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Reducing effects of ginkgo biloba (egb761) extract on skeletal muscle ischemia-reperfusion injury in rats

Abstract

Aim: The restoration of circulation following a period of ischemia results in the release of the metabolites that accumulate in the ischemic tissue into circulation with untoward effects on the organs. Many studies have been conducted to date into the prevention of such ischemia-reperfusion injuries. Ginkgo Biloba extract is known to have antioxidant effects, although there is a lack of data on its effects on a skeletal muscle ischemia-reperfusion model. The present study investigates the use of Ginkgo biloba extract (egb761) for the reduction of experimentally-induced skeletal muscle ischemia-reperfusion injury.

Material and Methods: A total of 32 Sprague-Dawley rats were divided into four groups as follows: Group 1 (n=8), control group; Group 2 (n=8), limb ischemia model; Group 3 (n=8), ischemia-reperfusion model; and Group 4 (n=8), Ginkgo biloba group. After the models were created in each group, soleus muscle, lung and liver tissues were removed. Tissue malondialdehyde (MDA) levels were measured biochemically to demonstrate lipid peroxidation. The percentage of viable cells in the soleus muscle was calculated through a histochemical examination.

Results: The comparison of soleus muscle MDA levels revealed no statistically significant difference between Group 1 and Groups 2 and 3, whereas the levels were significantly lower in Group 1 than those in Group 4 ($p<0.05$). Similarly, there was a significant reduction in Group 4 than in Group 3 ($p<0.05$). The analysis of lung tissue MDA levels revealed no significant difference between Group 1 and Group 2, whereas MDA levels were significantly increased in Group 3 than in Group 1 ($p<0.05$), while there was no difference between Group 1 and Group 4. The analysis of liver tissue MDA levels showed significantly increased levels in Groups 3 and 4 than in Group 1 ($p<0.05$). The percentage of viable cells was significantly decreased in Groups 2 and 3 than in Group 1 ($p<0.05$). There was a significant reduction in Group 4 than in the control group.

Conclusion: The results of the present study suggest that Ginkgo biloba (EGb761) extract may be used to reduce the local and systemic effects occurring after the restoration of blood flow following acute arterial occlusion. There is, however, an apparent need for large-scale, prospective, randomized trials to demonstrate the efficacy of Ginkgo biloba treatment.

Keywords: Ischemia, reperfusion injury, ginkgo biloba, rats

INTRODUCTION

Acute limb ischemia (ALI) is defined as the sudden decrease in oxygenated blood flow supplying an extremity. Unable to pass beyond the occlusion caused by a thrombus, embolism or injury, the resulting loss of blood flow results in a medical emergency that threatens both the extremity and the life of the patient (1).

The outcome following a sudden and complete occlusion of a limb artery is closely related to the timing of intervention

(2). Reperfusion within the first 4 hours after an occlusion is associated with the best outcomes, whereas interventions after 6 hours result in poor outcomes in terms of both saving the limb and preventing distant organ consequences (2).

Cellular injury occurring after reperfusion of ischemic but viable tissue is defined as ischemia-reperfusion injury (IRI) (2,3). IRI results from the release of free oxygen radicals, neutrophil activation, tissue infiltration and a decline in microvascular circulation (4). The acute IRI of a limb causes local and systemic

inflammatory changes that may impair limb function and threaten the life of the patient (5). Lung injury and injuries to distant organs, such as impairment in kidney, bowel, brain and splenic functions and even multiorgan failure are commonly encountered following reperfusion (5-9).

The common goal of almost all studies investigating the prevention of reperfusion injury following acute arterial occlusion has been to preserve the integrity of the limb and minimize or completely eliminate the life-threatening effects of systemic processes. Various methods and drugs have been proposed in these studies for the prevention of reperfusion injury. Ginkgo biloba (EGb 761) extract is one such drug, the antioxidant activity against free oxygen radicals of which has been demonstrated in the heart muscle, retina and bowel (10–14). The authors of the present study, on encountering no study in literature evaluating the use of Ginkgo Biloba extract for the prevention of skeletal muscle reperfusion injury, investigate the effects of Ginkgo Biloba extract on the prevention of skeletal muscle IRI. The aim of this study is to investigate the potential protective effect of Ginkgo Biloba extract in preventing IRI in different organs.

