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Research Article



Protective role of Ellagic acid in modulating Iron induced Nephrotoxicity in rats

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Abstract

Iron (Fe) is an environmental and industrial pollutant that affects various organs in human and animals. A body of evidence implicates that the free radical generation with subsequent oxidative stress is involved in the mechanism of Fe toxicity. Since the kidney is critical target of Fe toxicity we carried out this study to investigate the effect of Ellagic acid (EA), a polyphenolic compound on renal injury induced by Fe. In this study, Fe (30mg/kg body weight) was administered intraperitoneally for 10 days to induce toxicity. EA was administered orally (15, 30 and 60mg/kg body weight) for 10 days along with Fe. Fe induced renal damage was indicated by the increased activities of serum urea and creatinine along with reduced renal clearance. A significant increase in lipid peroxidation markers (TBARS and lipid hydroperoxide) was observed in kidney of Fe treated rats. Rats subjected to Fe toxicity also showed a decline in the levels of reduced glutathione (GSH), vitamin C and vitamin E together with significantly decreased levels of enzymic antioxidants superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). Administration of EA at 60 mg/kg body weight attenuated the Fe induced nephrotoxicity indicated by significantly decreased levels of serum urea and creatinine with normalization of creatinine clearance. On administration of EA, the depleted renal antioxidant defense system (enzymatic and non enzymatic antioxidants) was significantly increased in Fe treated rats. The biochemical observations were supplemented by histopathological examination of kidney section. Our results suggest that supplementation of EA might be helpful to alleviate the Fe induced oxidative injury in kidney.

Keywords: Ellagic acid, Iron, Antioxidant, Lipid peroxidation.

Introduction

Iron is an essential constituent of the body, being found in functional form in haemoglobin, myoglobin, the cytochromes, enzyme with iron sulphur complexes and other iron dependent enzymes. At the same time, excess iron in the body is associated with toxic effects and poses health problems (Britton et al., 1987). The toxic effects of iron overloading leads to chronic nephrotoxicity. Excess free iron causes free radical mediated peroxidation of membrane (Jacobs, 1998). Iron catalyzed oxidative stress is believed to be the main culprit involved in the pathogenesis of iron

induced cancer. The toxicity of Fe as an industrial pollutant, a food contaminant (Dabeka et al., 1995). Kidney is considered to be one of the most susceptible organ of Fe induced toxicity because it plays a principle role in the toxicokinetic of Fe, since it serves as a major organ of Fe excretion and acts as a site of accumulation (Vyskocil et al., 1994). Fe induced nephrotoxicity was manifested by toxic nephropathy with proteinuria, aminoaciduria and reduced renal clearance (Kagan et al., 2001). Increasing evidence suggest that excess generation of free radicals may

have an implication in the toxicity of Fe compounds (Chen et al., 2002). Fe is known to produce renal injury by the generation of free radicals and they tend to produce deleterious effects by reacting with all classes of macromolecules and critical cellular targets leading to their oxidative modification and increased lipid peroxidation. Another possible mechanism that might be operative in Fe induced toxicity is the depletion of endogenous antioxidants thereby reducing the protection against the free radical attack (Chen et al., 2003).

Flavanoids, which are polyphenolic antioxidants, occur naturally in vegetables and fruits. They are widely recognized as a naturally occurring antioxidant that can inhibit lipid oxidation in biological membrane. Ellagic acid 2,3,7,8 tetrahydroxy - benzopyrano [5,4,3, -cde] benzopyran -5-10-dione] is a naturally occurring phenolic constituent in certain fruits and nuts, such as raspberries, strawberries, walnuts, longan seed, mango kernel, pomegranate, etc [Soong 2006]. Ellagic acid has a wide variety of biological activities including potent antioxidant [Priyadarsini et al., 2002], anti inflammatory [Lino et al., 2002], anti carcinogenic and antifibrosis in bacterial and mammalian systems [Therisamma and Kuttan, 1996]. It is believed that ellagic acid either by countering the negative effects of oxidative stress by directly acting as an antioxidant or by activating or inducing cellular antioxidant enzyme systems.

In the light of above information, the present study was carried out to investigate the protective influence of ellagic acid on Iron induced nephrotoxicity and oxidative stress in rats.

