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Research Article

# Comparative study of assay of free radical damage in *in vivo*, *in vitro* and *ex vivo* conditions

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## ABSTRACT

**Background:** Interception of free radical events and damage in clinical situation remain arbitrary with poor understanding on antioxidant pharmacology.
**Methods:** Experiments on *in vitro* per-oxidation insult as well as assumed free radical damage situation in ischemia reperfusion are examined for profile of malondialdehyde rise and effect of *Gikgobiloba* treatment by different schedule.
**Results:** Effect of *Ginkgobiloba* was seen on ischemia reperfusion induced, hydrogen peroxide induced and ferric chloride induced methods. Antioxidant effect of *Ginkgobiloba* was consistent in all techniques.
**Conclusions:** Results reveal the significance of complementary experiment to elaborate issue of antioxidants.

**Keywords:** Antioxidant, *Ginkgobiloba*, Malondialdehyde

## INTRODUCTION

Reactive oxygen species (ROS) are involved in many cellular metabolic and signaling processes. Molecular oxygen can be reduced by one, two or four electron transfer to produce superoxide anion ($O_2^-$), peroxide anion ($HO_2^-$) and finally to hydroxyl ion ($HO^-$) respectively in biological system. Suboptimal antioxidant defense system leads to oxidative stress. The main component of oxidative stress are singlet oxygen ($_1O^2$), superoxide ($O_2^-$), hydroperoxyl ($O_2H$), Hydrogen peroxide ($H_2O_2$), hydroxyl radical (OH), hypochlorous acid (HOCL), and trioxocarbonate radical ($CO_3^-$).[1]

Critical sites of ROS attack are the membranes of intracellular organelles, e.g., the phospholipid-rich lysosomal membranes. Lipid peroxidation involves the process of oxidative destruction of lipids, localized mainly in the cell membranes. Lipid peroxidation, well correlated with oxidative stress intensity, is a chain reaction, in which polyunsaturated fatty acids are degraded to small, more reactive particles such as conjugated dienes, lipid hydroperoxides, and thiobarbituric

acid-reactive substances.[2] Therefore, their detoxication and elimination are necessary for normal physiologic cellular activity and survival. Living organisms have developed complex antioxidant systems to counteract ROS and to reduce organ damage. These antioxidant systems include enzymes such as superoxide dismutase, catalase, and glutathione peroxidase; macromolecules such as albumin, ceruloplasmin, and ferritin; and a variety of small molecules, including ascorbic acid, $\alpha$-tocopherol, $\beta$-carotene, ubiquinol-10, reduced glutathione, methionine, uric acid, and bilirubin.[3]

Free radical damage is significantly implicated in varieties of catastrophes as well as insidious pathologies.[4] In contrast, consensus on due management of free radical risk or damage remains to build. Perhaps the understanding of free radical pathogenesis in most instances is far from being comprehensive. Kinetic and dynamic interactions of antioxidant therapeutic agent with events involved in free radical damage need better investigation. In this context, free radical insult induced by different known methods and its mitigation by the agent may be worthwhile to compare. Present reports bears observation of some *in vitro* and *in vivo* maneuvers inducing oxidant insult and effect of *Ginkgobiloba*.

*Ginkgobiloba* comes from the family of one of the oldest living plant species dating back more than 200 million years. They were the first plant to regrow after the nuclear bomb detonated in Hiroshima and were free of sign of genetic mutation. The effect of *Ginkgobiloba* may be caused by single active ingredient or by the combined action of many active agents found in the extract. The most important substances are flavonoids (flavones glycoside) and terpenoids (ginkgolides and bilobalide). The most important flavonoids are glycosides of camferol, quercetin and isorhamnetin with glucose rhamnose. Perfusion with the *Ginkagobiloba* had a beneficial effect on ischemic/reperfused rat and Guinea pig heart *in vitro* and on ischemic rat's heart *in vivo*.[5]

Ischemia is a state of tissue oxygen deprivation accompanied by a reduced washout of the resulting metabolites.[6] Reperfusion is the restoration of blood flow to the ischemic tissue. Despite the unequivocal benefit of reperfusion of blood to an ischemic tissue, reperfusion itself can elicit a cascade of adverse reactions that paradoxically injure tissue.[7] Indeed, reperfusion injury has been well described in the literature to cause organ damage in the brain, heart, lungs, liver, kidneys, and skeletal muscle. The susceptibility of tissue to ischemia reperfusion injury (IRI) is a major obstacle to both reperfusions after an infarct and successful organ transplantation.