MATERIAL AND METHODS

Experimental Animals and Groups

Ethical permission for the study was obtained from the Uludağ University Experimental Animals Ethics Committee. A total of 32 female Sprague- Dawley rats (aged 10–12 weeks and weighing 350–400gr) were obtained from Uludağ University Experimental Animals Research Center. The animals were fed with standard rat pellets and maintained under standard conditions (12-hour light and dark cycle, $21\pm 2^{\circ}\text{C}$ constant ambient temperature, air-conditioned rooms with constant humidity). The 32 rats were divided into four groups as follows: Group 1 (n=8), control group; Group 2 (n=8), limb ischemia model; Group 3 (n=8), ischemia-reperfusion model; and Group 4 (n=8), Ginkgo biloba group.

Drug Administration

The animals in Group 4 received Ginkgo biloba extract 100 mg daily given in two separate doses (Tebokan fort® drops, Abdi İbrahim Pharmaceuticals Inc., Turkey) through an orogastric tube for five days, starting the day before the experiment (15-18). Due to lack of availability of the parenteral form of Ginkgo biloba extract in Turkey, oral drops were used at appropriate doses and durations.

Experimental Procedure

Ketamine HCl (Ketalar®) 30mg/kg and Xylazine HCl (Rompun®) 2mg/kg kept at $21\pm 2^{\circ}\text{C}$ in a room environment were administered into the left foreleg muscle.

The rats in Group 1 (n=8) underwent only anesthesia than soleus muscle, liver and lung tissues were removed after a 6-hour waiting period. After the induction of anesthesia, the rats in Group 2 (n=8) were subjected to right hindlimb ischemia through the application of a tourniquet at the level of hip joint. Soleus

muscle, liver and lung tissues were removed following a 4-hour waiting period without releasing the tourniquet. The tourniquet was released after 4 hours of ischemia and the limbs of the rats in Groups 3 (n=8) and 4 (n=8) were subjected to reperfusion for 2 hours. The soleus muscle, liver and lung tissues were removed at the end of the reperfusion period.

Biochemical Examination

The tissue malondialdehyde (MDA) levels were measured spectrophotometrically (UV 1202 Shimadzu) at 532nm to demonstrate lipid peroxidation in the soleus muscle, liver and lung tissues of the subjects.

Histopathological Examination

Histochemical staining with nitro blue tetrazolium (NBT) dye was used in the evaluation of the percentage of viable cells in the soleus muscle samples. Coronal sections were cut from four different sections of the muscle samples using a cryocut (Reichert Jung Cryocut 1800). The samples were incubated in a 50mg/100 ml NBT solution for 30 minutes at a temperature of 37°C and a pH of 7.4, and the sections were stained after dilution with Na succinate at a rate of 1/10. A microscopic examination was conducted in which viable cells appeared dark blue in color whereas necrotic cells appeared pale yellow in color. The percentage of viable cells in the sections cut from four different parts of each muscle sample was calculated using a planimetric method under a microscope at x100 magnification. The percentage of viable cells was calculated as the ratio of the viable cells within the entire magnification field.

Statistical Analysis

All data were uploaded to IBM SPSS Statistics for Windows (Version 22.0. Armonk, NY: IBM Corp.) for analysis.

All numerical data were expressed as mean $\pm$ standard deviation. A Kruskal Wallis test by ranks was used to compare the malondialdehyde levels of the groups (A); and a nonparametric Mann-Whitney U test (NWU) was used to compare the percentage of viable cells in Groups 2, 3 and 4 with those in Group 1. A p value of less than 0.05 was considered statistically significant.

RESULTS

All subjects completed the study. No mortalities were observed during the experiment. The comparison of soleus muscle MDA levels revealed no statistically significant difference between Group 1 and Groups 2 and 3, whereas the levels were significantly lower in Group 1 compared to the levels in Group 4 ($p<0.05$). Similarly, there was a significant reduction in Group 4 than in Group 3 ($P<0.05$) (Graph 1). The analysis of lung tissue MDA levels did not show a significant difference between Group 1 and Group 2, whereas MDA levels were significantly increased in Group 3 than in Group 1 ($p<0.05$), while there was no difference between Group 1 and Group 4 (Graph 2). The analysis of liver tissue MDA levels revealed significantly increased levels in Groups 3 and 4 than in Group 1 ($p<0.05$) (Graph 3). The percentage of viable cells was 70.5% in the muscle samples of the

control subjects that underwent only 6 hours of anesthesia. The percentage of viable cells was significantly decreased in Groups 2 and 3 than in Group 1 ($p<0.05$), and the levels were significantly decreased in Group 3 than in the control group ($p<0.05$). Unlike the other groups, the levels in the Ginkgo biloba-treated group were not from the levels in the control group. The percentages of viable cells in each group are presented in Table 1 and Graph 4.