Materials and Methods

Animals

Male albino wistar rats, 10-14 weeks old weighing 180-230g were used for the study. The animals were obtained from Central Animal House, Rajah Muthiah Medical College (RMMC) Annamalai University, Annamalainagar, India. Rats were housed in plastic cages under standard conditions with free access to drinking water and pellet diet. They were maintained in accordance with the guidelines of the National Institute of Nutrition (Indian Council of Medical Research, Hyderabad, India) and the Animal Ethical Committee, Annamalai University, approved the study. (Vide. No. 523).

Drugs and chemicals

Ellagic acid and Iron sulfate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The rest of the chemicals utilized were obtained from local firm (India) and were of analytical grade.

Experimental Design

The animals were randomly divided into six groups of six rats in each group.

Group 1: Control rats orally treated with dimethyl sulphoxide (0.2%) for 10 days.

Group 2: Control rats received EA (60 mg/kg body weight) dissolved in dimethyl sulphoxide (0.2%) for 10 days using intra gastric intubation.

Group 3: Rats received iron as iron sulfate (30 mg/kg body weight) intraperitoneally in isotonic saline for 10 days.

Group 4: Rats received Fe (30 mg/kg body weight) followed by oral administration of EA (15 mg/kg body weight) in dimethyl sulphoxide (0.2%) for 10 days.

Group 5: Rats received Fe (30 mg/kg body weight) followed by oral administration of EA (30 mg/kg body weight) in dimethyl sulphoxide (0.2%) for 10 days.

Group 6: Rats received Fe (30 mg/kg body weight) followed by oral administration of EA (60 mg/kg body weight) in dimethyl sulphoxide (0.2%) for 10 days.

At the end of the experimental period, animals in different groups were sacrificed by decapitation. Blood was collected and centrifuged for serum separation. The kidney was dissected out, weighed and washed using chilled saline solution. Tissue was minced and Homogenised (10% w/v) in appropriate buffer (pH 7.4), and centrifuged (3000 g for 10 Minutes). The resulting supernatant was used for enzyme assays.

Biochemical determination

Estimation of urea, creatinine and creatinine clearance

The level of urea and creatinine in serum samples were estimated spectrophotometrically according to the standard procedures using commercially available diagnostic kits (Sigma Diagnostics (I) Pvt Ltd, Baroda, India). Creatinine clearance was calculated from the

values of serum and urinary creatinine,time (last 24 h), and body weight .

Estimation of hemoglobin

The level of hemoglobin in blood was estimated by using cyanmethaemoglobin method described by Drabkin and Austin (1932).

Determination of lipid peroxidation

Lipid peroxidation in kidney was estimated colorimetrically by measuring thiobarbituric acid reactive substances (TBARS) and hydroperoxides by the method of Niehius and Samuelsson (1968) and Jiang et al., (1992), respectively. In brief, kidney homogenate (0.1 ml) was treated with 2 ml of TBA–trichloroacetic acid (TCA)–HCl reagent (0.37% TBA, 0.25 N HCl and 15% TCA, 1: 1:1 ratio) placed in a water bath for 15 min and cooled and centrifuged at room temperature; clear supernatant was measured at 535 nm against a reagent blank.

A 0.1ml kidney homogenate was treated with 0.9 ml of Fox reagent (88 mg of butylated hydroxytoluene, 7.6 mg of xylenol orange and 0.8 mg of ammonium iron sulphate were added to 90 ml methanol and 10 ml of 250 mM sulphuric acid) and incubated at 37 °C for 30 min. The colour that developed was read at 560 nm.

Determination of non enzymic antioxidants

Vitamin C concentration was measured by Omaye et al., (1979) method. To 0.5 ml of tissue homogenate, 1.5 ml of 6% TCA was added and centrifuged (3500 g, 20 min). To 0.5 ml of supernatant, 0.5 ml of DNPH reagent (2% DNPH and 4% thiourea in 9N sulphuric acid) was added and incubated for 3 hours at room temperature. After incubation, 2.5 ml of 85% sulphuric acid was added and colour developed was read at 530 nm after 30 min.

vitamin E was estimated by the method of Desai (1984). Vitamin E was extracted from tissue by addition of 1.6 ml ethanol and 2.0 ml petroleum ether to 0.5 ml plasma and centrifuged. The supernatant was separated and evaporated. To the residue, 0.2 ml of 0.2% 2, 2' - dipyridyl, 0.2 ml of 0.5% ferric chloride was added and kept in dark for 5 min. An intense red

colored layer obtained on addition of 4 ml butanol was read at 520 nm.