## METHODS

Inbred Charles Foster strain albino rat weighing 200-250 g of either sex, obtained from the central animal house of Institute of Medical Sciences, Banaras Hindu University, Varanasi were kept in departmental animal house at 26°C±2°C, 44-56% relative humidity and 10:14 hrs L: D cycle for 1 week before and during the experiments. Animals were provided with standard rodent pellet diet and water was given *ad libitum*. Principles of laboratory animal care (NIH publication no. 82-23 revised 1985) guidelines were followed. Approval from the Institutional Animal Ethical Committee was taken prior to the experimental work.

### In vivo *IRI*[8]

Albino rat of both sexes weighing between 200 and 250 g were used in experimental groups composed of six animals in each. Animals were housed at standard provision for 5 days before surgical intervention. Animal fasted overnight were anesthetized with pentobarbitone (40 mg/kg, i.p.). Abdomen was opened through bilateral flank incision, and left renal artery was occluded with non-traumatic vascular clamp. The renal artery occlusion was continued for 15 min, 30 min, 45 min, and 60 min in different groups after the period of ischemia, reperfusion was allowed for 5 min and insulted kidney was removed. The rat was killed by a lethal dose of anesthetic agent.

### In vitro *hydrogen peroxide* ($H_2O_2$) *induced peroxidation in kidney homogenate of rat*

Rats were killed by decapitation and kidney harvested and homogenated in v/v of normal saline. In the first set, 3 ml of buffer was added to 1 ml of kidney homogenate and MDA was estimated. In other four sets, 2 ml of buffer was added to 1 ml of kidney homogenate. Hydrogen peroxide was then added to yield different concentration (85 mM, 170 mM, 340 mM, and 680 mM) of 1 ml of $H_2O_2$, then MDA was estimated. The reaction mixture was kept for an interval of (5 min, 10 min, 15 min, 30 min, 40 min, 50 min, and 60 min.) and MDA was estimated.

### In vitro *ferric chloride* ($FeCl_3$) *induced lipid peroxidation in kidney homogenate of rat*

Rats kidney were prepared as above. In the first set, 3 ml of buffer was added to 1 ml of kidney homogenate and MDA was estimated. In other five groups, 2 ml of buffer was added to 1 ml of kidney homogenate and different concentration (0.05 mM, 0.25 mM, 0.5 mM, 1 mM, and 2.5 mM) of 1 ml of ferric chloride was added, then MDA was then estimated. The reaction mixture was kept for an interval of (5 min, 10 min, 15 min, 30 min, 40 min, 50 min, and 60 min.) and MDA was estimated.

### *Assay of tissue malondialdehyde*[9]

The reaction mixture contained 0.4 ml of tissue homogenate, 0.2 ml of sodium dodecyl sulfate, 1.5 ml of 20% acetic acid and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid. The mixture was finally made to 4 ml with distilled water and heated at 95°C for 60 min. After cooling with tap water, 1 ml of distilled water and 5 ml of mixture of n-butanol and pyridine were added, and the mixture was shaken

vigorously after centrifugation at 4000 rpm for 10 min., the absorbance of the organic layer was measured at 532 nM with Spectrophotometer. Malondialdehyde (in nM) estimation was done from the standard curve prepared with 1,1,3,3-tetra methoxy propane.