Table 1. Tissue malondialdehyde levels in all groups (nmol/g tissue) and the percentage of viable cells in the soleus muscle

	G1 (n=8)	G2 (n=8)	G3 (n=8)	G4 (n=8)
Soleus muscle MDA	76.6±20.8	82.4±36.1	77.6±26.0	59.1±14.7
Liver MDA	315±29.7	356.8±33.9	387.4±19.0	372.3±28.1
Lung MDA	157.0±26.0	174.2±2.5	189.5±10.8	166.4±19.6
Soleus muscle VC	70.5±7.5	31.62±8.6	27.1±5.4	69.37±2.6

MDA: Malondialdehyde, VC: Viable cells

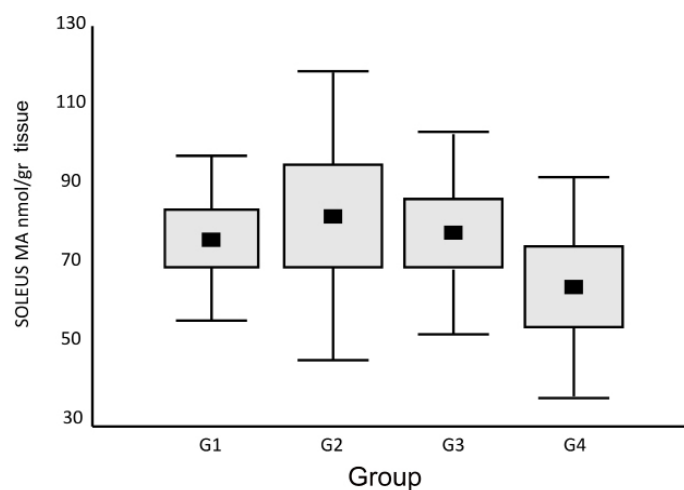


Figure 1. Between-group comparison of MDA levels in the soleus muscle

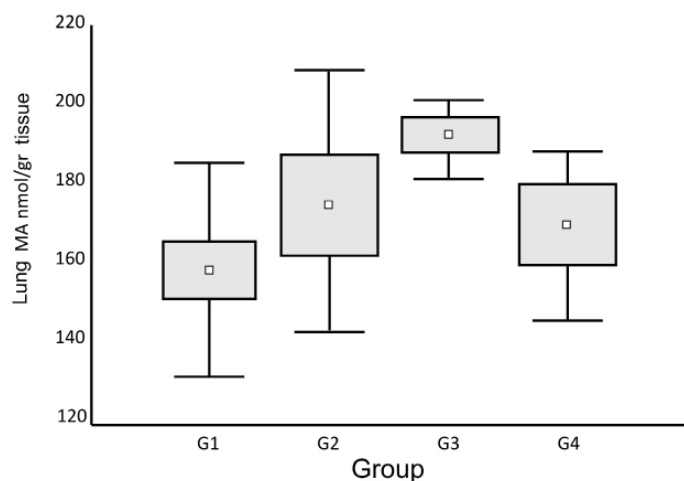


Figure 2. Between-group comparison of lung MDA levels

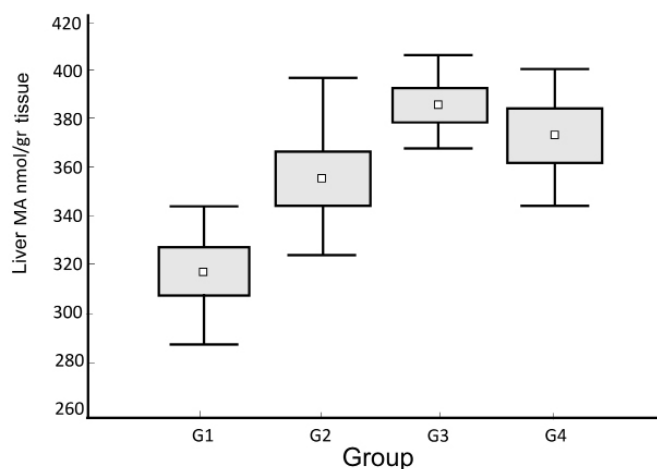


Figure 3. Between-group comparison of liver MDA levels

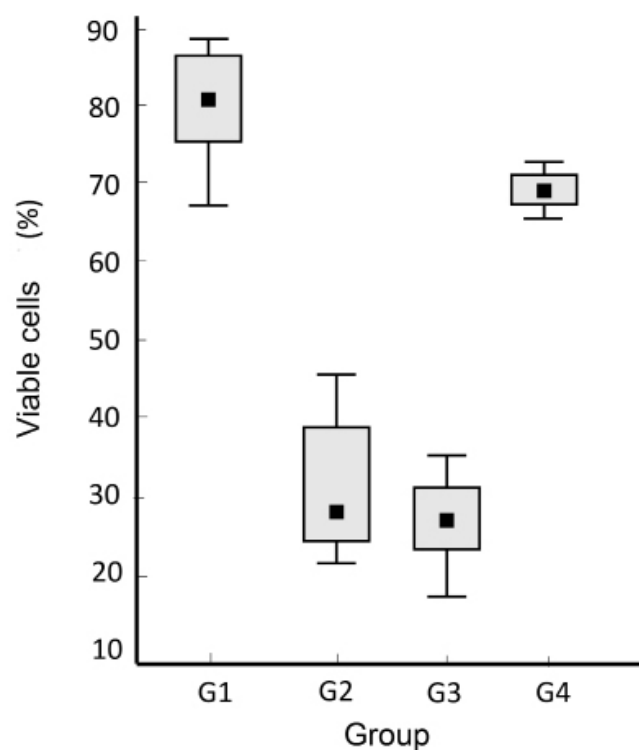


Figure 4. Between-group comparison of the percentage of viable cells (%)

DISCUSSION

A sudden blockage of blood flow to the limb is referred to as Acute Limb Ischemia (ALI). This situation requires emergency intervention (15), and can result from such conditions as embolism, thrombus formation and graft thrombosis, all of which require emergency intervention (16). Limbs can not recover without intervention (1). Surgical methods such as thromboembolectomy, bypass surgery and endovascular methods such as catheter-directed thrombolysis (CDT), percutaneous

thrombus aspiration, stent placement or combination therapies are used for the treatment of ALI (17).

The term “ischemia-reperfusion injury” has been used to describe changes occurring after the restoration of the blood flow to tissue following an ischemic period. Prolonged ischemia leads to necrosis of the muscle cells and the accumulation of K⁺ ions, myoglobin, lactic acid, creatine kinase and free oxygen radicals such as superoxide.