Reduced glutathione (GSH) was determined by the method of Ellman. 1.0 ml of tissue homogenate was treated with 0.5 ml of Ellman's reagent (19.8 mg of 5,5'-dithiobisnitro benzoic acid (DTNB) in 100 ml of 0.1% sodium nitrate) and 3.0 ml of phosphate buffer (0.2M, pH 8.0). The absorbance was read at 412 nm.

Assay of enzymic antioxidants

Superoxide dismutase (SOD) activity was determined by the modified method of NADH – Phenazinemetosulphate – nitroblue tetrazolium formazon inhibition reaction, which was measured spectrophotometrically at 560 nm (Kakkar et al., 1984). Catalase (CAT) was assayed colorimetrically as described by Sinha (1972) using dichromate-acetic acid reagent . GPx was assayed by the method of Rotruck et al. (1973) based on the reaction between glutathione remaining after the action of GPx and 5,5 -dithio bis-(2-nitro benzoic acid) to form a complex that absorbs maximally at 412 nm. The glutathione-S-transferase (GST) activity was determined spectrophotometrically by the method of Habig et al. (1974) in which 1-chloro-2, 4-dinitrobenzene used as substrate. The protein content in kidney homogenate was determined by the method of Lowry et al. using Folin's-phenol reagent and also Bovine Serum Albumin (BSA) as a standard.

Histological investigation

The kidney samples fixed for 48h in 10% formal-saline were dehydrated by passing successfully in different mixture of ethyl alcohol – water, cleaned in xylene and embedded in paraffin. Sections of liver (4-5 mm thick) were prepared and then stained with hematoxylin and eosin dye, which mounted in neutral DPX medium for microscopic observations.

Statistical analysis

All the data were expressed as mean \pm SD of number of experiments (n=6). The statistical significance was evaluated by one-way analysis of variance (ANOVA) using SPSS version 7.5 (SPSS, Cary, NC, and USA) and Duncans' Multiple Range Test (DMRT) obtained the individual comparisons. Values were considered statistically significant when $P < 0.05$.

Results**Kidney functional markers**

The changes in the level of serum urea, creatinine and creatinine clearance in control and experimental animals are shown in Table I. A significant increase in the levels of serum urea and creatinine and decreased

creatinine clearance were observed in Fe treated rats. Treatment with EA at 60 mg/kg significantly decreased the levels of serum urea and creatinine along with restoration of creatinine clearance in Fe treated rats when compared to other two doses (15, 30 mg/kg) of EA. Based on these finding 60 mg/kg of EA was fixed as a dose for further biochemical studies.

Table 1. Changes in the levels of kidney functional markers in control and experimental rats

Groups	Control	Normal +EA (60 mg/kg)	Normal + Fe (30 mg/kg)	Fe (30 mg/kg) +EA (15 mg/kg)	Fe (30 mg/kg) +EA (30 mg/kg)	Fe (30 mg/kg) +EA (60 mg/kg)
Urea (mg/dl)	38.78 ± 2.51 ^a	37.67 ± 2.44 ^a	65.41±5.30 ^b	59.73 ± 4.73 ^c	53.16± 4.86 ^d	45.49 ± 3.94 ^c
Creatinine (mg/dl)	0.99 ± 0.07 ^a	0.98 ± 0.07 ^a	1.48±0.08 ^b	1.38 ± 0.08 ^c	1.27 ± 0.07 ^d	1.15 ± 0.07 ^e
Creatinineclearance (mg/min)	0.31 ± 0.05 ^a	0.31 ± 0.05 ^a	0.10 ± 0.03 ^b	0.16 ± 0.03 ^c	0.22 ± 0.04 ^d	0.25 ± 0.04 ^d
Hemoglobin (g/dl blood)	14.63 ± 1.03 ^a	14.54 ± 1.14 ^a	8.63 ± 0.58 ^b	9.83 ± 0.73 ^b	11.23 ± 0.91 ^c	13.39 ± 1.09 ^a

Fe –Iron; EA –Ellagic acid. Values are given as mean ± S.D from 6 rats in each group. Values not sharing common superscripts letters (a-d) differ significantly at p<0.05 (DMRT).

