt Ltd, Cochin, India
11	Ethanol	Hong Yang Chemical Corporation, China

12	Sucrose	S D Fine Chemicals, Mumbai, India
13	ALP kits	Robonik India Pvt Ltd, Mumbai
14	Ketamine	Neon Pharmaceutical Ltd, India
15	TC kits	Robonik India Pvt Ltd, Mumbai
16	Xylazine	Indian Immunological, Guntur, India
17	TG kits	Robonik India Pvt Ltd, Mumbai

Experimental Models[29,30,31]

The healthy adult Albino rats (180-200g) of either sex will be divided in to four groups of sixanimals each as following.
Group-I (Normal) - Animals treated with vehicle (Normal saline).

Group- II (Pb alone) - toxic control, received lead (as lead acetate, $(Pb(CH_3COO)_2)$) only(100 mg/L through drinking water).

Group- III (Pb + EA 100 mg/kg) - Animals treated with ellagic acid (100 mg/kg, p.o) alongwith lead for 8 weeks.
Group- VI (Pb + EA 200 mg/kg) - Animals treated with ellagic acid (200 mg/kg, p.o) alongwith lead for 8 weeks.

Group-I animals are treated with normal saline by oral route daily for 8 weeks. Group II animals treated with lead 100 mg/L through drinking water daily for 8 weeks. Group- III animals are treated with ellagic acid (100 mg/kg) along with lead by oral route daily for 8 weeks. Group- VI animals are treated with ellagic acid (200 mg/kg) along with lead by oral route for 8 weeks.

After 24 hour after the last administration the rat was anesthetized with ketamine and xylazine. The recordings of ECG waves were measured on the physiography. Heart rate, QRS interval, PR interval, QT segment and RR interval were measured. The blood was collected by retro-orbital puncture and the serum was separated by centrifugation. After that the rats were sacrificed by mild ether anesthesia. Three hearts from each group were homogenized with sucrose solution (0.25M) to prepare the heart tissue homogenate (HTH). Different biochemical analysis was performed in isolated serum and in HTH. Remaining three heart samples from each group wereembedded for histological examination.

The parameters estimated were:

- Antioxidant Superoxide dismutase (SOD), reduced glutathione (GSH) and catalase willbe determined in heart tissue homogenate.

Procedure For Estimation Of Antioxidants

Preparation of Heart Tissue Homogenate

The hearts removed after the experiment was made free of the adjacent vessels and fatty tissue mass with the help of scissors. Hearts were then cut open, rinsed with saline (0.9% NaCl) and dried using filter paper. The weight of the heart was then recorded. Thereafter the heart was homogenised in ice cold 0.25M sucrose solution using a mortar and pestle. The homogenate thusobtained was centrifuged at 5000 rpm for 15 min. The supernatant was decanted and used for theestimation of SOD and catalase.[32]

Estimation of SOD in heart tissue homogenate[33]

Principle:

SOD is a metalloproteinase and is the first enzyme involved in the antioxidant defence againstROS by lowering the steady state level oxygen. SOD scavenges the superoxide ions produced as cellular byproducts. SOD is a major defence for aerobic cells combating the toxic effect ofsuperoxide radicals.
Reagents:

a) Sodium Pyrophosphate buffer (0.052 M, pH 8.3): 2.31 g in 100 ml of distilled Water adjusted

b) 186 µM Phenazinemethasulphate (PMS): 5.7 mg in 100 ml of distilled water.

c) 300 µM nitro blue tetrazolium (NBT): 2.45 mg in 1 ml of distilled water.

d) 780 µM NADH: 5.53 mg in 1 ml distilled water.

e) Glacial acetic acid.

f) n-butanol

Estimation of superoxide dismutase (SOD)

To 0.4 ml of the homogenate (supernatant), 1.2 ml of sodium pyrophosphate buffer, 0.1 ml of Phenazinemethasulphate (PMS), 0.3 ml of nitro blue tetrazolium (NBT) were mixed and distilledwater was added to make up the volume up to 3 ml. The reaction was started by addition of 0.2 ml of NADH. It was incubated at 30°C for 60 sec. The reaction was stopped by addition of glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4 ml of n- butanol. The mixture was allowed to stand for 10 min, centrifuged and butanol layer will be taken out. Colour intensity of the purple colourchromogen was measured at 560 nm against butanol. Increase in absorbance (optical density) is directly proportional to concentration of SODand vice versa and % increase in SOD was calculated.
% Increase in SOD = (Abs of control-Abs of test / Abs of control) x100.

