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# Effect of *Ginkgo biloba* on Renal Ischemia-Reperfusion Induced Oxidant Stress in Rats.

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## ABSTRACT

*Ginkgo biloba* extract was evaluated for prophylactic value against renal ischemia-reperfusion induced oxidant stress in rat subjected to transient unilateral renal artery ligation. Oral *Ginkgo biloba* treatment (2mg/kg) was administered by different schedule before experimental induction of ischemia-reperfusion injury. Malondialdehyde has been taken as indicator for free radical damage produced by ischemia-reperfusion injury. Three hour pretreatment by the drug has most prominent inhibitory effect on consequent tissue and plasma malondialdehyde level of lipid peroxidation. Further, the relative inhibition of malondialdehyde level in tissue and plasma allow to infer drug effect on generation, but not the clearance of malondialdehyde. No significant difference of outcome from 1, 2 and 3 days treatment indicate no cumulation and thus safety in that respect. *Ginkgo biloba* treatment appears both effective and safe for prophylaxis of reperfusion injury following ischemia catastrophies.

## INTRODUCTION

Ischemia reperfusion injury is the major reason for organ dysfunction or even non function following transplantation, mainly of marginal organs. Ischemia-reperfusion injury also restricts the long-term survival of transplanted organs. Ischemia-reperfusion injury affects $O_2$-dependent cells of tissues and organs, such as heart, brain, liver, kidney, and intestine. Renal ischemia–reperfusion injury is a major cause of acute renal failure and kidney allograft dysfunction[1]. Ischemia-reperfusion injury is a multifaceted phenomenon linking not only intracellular injury processes but also an injurious inflammatory response. Both the intracellular injury processes and the injurious events of the inflammatory response are interrelated[2]. Ischemia is a state of tissue oxygen deprivation accompanied by a reduced washout of the resulting metabolites[3]. Hypoxic cell injury predominates in the ischemic phase. Anoxic injury starts with a decrease in mitochondrial energy production.

Reperfusion is the restoration of blood flow to the ischemic tissue. Upon resupply of blood, the inflammatory response is initiated. In this inflammatory response, macrophages, endothelial cells, neutrophils, lymphocytes, platelets, parenchymal cells, as well as noncellular elements, including the complement system, the blood coagulation cascade, reactive oxygen species, nitric oxide, and pro- and anti-inflammatory cytokines in addition to other mediators may be involved and the microvascular perfusion might be impaired[2]. 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[4]. 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.

Molecular oxygen can be reduced by one, two or four electron transfer to produce superoxide anion ($O2^-$), 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 ($O_2H\cdot$), Hydrogen peroxide ($H_2O_2$), hydroxyl radical ($OH\cdot$), hypochlorus acid (HOCL) and

trioxocarbonate radical ($CO_3^{\cdot-}$). Increased activity of nitroxidant and suboptimal anti nitroxidant defence system leads to nitroxidative stress. Main component of nitroxidative stress is peroxinitrite anion ($ONOO^-$), peroxynitrous acid ($ONOOH^-$), nitronium ion ($NO_2^+$), nitrogen dioxide radical ($NO_2^{\cdot}$), nitryl chloride ($NO_2Cl$) and nitrite ion ($NO_2^-$). NO and peroxynitrite (ONOO–) are major reactive nitrogen species in biological systems[5].

Reactive oxygen species (ROS) are involved in many cellular metabolic and signaling processes and thought to have a role in aging[6]. ROS easily react with most biological macromolecules causing their degradation and destruction. ROS can cause cellular injury through their actions on phospholipids, proteins, and nucleic acids. High oxidative stress leads to depletion of enzymatic and nonenzymatic antioxidants[7]. The unsaturated lipid molecules of cell membranes are particularly susceptible to this damaging free radicals process and readily contribute to the uncontrolled chain reaction.

Therefore, their detoxification and elimination are necessary for normal physiologic cellular activity and survival. 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 (TBARs)[8]. Living organisms have developed complex antioxidant systems to counteract reactive oxygen species 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[9].

The aim of the present study was to determine the role of *Ginkgo biloba* in the treatment of rat model of Ischemia reperfusion injury, an oxidant-mediated disorder. Plant extracts may be an alternative to currently used antioxidant for controlling disease caused by excessive oxidative stress, because they constitute a rich source of bioactive chemicals. The most important of these bioactive constituents of plants are phenols, alkaloids, tannins, glycosides, flavonoids and essential oils. They could lead to the development of new classes of possibly safer disease control agents. *Ginkgo biloba* 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 *Ginkgo biloba* may be caused by single active ingredient or by the combined action of many active agent found in the extract. The most important substances are flavenoids (flavones glycoside) and terpenoids (ginkagolides and bilobalide). The most important flavenoids are glycosides of camferol, quercetin and isorhamnetin with glucose rhamnose. Perfusion with the *Ginkagobiloba* had beneficial effect on ischemic / reperfused rat and Guinea pig heart *in vitro* and on ischemic rats heart *in vivo*[10].