Lipid peroxidation and antioxidants

The levels of lipid peroxidation and nonenzymic antioxidant in kidney of control and experimental rats are illustrated in Table 2. The levels of TBARS and hydroperoxides were significantly (p < 0.05) increased

while the levels of nonenzymic antioxidants (GSH, vitamin C and vitamin E) were significantly decreased (p < 0.05) in kidney of Fe treated rats. Administration of EA significantly (p < 0.05) decreased the lipid peroxidation with significant increase in the levels of nonenzymic antioxidants.

Table 2. Changes in the levels of lipid peroxidation and nonenzymatic antioxidant status in kidney of experimental rats

Parameters	Normal	Normal + EA (60mg/kg)	Normal + Fe (30mg/kg)	Fe (30mg/kg) + EA (60mg/kg)
TBARS (mM /100 g tissue)	18.83±1.03 ^a	18.01 ± 0.84 ^a	30.35 ± 2.18 ^b	20.91 ± 1.20 ^c
Hydroperoxides (mM / 100g tissue)	0.69 ± 0.05 ^a	0.67 ± 0.05 ^a	0.99 ± 0.07 ^b	0.79 ± 0.06 ^c
Vitamin C (mole / mg tissue)	0.97 ± 0.09 ^a	1.03 ± 0.09 ^a	0.62 ± 0.06 ^b	0.84 ± 0.07 ^c
Vitamin E (mole / mg tissue)	0.71 ± 0.04 ^a	0.75 ± 0.04 ^a	0.48 ± 0.01 ^b	0.57 ± 0.03 ^c
GSH (mg/ g tissue)	3.26 ± 0.19 ^a	3.38 ± 0.19 ^a	2.11 ± 0.18 ^b	2.68 ± 0.22 ^c

Fe –Iron; EA –Ellagic acid. Values are given as mean ± S.D from 6 rats in each group. Values not sharing common superscripts letters (a-c) differ significantly at p<0.05 (DMRT).

Table 3 represents the status of enzymic antioxidants in kidney of control and experimental animals. Exposure to Fe significantly ($p < 0.05$) decreased the

activities of SOD, CAT, GPx and GST in kidney. Administration of EA to Fe treated rats restored the activities enzymic antioxidants to that of the controls.

Table 3. Changes in the activities of kidney antioxidant enzymes in control and experimental rats

Parameters	Normal	Normal + EA (60mg/kg)	Normal + Fe (30mg/kg)	Fe (30mg/kg) + EA (60mg/kg)
SOD (units [#] / mg protein)	13.91±0.85 ^a	14.41 ± 0.97 ^a	9.09 ± 0.51 ^b	11.34 ± 0.67 ^c
CAT (units [#] / mg protein)	50.25±3.66 ^a	53.04 ± 3.86 ^a	33.05 ±1.74 ^b	42.27 ± 2.89 ^c
GPX (units [#] / mg protein)	7.10±0.31 ^a	7.16± 0.39 ^a	4.92 ± 0.21 ^b	6.68 ± 0.27 ^c
GST (units [#] / mg protein)	6.41±0.33 ^a	6.53 ± 0.45 ^a	4.12 ± 0.16 ^b	5.70 ± 0.28 ^c

Fe –Iron; EA –Ellagic acid. Values are given as mean ± S.D from 6 rats in each group. Values not sharing common superscripts letters (a-c) differ significantly at $p < 0.05$ (DMRT).

[#] Units of enzyme activities are expressed as:

SOD - One unit of activity was taken as the enzyme reaction, which gave 50% inhibition of NBT reduction in one minute.

CAT - μ moles of hydrogen peroxide consumed / minute.

GPx - μ g of glutathione consumed / minute.

GST- μ moles of CDNB-GSH conjugate formation / minute.

Histological investigation

The kidney of control (Figure 1) and EA alone (Figure 2) treated rats showed a normal structure of kidney. Fe exposure caused changes in kidney architecture characterized by focal areas of hemorrhages and necrosis (Figure 3). Fe along with EA significantly reduced the hemorrhagic conditions (Figure 4).

Discussion

Renal injury following Fe exposure is well characterized and Fe is known to produce renal injury by binding to low molecular weight metallothionein like proteins leading to the generation of free radicals (Dhir et al., 1991). In the present study, an increased level of serum urea and creatinine along with decreased creatinine clearance in Fe treated rats reflects the renal damage. The measurements of creatinine and creatinine clearance have often been extensively used as a tool in the diagnosis of chronic renal failure (Sesso et al., 1996). The significant increase in the levels of renal functional markers upon

Fe exposure may result from the cellular damage due to excess free radical production.