*Ginkgobiloba* treatment: *Ginkgobiloba* extract (Ginkoba-Microlab) (2 mg/100 g) was administered orally in aqueous 1% gum acacia suspension. Pretreatment schedule followed as under:

Single dose at 1 hr before renal artery ligation, 3 hrs before renal artery ligation, and day before renal artery ligation. Multiple dose twice daily dose on preceding 2 days and thrice daily dose on preceding 2 days.

### *Statistical analysis*

Statistical analysis of data was performed using ANOVA test.

**Table 1: Renal tissue MDA indices by varying period of ischemia.**

| Time period | Tissue (MDA in nM) |
|---|---|
| Normal kidney, 0 min | 2.3±0.28 |
| RAO, 15 min | 2.5±0.10 |
| RAO, 30 min | 2.4±0.10 |
| RAO, 45 min | 5.0±0.40* |
| RAO, 60 min | 6.4±0.52* |

*p<0.01

## RESULTS

The technique of renal ischemia reperfusion yielded prominent lipid peroxidation at 45 min and more of occlusion. Hydrogen peroxide even in smallest standard concentration of 85 mM enhanced peroxidation quickly in 5 min assay. As is evident from Tables 1-6, more lipid peroxidation occurred by 40 min and was hydrogen peroxide concentration dependent. Ferric chloride peroxidation had similar trend with most peroxidation occurring by 45 min. *Ginkgobiloba* pretreatment whether 1 hr before or insidious reduced hydrogen peroxide induced peroxidation (Figure 1). Similar inhibition was also seen with ferric chloride induced lipid peroxidation. *Ginkgobiloba* treatment reduced IRI, but inhibitory effects at 3 hrs pretreatment were more prominent than 1 hr pretreatment. MDA concentration was evaluated in hydrogen peroxide (85 mM) induced lipid peroxidation in rat kidney homogenate. The concentration of MDA was found to increase with the time and maximum injury occurred at 40-60 min as evident from Table 2 & Figure 2. Similar trends were found

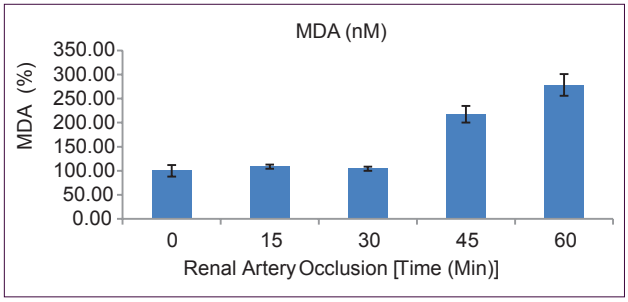


**Figure 1: Renal tissue MDA indices (percentage) by varying period of ischemia.**

**Table 2: Effect of different concentration of $H_2O_2$ for induction of lipid peroxidation in rat kidney homogenate at different time interval.**

| $H_2O_2$ (mM) | MDA (in nM) time (in min) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 5 min | 10 min | 15 min | 30 min | 40 min | 50 min | 60 min |
| 0.00 | 2.58±0.2 | 2.50±0.01 | 2.60±0.07 | 2.61±0.1 | 2.60±0.05 | 2.58±0.07 | 2.54±0.01 |
| 85 | 6.18±0.1 | 9.1±0.09* | 12.5±0.07* | 12.5±0.1* | 19.1±0.07* | 19.2±0.03* | 19.4±0.04* |
| 170 | 7.78±0.02* | 11.3±0.07* | 15.1±0.03* | 9.3±0.1* | 20.51±0.09* | 25.3±0.01* | 26.1±0.07* |
| 340 | 12.5±0.03* | 18.1±0.03* | 25.1±0.01* | 28.1±0.09* | 28.7±0.07* | 28.7±0.09* | 28.9±0.1* |
| 680 | 15.3±0.1* | 24.1±0.01* | 31.1±0.03* | 33.3±0.07* | 40.1±0.03* | 41.7±0.03* | 41.3±0.1* |

