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The effect of *Ginkgo biloba* EGb 761 on intestinal anastomotic healing in rats with ischemia-reperfusion induced in the lower extremities

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Abstract

Background/Aim: Remote ischemia-reperfusion (I-R) injury for anastomotic healing is a newly identified risk factor and there is a wide array of studies being conducted. This study aimed to reveal the negative effects of lower I-R on the colonic anastomotic healing process and examine the effects of *Ginkgo biloba* EGb 761 treatment, a platelet activating factor (PAF) antagonist, on anastomotic healing through the inhibition of pathological mechanisms of mediators causing these negative effects.

Methods: Thirty-six Wistar-Albino rats were divided into sham, I-R and I-R+EGb 761 groups, each consisting of 12 rats. In the subjects in the sham group, an end-to-end anastomosis was performed by transecting the descending colon following midline laparotomy. In the I-R group, unilateral lower extremity ischemia was created by occluding the femoral artery and collaterals using a tourniquet from the most proximal segment of the left extremity. Then, the descending colon was transected, anastomosis was performed, and reperfusion was created by a tourniquet application at the 30th minute of ischemia. Different from the I-R (control) group, the subjects in the I-R+EGb 761 group were given two equal doses of 64 mg/kg/d *Ginkgo biloba* EGb 761 by the orogastric route until 10 days after surgery. After all subjects were sacrificed on the 10th day of surgery, the descending colon segment containing the anastomosis area was resected and samples were taken for bursting pressure and hydroxyproline measurements.

Results: In the I-R group, anastomotic bursting pressure and perianastomotic hydroxyproline values were significantly lower compared to the sham group and the I-R+EGb 761 group. However, there was no statistically significant difference in these parameters between the sham and the I-R+EGb 761 groups.

Conclusion: Colonic anastomotic bursting pressure and peri-anastomotic hydroxyproline values in the sham group were significantly decreased by lower extremity I-R, and this change was prevented with the use of EGb 761.

Keywords: Remote ischemia-reperfusion, Ginkgo biloba EGb 761, PAF antagonist

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Introduction

Among the factors responsible for mortality and morbidity after gastrointestinal system surgery, anastomotic leaks have a leading role [1, 2]. A successful anastomotic healing process depends on many factors. Intestinal anastomoses should be performed to healthy bowel ends, without tension, with appropriate suture materials and technique, causing minimal tissue damage, clear of contaminated material, fibrin, etc., and should be reinforced with a serosal patch or omentum, when necessary. There should be no distal obstruction and the patient should be nutritionally prepared to decrease post-operative anastomotic complications. Blood supply and oxygenation of anastomosis are the most important factors for successful anastomotic healing. Although the negative effects of tissue ischemia on wound healing are well known, the role of reperfusion in anastomotic healing has not yet been explained in detail [2].

When a tissue in an organism undergoes ischemia, a series of chemical events occur, leading to cellular dysfunction and necrosis. It is especially important to ensure the blood supply in ischemic tissues to provide sufficient energy for the cell and remove toxic metabolites. Reperfusion is the reorganization of blood flow to the tissue by eliminating the factor causing ischemia. Reperfusion has two positive effects on ischemic tissue: Meeting the energy needs and removing toxic metabolites [2-4].

However, this 'inevitable re-flow' (reperfusion) phenomenon paradoxically triggers a series of metabolic events that cause further damage to tissues [2,4-5]. Reperfusion injury occurs through a complex mechanism accompanied by free oxygen radicals, endothelial factors, and neutrophils. The release of many vasoactive mediators, cytokines, endothelin, and free toxic oxygen radicals leads to leukocyte activation, endothelial dysfunction, and tissue edema in the reperfused tissue. The cardiovascular and pulmonary systems are also affected after ischemia-reperfusion (I-R). All these factors can disrupt anastomotic perfusion, and the released mediators can cause adverse effects around the anastomosis [2, 6-8].