Administration of EA attenuated Fe induced nephrotoxicity, which is indicated by significantly decreased levels of serum urea and creatinine with normalization of creatinine clearance. This can be attributed to the free radical quenching ability of EA suggesting that it may be quite effective in improving renal function under Fe toxic conditions.

Fe exposure has been reported to produce hematological effects by increasing the peroxidation reactions in erythrocytes mediated by ROS (Chen et al., 1999). The oxidative threat induced by Fe may initially alter the membrane skeleton of erythrocyte, followed by its deformation thus promoting hemolysis and resulting in decreased levels of Hb and anemia (Zeromski et al., 1995). Treatment with EA minimizes the toxic effects of Fe on Hb through its antioxidant mechanism, which was evident from increased concentration of Hb in blood in EA treated animals. In this context, the antioxidant effect of EA on erythrocytes and isolated rat hepatocytes has already been described (Anon et al., 1992).

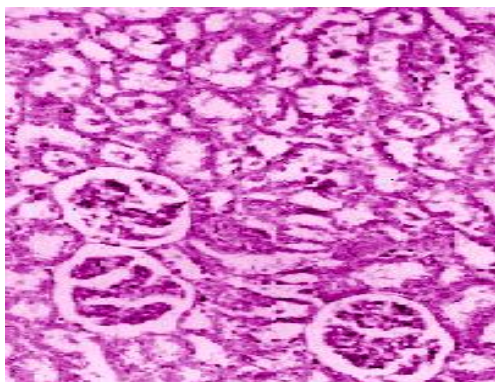


Figure 1. Control rat kidney H&E x 20. Normal architecture of kidney

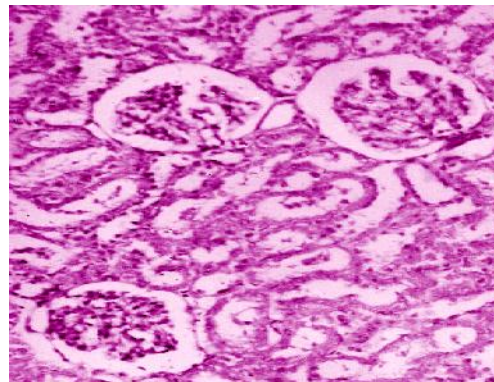


Figure 2. Normal + Ellagic acid (60mg)-treated rat kidney H&E x 20. Normal glomeruli with tubules

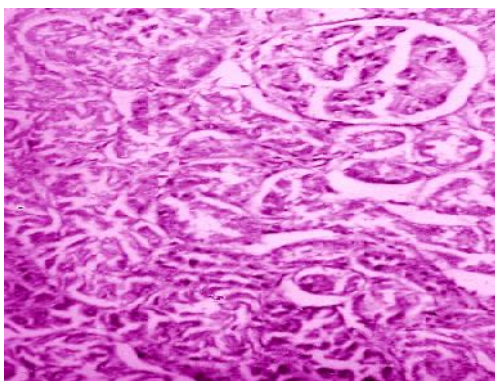


Figure 3. Normal + Fe (30mg) treated rats kidney H&E x 20. hemorrhages and necrosis

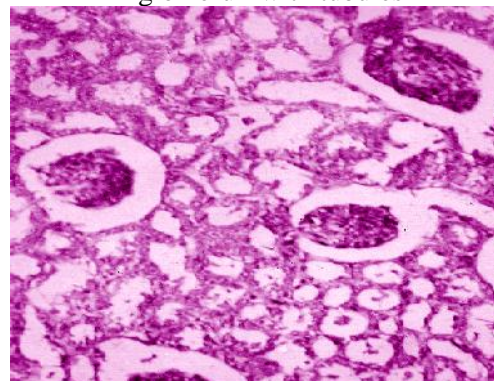


Figure 4. Fe + Ellagic acid treated rat kidney H&E x 20. Near normal.

LPO is the oxidative degradation of PUFA and it is catastrophic for living system because its occurrence in biological membrane causes impaired membrane function, impaired structural integrity, decreased fluidity and inactivation of membrane bound enzymes (Valko et al., 2005). LPO is an earlier intracellular event following Fe exposure and regarded as an important mechanism by which Fe exerts its toxic effect on kidney (Sunderman et al., 1985). Oxidative injury induced by Fe can be detected by measuring the lipid peroxidation markers such as TBARS and lipid hydroperoxides. In our study, we observed increased levels of LPO markers in Fe treated rats that might be due to excess free radical generation by Fe which in turn has the ability to react rapidly with membrane lipids leading to enhanced LPO.