Distant organ injuries, such as impairments in the lung, kidney, bowel, brain and splenic function, multiorgan failure and even sudden death can occur with the release of these metabolites into the body after reperfusion (5–9). Ischemia-reperfusion injury is associated with endothelial cell inflammation, increased vascular permeability, imbalance of vasodilator and vasoconstrictor factors, and activation of the complement system and clotting (21). These changes occurring after an ischemic period have been well documented, although studies have shown that fundamental functional and cellular impairment occurs after reperfusion, which is an important complication affecting the course of ischemia (20,22).

Ginkgo biloba leaves have been long used, particularly in Chinese medicine, for the treatment of vascular diseases (23). Ginkgo biloba leaves contain flavonoid glycosides and terpenoids that are reported to have strong free radical-scavenging and antioxidant effects (24), and to have protective effects against myocardial and brain ischemia-reperfusion injury through the inhibition of platelet-activating factor antagonists and inducible nitric oxide (NO) synthase (iNOS) expression, as well as NO production (14,25).

The skeletal muscle ischemia-reperfusion model described by Hardy et al. was used in the present study (26), in which a plastic band was tightened around the limb at the hip level to reduce blood flow to the limb by 98% (27). The submaximal decrease in arterial blood flow achieved using this method resembles acute arterial occlusion in humans.