Although many mediators have been defined in intestinal and distal organ dysfunction related to I-R, it is known that platelet activating factor (PAF) and free toxic oxygen radicals play a particularly important role in this process [3, 5, 9]. Many experimental studies have shown that *Ginkgo biloba* extract EGb 761 and other PAF antagonists can reduce or even prevent a possible injury in local and remote organ systems due to intestinal I-R [5-7, 9-12]. As a *Ginkgo biloba* extract, EGb 761, prepared in a form suitable for experimental use, is a PAF antagonist with strong effects that scavenges toxic free radical activity [13]. While providing vascular relaxation, it prevents platelet aggregation and stabilizes lysosomal membranes. This extract is used for therapeutic purposes in many ischemic events, including cerebrovascular and peripheral vascular insufficiency. EGb 761 has different effect potentials in different organs and systems and exhibits protective effects in neurodegenerative, sensory and vascular diseases [13, 14]. This molecule, which can act systemically at molecular, cellular, and textural levels or in the whole organism, has no particular one-way (activator or inhibitory) effect; rather, it is a regulatory compound that helps the adaptation of an organism to the current environment [10, 13, 15].

Considering the positive effects of EGb 761 on various pathophysiological mechanisms in remote organ I-R injury, the current study aimed to analyze the effects of this compound on intestinal anastomosis healing in rats with I-R injury induced in the lower extremity.

Materials and methods

Materials

Ethics approval was obtained from the Local Ethics Committee of the Turkish Ministry of Health Haydarpaşa Research and Teaching Hospital (date: 26.08.2004 number: 840098-604.01.01-E.3767), and the study was conducted at the Experimental Research Center (TADEM) of Taksim Training and Research Hospital between January 2005 and June 2005. Thirty-six male Wistar-Albino rats, weighing between 250-300 grams, raised in the Experimental Animal Production and Research Laboratory of Taksim Training and Research Hospital were used. The rats were housed in appropriate cages in the animal laboratory at a temperature of 22 ± 2 °C, a humidity of 50-60% and under 12 hours of light and dark cycles by turning the lights on at 8 a.m. and turning them off at 8 p.m. The rats were fed a standard commercial pellet diet and provided tap water. All rats were handled according to the 'Care Principles of Experimental Animals' specified by the National Association of Medical Research and the 'Guidelines for the Care and Use of Laboratory Animals' revised by the Institute of Laboratory Animal Resources.

Ginkgo biloba extract EGb 761 (Tebokan® Fort Damla) was purchased from Abdi İbrahim Pharmaceuticals and kept under suitable conditions. Biochemical measurements were performed at the Biochemistry Clinic of Taksim Training and Research Hospital. Surgical instruments of the hospital were used after completing routine sterilization procedures. Intestinal samples were stored at -20°C in a deep freezer until biochemical evaluations.

Methods

All rats used in the study were kept in the same laboratory environment for a week before the experiment. They were fed standard laboratory diet and water. The rats to be operated were fasted with only water intake on the night before surgery.

For anesthesia, following ether induction, 65 mg/kg sodium pentothal (Pental® 1gr, İbrahim Etem Ulagay Pharmaceuticals, İstanbul, Turkey) was intraperitoneally administered. One-third of this dose was repeated intramuscularly when necessary. The surgery and injections were performed under general anesthesia. During the anesthesia, the subjects were monitored under room air conditions without the need for respiratory support. Surgery was performed under a heating lamp in order to prevent hypothermia that may develop during the experiment.

Care was taken to perform standard surgical procedures and follow sterilization rules. To perform the surgical procedure under ideal conditions, fixing boards were used. All surgical procedures were performed with a loop. Laparotomies were

performed with a 3.5-4 cm midline incision after the abdominal skin was shaved, cleaned, and covered with povidone iodine. To reduce heat loss from the tissues, the intestines were covered with gauze pads soaked in warm and sterile saline. Five milliliters of Ringer's lactate were subcutaneously administered to all subjects following the surgical procedure to prevent dehydration.