*p<0.01

**Table 3: Effect of different concentration of $FeCl_3$ for induction of lipid peroxidation in rat kidney homogenate.**

| $FeCl_3$ (mM) | MDA (in nM) time (in min) | | | | |
|---|---|---|---|---|---|
| | 5 | 15 | 30 | 45 | 60 |
| 0.00 | 2.58±0.01 | 2.50±0.07 | 2.54±0.03 | 2.60±0.03 | 2.54±0.1 |
| 0.05 | 3.87±0.03* | 4.8±0.03* | 5.7±0.07* | 7.5±0.08* | 8.1±0.07* |
| 0.25 | 5.1±0.08* | 7.5±0.03* | 7.9±0.1* | 14.1±0.03* | 8.1±0.07* |
| 0.50 | 7.4±0.07* | 7.8±0.07* | 12.1±0.05* | 15.3±0.07* | 17.1±0.08* |
| 1.0 | 7.6±0.03* | 9.8±0.01* | 12.1±0.03* | 19.3±0.04* | 18.3±0.03* |
| 2.5 | 7.7±0.01* | 12.1±0.08* | 16.8±0.01* | 22.7±0.03* | 22.1±0.07* |

*p<0.01

**Table 4: Comparative profile of tissue MDA in rat treated with *Ginkgobiloba* prior to *in vitro* $H_2O_2$ induced homogenate insult and rat with only *in vitro* insult.**

| Intervention | Tissue (MDA in nM) |
|---|---|
| Without insult | 2.58±0.2 |
| $H_2O_2$ induced insult (85 mM) | 6.0±0.01 |
| Insult + Gb 1 hr before | 3.0±0.0* |
| Insult + Gb 3 hrs before | 2.8±0.02* |
| Insult + 1 day o.d. | 4.33±0.21* |

*p<0.01

with 170 mM, 340 mM and 680 mM of hydrogen peroxide (Table 2 & Figure 2). MDA concentration was evaluated in ferric chloride (0.05 mM) induced lipid peroxidation in rat kidney homogenate. The concentration of MDA was found to increase with the time and maximum injury occurred at 60 min as evident from Table 3 & Figure 3. Similar trends were found with 0.25 mM, 0.5 mM, 1.0 mM and 2.5 mM of hydrogen peroxide (Table 3 & Figure 3). Comparative profile of tissue MDA in rat treated with *Ginkgobiloba* prior to *in vitro* $H_2O_2$ induced homogenate insult and rat with only *in vitro* insult was estimated. Maximum reduction in MDA concentration occurred with 1 day once daily dose of *Ginkgobiloba* pretreated