The end-products of lipid peroxidation are aldehydes, hydrocarbon gases and malondialdehyde. It is extremely difficult to confirm the presence of free oxygen radicals due to their short half-life. Accordingly, the demonstration of biological free oxygen reactions is based on the demonstration of malondialdehyde (MDA) as the end-product of lipid peroxidation (28). We used the MDA method because we thought that demonstrating lipid peroxidation would be sufficient to show cell damage and it was easy to access.

The percentage of viable cells in the soleus muscle samples obtained from the subjects was 70.5±7% in Group 1. The loss in percentage of viable cells in Group 1 can be attributed to the death of a certain number of cells during harvesting from the soleus muscle or the staining of the sections cut from the tissue samples frozen at 80°C. The NBT staining method has the highest accuracy rate following 12–16 hours of reperfusion,

that is, after the complete loss of cytoplasmic enzyme activity, and the rate of false positive results may be increased in samples harvested before this time. A comparison of the control group (Group 1) and the other groups can be considered appropriate considering the fact that all samples in all groups were stained at the same time. While the percentage of viable cells in Group 1 was 70.5±7.4%, the percentage of 31.6±8.6% recorded in Group 2 suggests a significantly ($p<0.05$) decreased percentage of viable cells in Group 2, implying that cytochrome enzyme activity, indicating the viability of the tissue, decreased as a result of ischemia. Although there was no significant difference between Group 1 and Group 4, the finding of a significant ($p<0.05$) suggests that Ginkgo biloba extract may have protected tissue viability. The lack of difference in the soleus muscle MDA levels of Group 2 and Group 1 suggests that ischemia plays no role in lipid peroxidation. The lowest levels of lipid peroxidation correspond to 2–4 hours and the highest levels correspond to 18 hours after reperfusion (29). The MDA levels were lower in Groups 3 and 4 because the levels were analyzed 2 hours after reperfusion. The significantly decreased MDA levels in Group 4 ($p<0.05$) than in Group 3 after an equal period of reperfusion suggests that Ginkgo biloba extract has the ability to reduce lipid peroxidation in muscle tissue subjected to lipid peroxidation.

The lack of difference in lung tissue MDA levels between Group 2 and the control group suggests that ischemia does not initiate lipid peroxidation. A comparison of Group 1 and Group 3 revealed a significant increase in liver MDA levels ($p<0.05$). It can be speculated that reperfusion triggers lipid peroxidation in the early period and endogenous protective mechanisms are depleted in the early period or remain ineffective. Lung injuries occurring after reperfusion following limb ischemia can be attributed to the effects of toxic substances on the lung tissue produced after lipid peroxidation (30). Injuries occurring during reperfusion can be largely attributed to the activity of polymorphonuclear leukocytes. Such lung injuries start 30–45 minutes after reperfusion, with the early effects being a result of neutrophilic activation (30). The fundamental causes of lung injury are the previously-explained cascades of events resulting from the interaction of neutrophils with the endothelium. The lack of any significant increase in lung tissue MDA levels in Group 4 than in Group 1 suggests that the Ginkgo biloba extract reduced ischemia-reperfusion injury in the lung tissue.

Liver tissue MDA levels did not differ significantly between the control group and Group 2, while the levels were significantly increased in Groups 3 and 4 ($p<0.05$). This finding suggests that the ischemia itself did not trigger lipid peroxidation in the liver, but that the lipid peroxidation occurred because of the reperfusion. No significant difference was found between Group 3 and Group 4. Considering the fact that the effects of Ginkgo biloba extract are three times lower in the liver than in the plasma, and the finding of slightly lower levels in Group 4 than in Group 3, despite the lack of statistical significance, suggest that Ginkgo biloba extract has limited activity against lipid peroxidation in

the liver (31).

CONCLUSION

The results of the present study suggest that Ginkgo biloba (EGb761) extract may be considered a beneficial additional intervention for the reduction of the local and systemic effects occurring after the restoration of blood flow to the limb following acute arterial occlusion. Studies involving larger groups using parenteral forms of the drug, and considering endogenous free radical scavengers together with longer ischemia-reperfusion periods, may provide further beneficial information.

Conflict of Interests: The author declares that there are no conflict of interests.

Financial Disclosure: There are no financial supports.

Ethics committee approval: Ethical permission for the study was obtained from the Uludağ University Experimental Animals Ethics Committee.

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