*Ginkgo biloba* extract has got established for therapy of microcirculatory dysfunction and thrombotic risk. It is shown to antagonize platelate activating factor[11]. Antioxidant effect of *Ginkgo biloba* extract has been shown as additional beneficial property[12]. The latter would be particularly relevant to mitigate free radical damage ensuing with reperfusion of ischemic tissue. The damage may be ultimately indicated in terms of lipid per-oxidation in affected tissue and in circulation, assayable by malondialdehyde level[13]. Key consideration for any prophylactic or mitigating therapy of such condition with *Ginkgo biloba* may be the time course of drug action. The issue is complex being determined by pathophysiological event and kinetic and/or dynamic drug behavior. Present report attempt to examine the same, employing different regimens of *Ginkgo biloba* pretreatment in rats subjected to experimental renal ischemia-reperfusion injury by transient unilateral renal artery occlusion.

## MATERIALS AND METHODS

Inbred Charles Foster (CF) strain albino rat weighing 200-250g 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 $26^0 \pm 2^0$C, 44-56% relative humidity and 10:14 hr L:D cycle for one 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. Experimental group composed of six animals in each.

Animal fasted overnight were anaesthetized with pentobarbitone (40 mg/kg, i.p.) through lateral flank incision, left kidney was accessed. The left renal artery was occluded with non traumatic vascular clamp for 1 hour period. Consequently the clamp was released and allowing 5 minute period thereafter (reperfusion), blood sample was drawn as maximal aspiration with intracardiac syringing. The left kidney subjected to procedure was removed, briskly rinsed in cold ($4^0$C) normal saline and taken in previously weighed beaker with normal saline and 1 drop of 1% beta hydroxyl toluene solution. The same was weighed again to deduce the weight of tissue.

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Control group of animals were shame operated without renal artery maneuver. After uneventful 1 hour post surgical period sample of blood and left kidney were similarly harvested. All the animals were then scarified by cervical dislocation.

### *Ginkgo biloba treatment*

*Ginkgo biloba* extract (Ginkoba-Microlab) (2 mg/100gm) was administered orally in aqueous 1 % gum acacia suspension. Pretreatment schedule followed as under: Single dose, at one hour before renal artery ligation, three hour 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.

### *Malondialdehyde estimation* [14]

Plasma malondialdehyde: The reaction mixture contained 0.4 ml of plasma, 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$^0$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 (Systronic). Malondialdehyde (in nM) estimation was done from the standard curve prepared with 1,1,3,3-tetra methoxy propane.

*Tissue malondialdehyde*: 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$^0$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 (Systronic). Malondialdehyde (in nM) estimation was done from the standard curve prepared with 1,1,3,3-tetra methoxy propane.

### Statistical analysis

Statistical analysis of data was done using ANOVA test .

## RESULTS

Plasma and tissue malondialdehyde profile expressed as nanomoles per liter using 1,1,3,3-tetra methoxy propane standard curves are displayed in table 1 and figure 1. On the basis of above experiment (effect of *Ginkgo biloba* treatment for different time schedule on experimentally induced renal ischemia-reperfusion injury in albino rat) following results may be drawn.

*Ginkgo biloba* was given 1 hour before induction of renal ischemia-reperfusion injury reduced malondialdehyde level 65% in blood 25% in tissue. Administration of same dose 3 hour before induction of renal ischemia-reperfusion injury decreased malondialdehyde level by 95% in blood and 56% in tissue. However, *Ginkgo biloba* given 1 day before also reduces blood and tissue Malondialdehyde level about 57% and 35% respectively.

*Ginkgo biloba* was given for 2 days (Twice daily) and 3 days (thrice daily) does not show any superiority over *Ginkgo biloba* given one day before induction of renal ischemia-reperfusion injury.