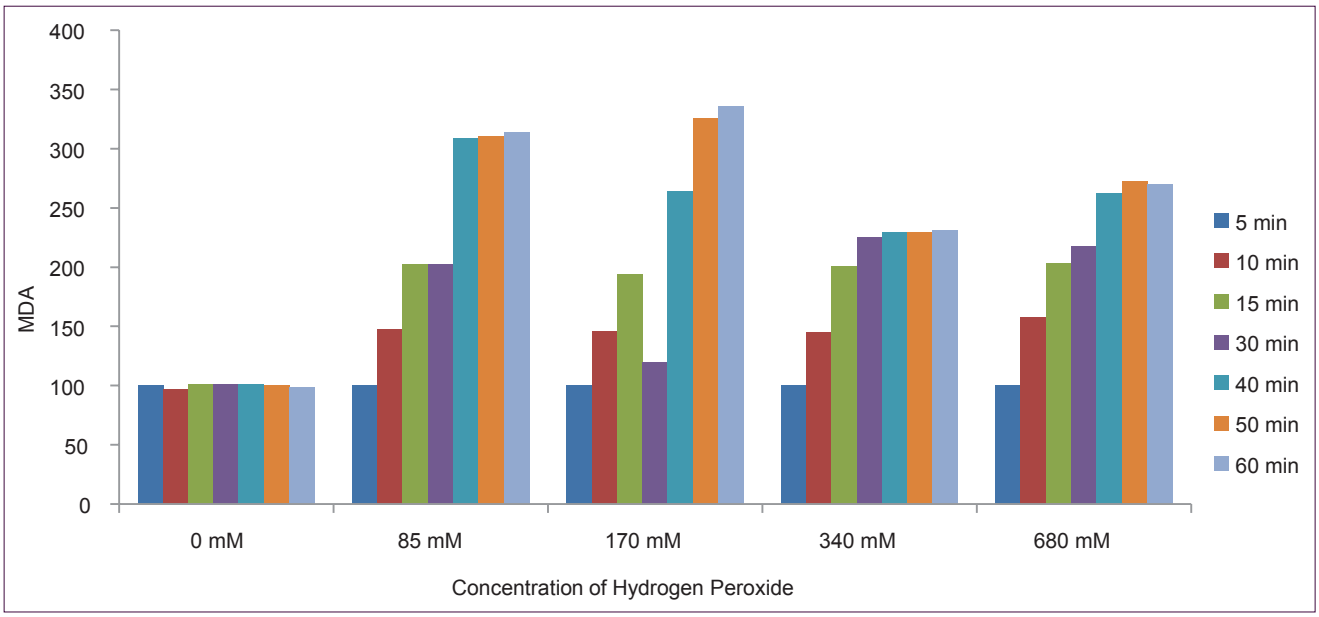


**Figure 2: Effect of Different Concentration of H2O2 for induction of lipid peroxidation in rat kidney homogenate at different time interval (percentage).**

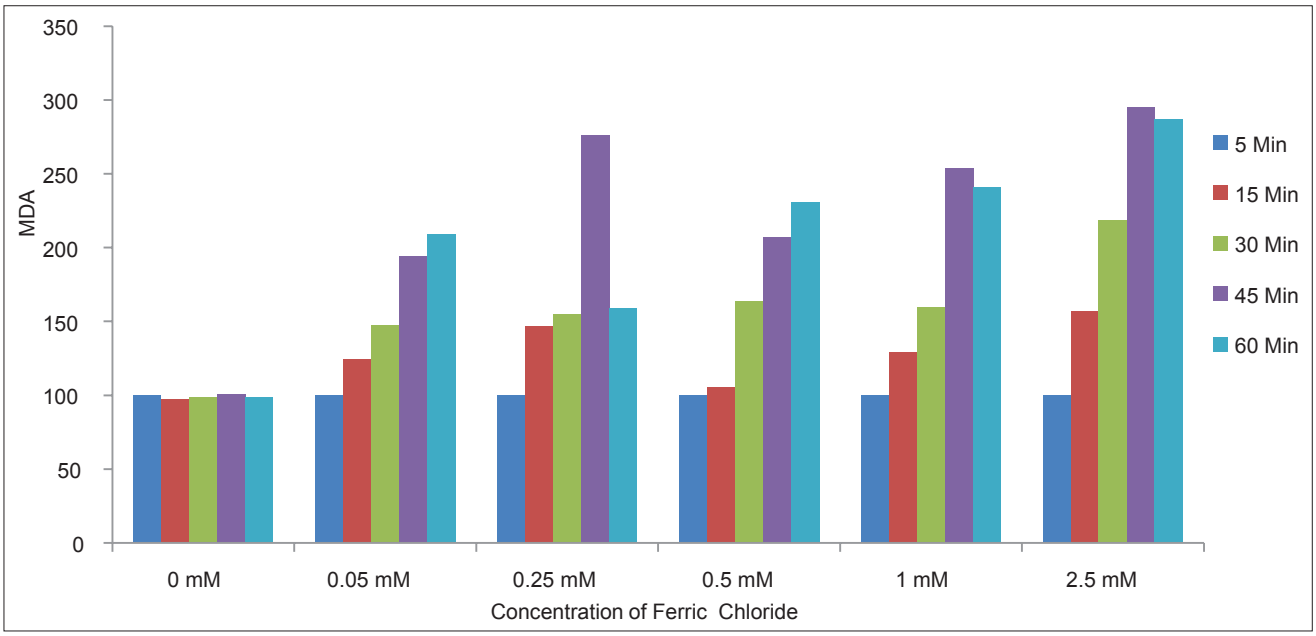


**Figure 3: Effect of different concentration of FeCl3 for induction of lipid peroxidation in rat kidney homogenate at different time duration (percentage).**