Estimation of Catalase in homogenate[100]

Principle:

Catalase is a hemeprotein, localized in the microperoxisomes. It reduces hydrogen peroxide produced by dismutation reaction and prevents generation of hydroxyl radicals thereby protecting the cellular constituents from oxidative damage in peroxisome. The enzyme catalyses the decomposition of H2O2 to water and oxygen and thus protecting the cell from oxidative damage by H_2O_2.

Reagents:

a) 0.2 M phosphate buffer (pH 8.0): 50 ml potassium di-hydrogen orthophosphate (0.2M) + 46.1ml NaOH (0.2M), volume made up to 200 ml and pH adjusted to 8.0
b) 2 M Hydrogen peroxide: 22.45 ml of H2O2, volume made up to 100 ml.

c) 5% potassium dichromate: 5 g in 100 ml of distilled water. d) Dichromate-acetic acid reagent-5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratios.

Estimation of catalase

The reaction mixture (1.5 ml, vol.) contained 1.0 ml of phosphate buffer, 0.1ml of tissuehomogenate (supernatant) and 0.4 ml of H2O2. The reaction was stopped by the addition of 2.0 ml of dichromate-acetic acid reagent and was incubated at 100°C for 2 min. Colour intensity of green colour was measured calorimetrically at 620 nm. Increase in Absorbance (optical density) is directly proportional to concentration of catalase and % increase in catalase was calculated.
% Increase in catalase = (Abs of control- Abs of test / Abs of control) x 100

Estimation of Reduced Glutathione (GSH)

Principle:

The general thiol reagent, 5, 5'-dithiobis [2-nitrobenzoic acid] (DTNB, Ellman's Reagent) reacts with GSH to form the 412nm chromophore, 5-thionitrobenzoic acid (TNB) and GS-TNB. The GS-TNB is subsequently reduced by glutathione reductase and β-nicotinamide adenine dinucleotide phosphate (NADPH), releasing a second TNB molecule and recycling the GSH; thus amplifying the response. Any oxidized GSH (GSSG) initially present in the reaction mixtureor formed from the mixed disulfide reaction of GSH with GS-TNB is rapidly reduced to GSH.

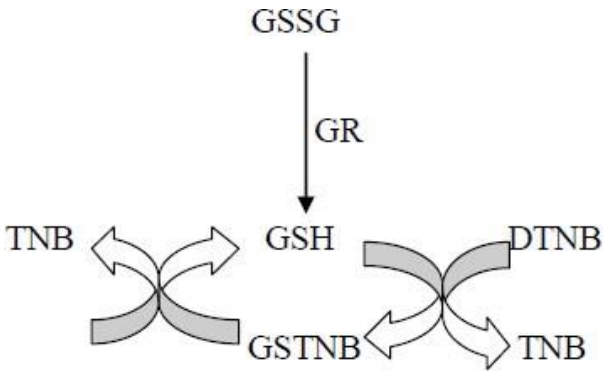

Figure 2: GSH recycling mechanism

Procedure:

Reduced glutathione (GSH) was measured in accordance with Ellman's method. 1ml of blood sample was withdrawn from each rat and treated with 1ml of 5% (w/v) TCA in 1 mM EDTA; then centrifuged at 2000 g for 10 mins. After that 1ml of the filtrate was mixed with 5ml of 0.1Mphosphate buffer, pH=8.0 and 0.4ml of 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) (0.01% in phosphate buffer; pH=8) was added to it. The absorbance of the solutions was measured at 412nm against blank prepared from 6.0ml of phosphate buffer and 0.4ml of DTNB.