**Table 1. Comparative profile of plasma and tissue malondialdehyde in rats treated with *Ginkgo biloba* for different time duration prior to ischemia reperfusion injury and in rats without *Ginkgo biloba* (n=6 in each group)**

| S.No. | Drug Schedules Treatment | Malondialdehyde in nanomoles per litre (Mean±SEM) | |
|---|---|---|---|
| | | Blood | Kidney tissue |
| 1. | Sham | 3.91 ±0.23 | 2.3±0.28 |
| 2. | Isc / rep | 11.8±1.13 | 6.4±0.52 |
| 3. | 1 hr before | 4.08±0.08** | 4.0±0.1** |
| 4. | 3 hr before | 1.0±0.1** | 2.8±0.1** |
| 5. | For 1 day (o.d.) | 5.0±0.08** | 4.16±0.1** |
| 6. | For 2 day (b.d.) | 5.33±0.3** | 4.15±0.38** |
| 7. | For 3 day (t.d.s.) | 4.58±0.69** | 4.33±0.2** |

*P<0.05 shown as, *P<0.01 shown as***

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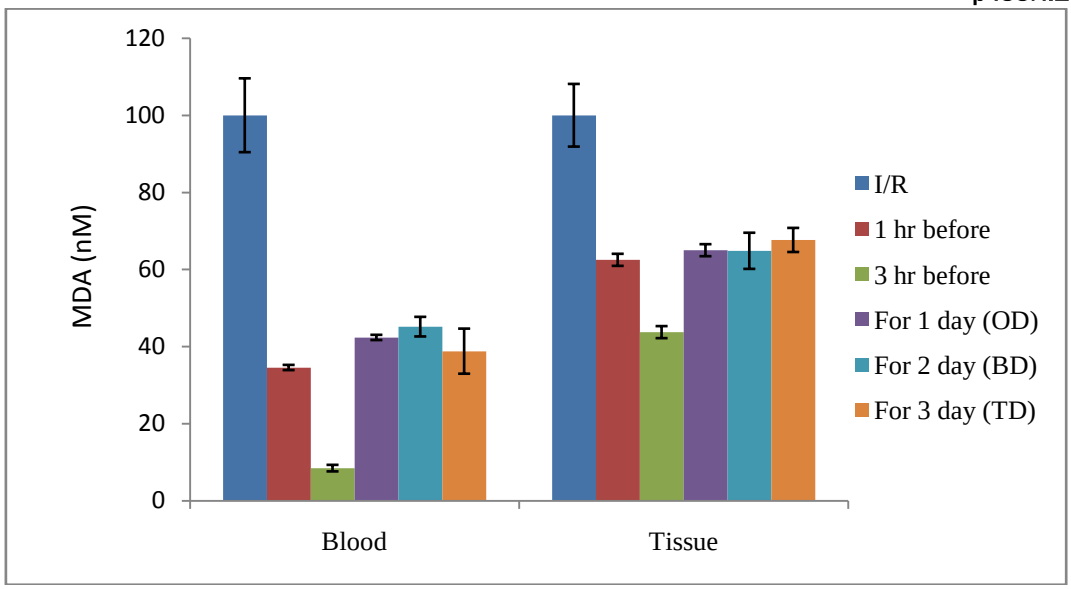


**Figure 1. Comparative profile (Percentage) of plasma and tissue malondialdehyde in rats treated with *Ginko biloba* for different time duration prior to ischemia reperfusion injury and in rats without *Ginko biloba* (n=6 in each group) Series 1: Blood, Series 2: Tissue.**

## 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 (eg, by calpain-mediated proteolysis of cytoskeletal proteins). Increased cellular sodium may cause osmotic swelling, which may contribute to disruption of the plasma membrane (2). 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 O2 by the damaged enzymes, resulting in the formation of reactive oxygen species, thus initiating a reactive oxygen speciesmediated injury to these cells[15].

The experiment of renal artery ligation induced renal ischemia-reperfusion injury yielded significant rise in tissue and plasma level of lipid peroxidation marker malondialdehyde, validating the employed procedure. All regimen of *Ginkgo biloba* pretreatment significantly reduced such rise indicating a protective effect. Inhibition of lipid peroxidation was most marked at 3 hour after treatment, both in tissue and plasma. That should reflect the optimal course for buildup of drug concentration at site of injury. Tissue malondialdehyde levels which may indicate ischemia reperfusion injury induced generation of free radical stress consistently and more prominently. Plasma malondialdehyde level may be taken to be result of malondialdehyde shed by injured tissue. *Ginkgo biloba* effects were more prominent on plasma malondialdehyde levels.

No significant difference in inhibitory profile of *Ginkgo biloba* given by serial more intensive schedule of 1, 2 and 3 days reveals lack of cumulative profile of studied schedule. Clinical inference of the finding is that *Ginkgo biloba* treatment has protective value in mitigating reperfusion injury following ischemic episodes and may be safe even when used in high doses due to low possibility of cumulation.

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