rats (Table 4 & Figure 4). Comparative profile of tissue MDA in rat treated with *Ginkgobiloba* prior to *in vitro* $Fecl_3$ induced homogenate insult and rat with only *in vitro* insult was estimated. Maximum reduction in MDA concentration occurred with 1 day once daily dose *Ginkgobiloba* pretreated rats (Table 5 & Figure 5). Comparative profile of tissue MDA

**Table 5: Comparative profile of tissue MDA in rat treated with *Ginkgobiloba* prior to *in vitro* $FeCl_3$ induced homogenate insult and in the rat with only *in vitro* insult.**

| Intervention | Tissue (MDA in nM) |
|---|---|
| Without insult | 2.58±0.2 |
| $FeCl_3$ induced insult (1 mM) | 7.6±0.03 |
| Insult + Gb 1 hr before | 3.8±0.01* |
| Insult + Gb 3 hrs before | 3.1±0.2* |
| Insult + 1 day o.d. | 5.1±0.21* |
| Insult + 2 days b.d. | 4.9±0.1* |
| Insult + 3 days t.d.s. | 4.6±0.36* |

*p<0.01

**Table 6: Comparative profile of tissue MDA in rat treated with *Ginkgobiloba* prior to induction of ischemia reperfusion injury *in vitro* and in the rat with only ischemia reperfusion injury.**

| Intervention | Tissue (MDA in nM) |
|---|---|
| Without insult | 2.58±0.2 |
| Isc/rep induced insult (for 1 hr) | 6.4±0.52 |
| Insult + Gb 1 hr before | 4.0±0.01* |
| Insult + Gb 3 hrs before | 2.8±0.1** |
| Insult + 1 day o.d. | 4.16±0.1** |
| Insult + 2 days b.d. | 4.75±0.38* |
| Insult + 3 days t.d.s. | 4.33±0.2** |