The concentrations of reduced glutathione were determined from standard curve, which was constructed as follows. Different aliquots of standard reduced glutathione stock solution were taken in 10ml volumetric flasks. To each solutions 10ml of 0.4ml DTNB solution was added and volume was adjusted up to the mark with phosphate buffer; pH=8. The absorbance of each solution was measured at 412nm against a blank containing 9.6ml of phosphate buffer; pH=8 and0.4ml DTNB solution. By plotting the absorbance against concentration a straight line passing through the origin of grid was obtained. The best fit equation was A=0.000531M; where M=nanomoles of reduced glutathione, A=absorbance. R=0.0991, SEM=0.0059 and F=574.07 (dF=1, 10)

Statistical Analysis

Results are expressed as mean ± SE. Statistical significance was assessed using One-way Analysis of variance (ANOVA) followed by Tukey-Karmer multiple comparison tests. $P<0.05$ was considered significant.

RESULTS

Effect On Antioxidant Activity

Extremely significant ($P<0.001$) decrease was observed in SOD, and GSH level in Pb group compared to normal group. Treatment groups such as Pb+EA(100mg/kg) and Pb+ EA (200mg/kg) showed extremely significant ($P<0.001$) increase in SOD, and GSH level compared to Pb group.

Extremely significant ($P<0.001$) decrease was observed in catalase level in Pb group comparedto normal group. Pb+EA(100mg/kg) showed extremely significant ($P<0.001$) and Pb+ EA(200mg/kg) group showed moderately significant ($P<0.01$) increase in catalase compared to Pb group.

Table 2: Effect on heart tissue homogenate level of SOD, Catalase and GSH in Pb induced cardiotoxicity.

Treatment	Heart Tissue Homogenate (U/L)		
	SOD	Catalase	GSH
Normal	86.61±2.73	54.63±1.71	87.07±1.79
Pb alone	22.80±2.15***	21.29±1.51***	35.97±2.41***

Pb+EA(100mg/kg)	43.42±3.29$^{***###}$	31.99±0.90$^{***###}$	50.51±1.23$^{***###}$
Pb+EA(200mg/kg)	61.18±1.77$^{***###+++}$	39.74±1.57$^{***###++}$	65.52±2.02$^{***###+++}$

All the values are in Mean ± SEM, n=6, ***P<0.001 when compared to normal group, ###P<0.001 when comparedto Pb group and ++P<0.01, +++P<0.001 when compared to Pb+EA(200mg/kg) group. Pb – Lead; EA – Ellagic acid.