EA decreased the levels of LPO in kidney of Fe treated rats. The protective mechanism exerted by EA might be due to its ability to directly interact with

ROS thereby decreasing free radical mediated LPO. It is well established that EA effectively scavenges free radicals because of their hydroxyl groups, possessing electron donating properties, which thereby prevent the membrane from free radical attack. Further, the metal chelating ability of EA may also contribute significantly to its beneficial role by reducing Fe accumulation in kidney, a major causative factor for renal injury. Another possible mechanism by which EA attenuates LPO could be due to its potentiality to act as a chain breaking antioxidant thus terminating the chain reaction of LPO (Nardini et al., 1998).

In order to preserve the biological system from detrimental oxidative processes caused by free radicals both enzymic and non-enzymic antioxidant defense mechanisms are operative in the cell. Depletion of cellular antioxidants contributes significantly to the development of Fe induced nephrotoxicity. GSH is a

major antioxidant that offers protection against the free radical attacks. It is a sulfhydryl peptide involved in the protection of normal cell structure and function by maintaining redox homeostasis, quenching of free radicals, participation in detoxification reactions and regeneration of other antioxidants (Bray and Tabor, 1993). Determination of changes in the concentration of GSH provides an alternative method of monitoring oxidative damage within the cell. Depletion of GSH in Fe toxic conditions may play a major role in the overall toxic manifestations of Fe (Ahmed et al., 1999). It is consistent with our findings, which showed decreased levels of GSH. It has been suggested that Fe may bind exclusively to the sulfhydryl group of GSH with high affinity leading to its inactivation resulting in impaired cellular defense against ROS and finally peroxidative damage (Cartana et al., 1992).

Vitamin C and vitamin E are the other major non-enzymic antioxidants having synergetic action in scavenging oxygen derived free radicals, and these vitamins are likely to be most susceptible to free radical oxidation (Buettner, 1993). Vitamin E is a major chain breaking antioxidant, which inhibits LPO. Vitamin C is the most potent water-soluble antioxidant that scavenges a wide variety of free radicals. In our study, we observed decreased levels of Vitamin C and E in kidney of Fe treated rats and it correlates with previous reports that show decreased levels of these antioxidants upon Fe administration (Salinkow et al., 2004; Chen et al., 2002). The observed decrease in the levels of antioxidants might be due to their increased utilization for scavenging excess free radicals induced by Fe.

Administration of EA restored the levels of GSH, vitamin C and E. It might be due to excellent antioxidant activity of EA with mechanism involving both free radical scavenging and metal chelation. The above effect of EA minimizes the usage of endogenous antioxidants in scavenging free radicals thus restoring their near normal levels. Further, EA has been shown to act synergistically with vitamin E by delaying its consumption and by recycling vit E from tocopheroxy radical (Nardini et al., 1997).

A remarkable depletion of enzymic antioxidants such as SOD, CAT, GPx and GST which plays a crucial role in protection against free radicals have also been observed in Fe toxicity. The decreased levels of these antioxidants upon Fe administration might be due to direct binding of the metal to the active site of the

enzyme or as a consequence of increased free radical production thereby leading to irreversible inhibition in their activities. This may be the valuable reason for the increased lipid peroxidation indices in our study.

EA efficiently modulates the antioxidant status in tissues, suggesting the enhancing effect of EA on cellular antioxidant defenses. Further, EA can double the antioxidant capacity of plasma even in their micromolar concentration. Thus, the antioxidant effect of EA may involve dual actions: direct action on free radical scavenging and indirect action through the induction of antioxidant enzymes.

Histological investigation of present study suggest that the focal areas of hemorrhage in Fe treated rats reflects the accumulation of free radical as well as increased oxidative stress, the basis for cellular damage. Administration of EA reduced the histological alteration caused by Fe, which may be due to antioxidant property of EA and therefore be important in protecting cell against toxicity.

On the basis of above results, we can infer that the antioxidant activity of EA with mechanism involving free radical scavenging and metal chelation might be responsible for the protection against Fe induced peroxidative damage. Hence EA is suggested to be helpful in alleviating the Fe induced oxidative stress in kidney.

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