*p<0.05, **p<0.01

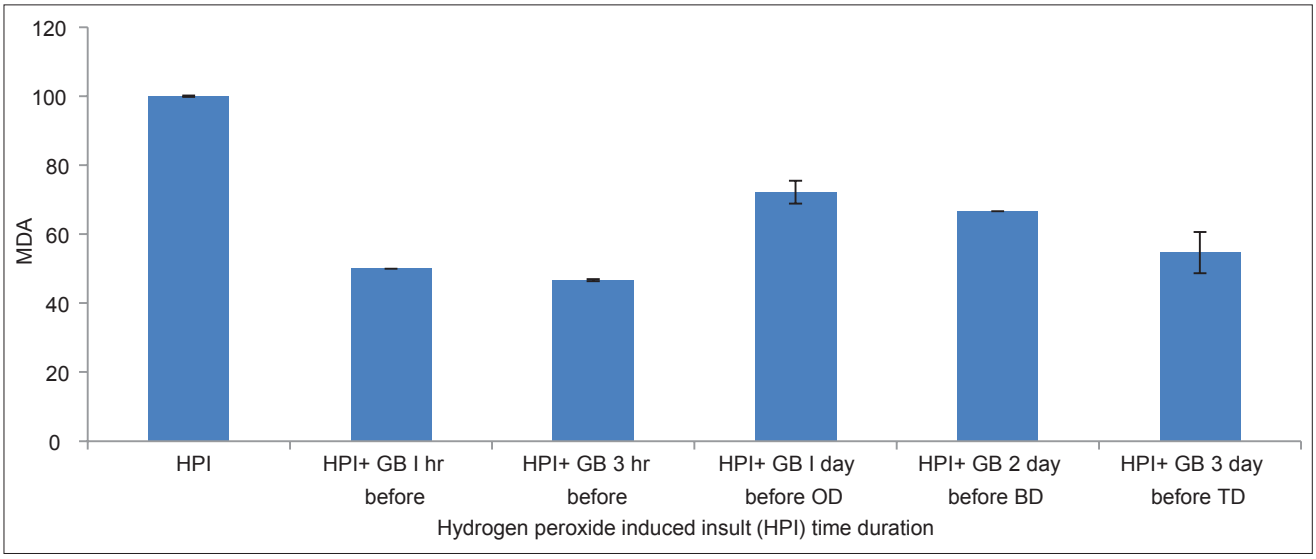


**Figure 4: Comparative profile of tissue MDA in rat treated with Ginkgobiloba prior to in vitro H2O2 induced homogenate insult and rat with only in vitro insult (percentage).**

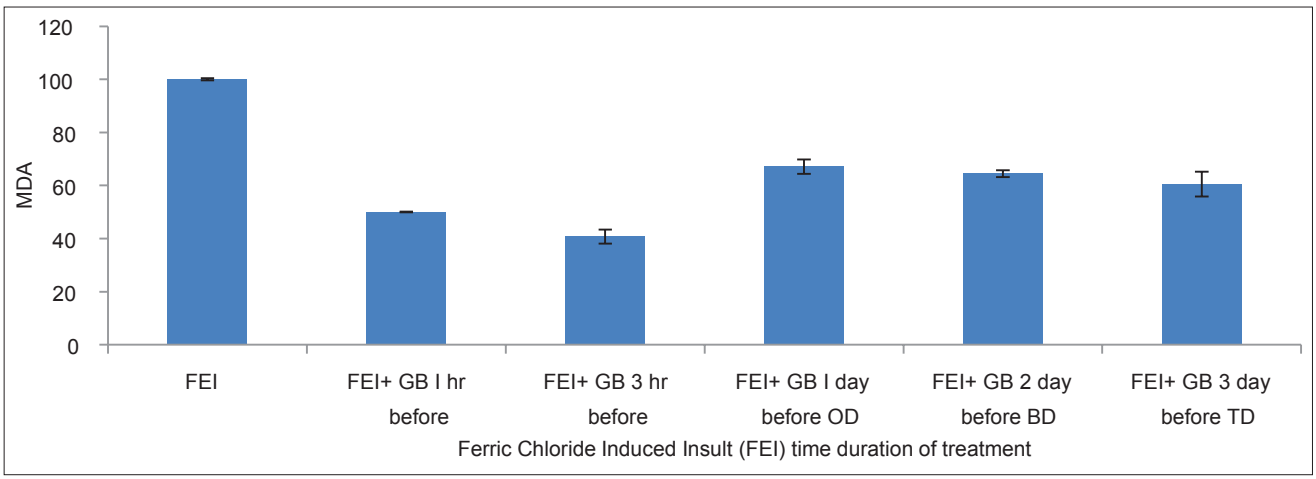


**Figure 5: Comparative profile of tissue MDA (percentage) in rat treated with Ginkgobiloba prior to in vitro FeCl3 induced homogenate insult and in rat with only in vitro insult.**