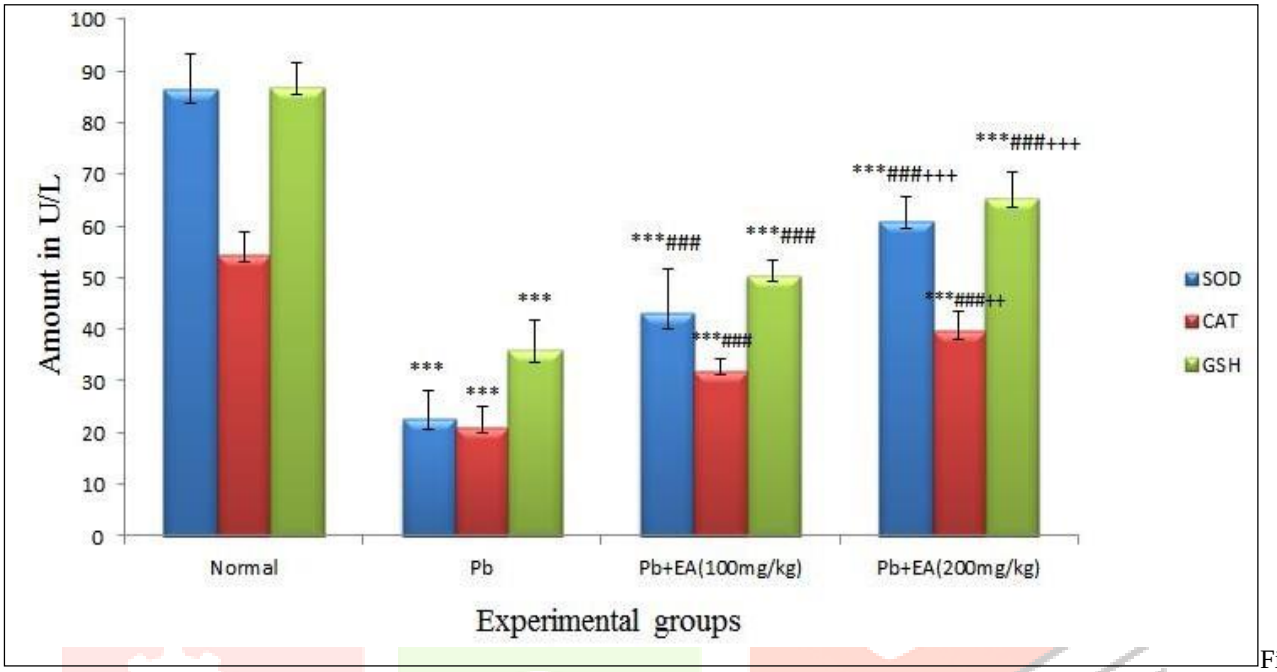

Figure 3:

Effect on SOD, Catalase and GSH level in Pb induced cardiotoxicity

All the values are in Mean ± SEM, n=6, ***P<0.001 when compared to normal group, ###P<0.001 when compared to Pb group and ++P<0.01, +++P<0.001 when compared to Pb+EA(200mg/kg) group. Pb – Lead; EA – Ellagic acid.

DISCUSSION

The current research was designed to evaluate the cardioprotective activity of Ellagic acid against lead (Pb) induced cardiotoxicity. The study revealed a highly significant protective actionof Ellagic acid against cardiac injury caused by the exposure to lead (Pb).

Heavy metals are the metallic elements found naturally in the earth's crust. These elements have relatively high atomic weight and density than water. Lead (Pb) is a naturally occurring bluish- gray toxic heavy metal found in all parts of the environment.[37] Long-term exposure to lead can produce lead toxicity in body. Lead toxicity is particularly insidious hazard causing irreversible health effects. It interferes with a number of body functions and produces adverse effects on central nervous, hematopoietic, hepatic and renal system, and results in serious disorders.[38]

Ellagic acid is a natural polyphenol compound obtained mainly from strawberry (*Fragaria* x *ananassa*) belonging to family rosaceae. Strawberry achenes and leaves are rich in EA. Ellagic acid rich foods and

formulations are exerts beneficial health effects in human health. It is polyphenol antioxidant compound possess free radical scavenging properties. The other biological activities of EA include anti-inflammatory, antidiabetic, anti-proliferative, neuroprotective, and prebiotic effects.[36]

Cardiotoxicity is caused due to generation of oxygen derived free radicals. They cause direct injury to cell membranes, which kills cardiac membranes. Oxidative stress and ionic mechanism are the two major ways for lead metal toxicity. The exposure to Pb from the polluted environment causes this heavy metal to attack on cells and initiate the production of free radicals and the generation of antioxidants to detoxify the reactive intermediates. The imbalance between

production of ROS and antioxidants is the main reason for the development of oxidative stress inliving cells.[37]

In present study, animals treated with only Pb showed significant decrease in Superoxide dismutase (SOD), catalase and Reduced Glutathione (GSH) values, which indicates the induction of cardiac toxicity. Treatment with EA low and high doses displayed increase in the levels of the anti-oxidant enzymes indicating protective activity of EA against Pb induced cardiotoxicity.

Cardiac damage causing a release of cardiac biomarkers to blood serum. The increase in the level of cardiac biomarkers in the blood used as a biomarker to identify the damage.[38]

CONCLUSION

The present investigation demonstrated the cardioprotective action of ellagic acid against cardiotoxicity induced by lead (Pb). Administration of lead causes cardiac damage detected by changes levels of antioxidant enzymes.Treatment with ellagic acid showed dose dependent cardioprotective activity against Pb induced cardiotoxicity. High dose of ellagic acid (200 mg/kg) showed maximum cardioprotection.

The efficacy of Ellagic acid could be attributed to its potential antioxidant property, free radical scavenging activity, and metal ion chelating capacity.

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