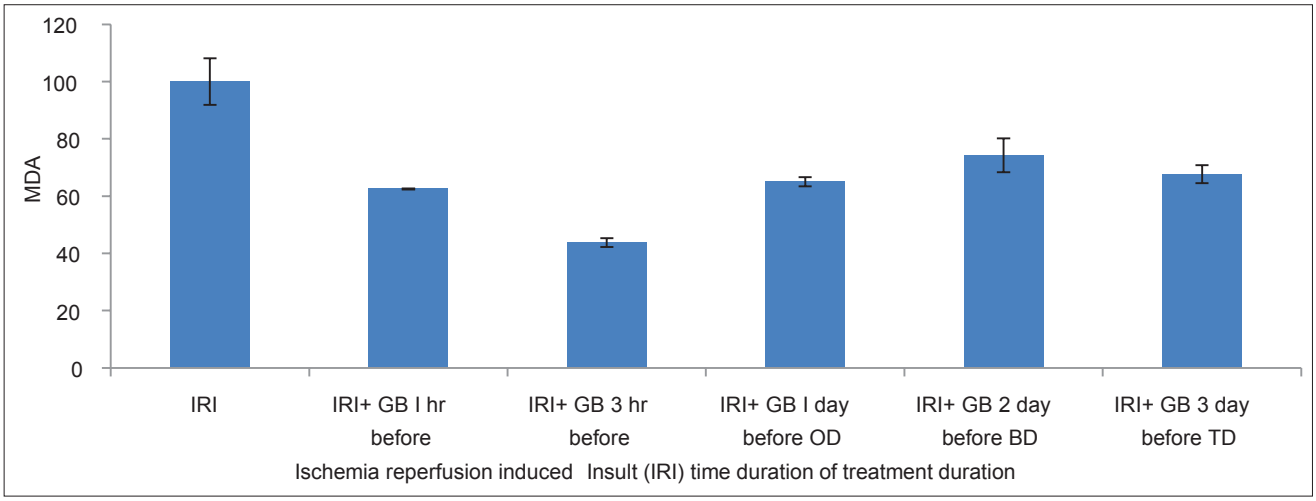


**Figure 6: Comparative profile of tissue MDA (percentage) in rat treated with Ginkgobiloba prior to induction of ischemia reperfusion injury in vitro and in rat with only ischemia reperfusion injury.**

in rat treated with *Ginkgobiloba* prior to induction of IRI *in vitro* and in the rat with only IRI was estimated. Maximum reduction in MDA concentration occurred with 2 days twice daily dose *Ginkgobiloba* pretreated rats (Table 6 & Figure 6).

## DISCUSSION

Anoxic injury starts with a decrease in mitochondrial energy production. The cytosolic pH decreases directly owing to ATP degradation, to increased glycolytic rate, and possibly to liberation of $H^+$ from damaged lysosomes. The cytosolic pH decreases directly owing to ATP degradation, to increased glycolytic rate, and possibly to liberation of $H^+$ from damaged lysosomes. Almost in parallel, cellular ion homeostasis becomes impaired, eventually resulting in increased cytosolic $Na^+$ and $Ca^{2+}$ concentrations. An increase in cytosolic $Ca^{2+}$ concentration may activate hydrolases, such as phospholipases (especially phospholipase A2) and proteases (calpains and others). The hydrolases may further enhance the injury process by degradation of their substrates (e.g., by calpain-mediated proteolysis of cytoskeletal proteins). Increased cellular sodium may cause osmotic swelling, which may contribute to disruption of the plasma membrane.[10] Upon resupply of blood, the inflammatory response is initiated. In the ischemic phase electron-transferring enzymes, such as those of the mitochondrial respiratory chain, may be damaged. Upon reperfusion of the still viable cells, electrons are transferred to $O_2$ by the damaged enzymes, resulting in the formation of ROS, thus initiating a ROS mediated injury to these cells.[11]

In contrast to most report, lipid peroxidation raised prominently at 45 min of ischemia reperfusion insult and not 30 min. More prominent *Ginkgobiloba* inhibition of free radical damage at 3 hrs than 1 hr suggest pharmacokinetic basis.[12] Both simple *in vitro* technique of lipid peroxidation by hydrogen peroxide and ferric chloride appear useful for quick screening of antioxidant effects. Comparative study of antioxidants of varying lipid solubility is in progress to further elaborate utility of these methods. The technique does not discriminate against inhibiting generation and neutralizing ROS. Renal ischemia reperfusion experiment led to 24 hrs mortality of 70% in uncontrolled laboratory condition and hydration. The same is being attempted to correlating study of renal function alteration as increment of MDA level is in progress to neutralize are production inhibitory drug effects and their protective significance in renal protection may then be elaborated.

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*Ethical approval: The study was approved by the Institutional Animal Ethical committee*

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