Experimental models

Sham model: No procedure was performed to cause ischemia in this group, and it was taken as the basis for pathological evaluations and bursting pressure. Colon anastomosis was performed following a 4 cm midline incision after anesthesia induction to create the control group model.

I-R model: To create the remote I-R model, after anesthesia induction, unilateral lower extremity ischemia (LEI) was created by compressing the femoral artery and collaterals with a tourniquet from the upper third of the left thigh. The lack of distal pulses and pale leg color indicated ischemia. Following an ischemia period of 20 minutes, a full-thickness incision and anastomosis were made on the descending colon with a 3.5-4 cm midline incision. At the end of the 30th minute, the tourniquet compressing the femoral artery was loosened and reperfusion was achieved. The return of leg color to normal and the ability to record distal pulses with a manual Doppler device were considered reperfusion.

Bowel anastomosis model: Following a midline laparotomy, the descending colon was prepared and cut, and anastomosis was performed with 6/0 propylene (Prolene, Ethicon UK®) over a single layer using an average of 8-10 sutures over one layer. The abdomen was covered with 3/0 silk material over two layers.

Experimental groups

A total of 36 subjects were included in the study and divided into three groups of 12 rats each.

Group 1 (Sham group): In this group, anastomotic healing was investigated without an ischemia model. Intestinal anastomosis was performed and the abdomen was closed after laparotomy.

Group 2 (I-R group): In this group, the effects of remote I-R injury on anastomotic healing were investigated. LEI was induced with a tourniquet, and intestinal anastomosis was performed following laparotomy. After the 30-minute ischemia period, reperfusion was created by loosening the tourniquet.

Group 3 (I-R + EGb 761 group): In this group, the effects of *Ginkgo biloba* EGb 761 on anastomosis healing were investigated. Different from the I-R group, this group was administered two equal doses of 64 mg/kg/d *Ginkgo biloba* EGb 761 through the orogastric route until 10 days after surgery.

In all three groups, on the 10th day of surgery, laparotomy was performed again and the colon segment covering the anastomosis area was resected for bursting pressure measurements and biochemical examinations (hydroxyproline level). Subsequently, the subjects were sacrificed.

Sampling

In all groups, 10 days after the operation, the descending colon segment that was anastomosed was identified by a re-laparotomy after ether induction. A large segment of the colon including the anastomosis line was resected without damaging the anastomotic line, preserving the surrounding adhesions. Feces in the colon segment was cleared by administering physiological saline from the proximal end. Subsequently, the removed segment was cut at 2 cm proximal and distal of the anastomotic line to obtain a standard bowel length for bursting pressure measurements and biochemical examinations (hydroxyproline level).

Measurements

Anastomotic bursting pressure measurements were performed with the modification of a technique previously described in the literature in detail [2]. Accordingly, an 18-F plastic catheter was placed at the proximal end of the anastomosis line and tied over the catheter with 2/0 silk, ensuring no leakage. At the distal end, it was tied with 2/0 silk sutures so that the fluid would not leak. The catheter placed at the proximal end was connected to the infusion pump (Abbott Lifecare 5000®) set to deliver 2 ml of fluid per minute via a three-way tap. Another catheter placed in the three-way tap was connected to the mercury manometer instrument. In all subjects, bursting pressures were recorded in mmHg by observing fluid leakage from the anastomotic line or pressure drop on the manometer. The measurement of hydroxyproline was carried out in the biochemistry laboratory of Taksim Education and Research Hospital using a model based on the principle of the hydrolysis of this substance from collagen, its oxidization with chloramine T, and then the reaction with the Erlich reagent to form colored chromophore compounds.

Statistical analysis

Data were analyzed using the IBM-SPSS for Windows version 20.0 software package (IBM Corp., Armonk, NY, USA). The mean bursting pressures and tissue hydroxyproline concentrations of the corresponding groups were calculated and expressed as median (SD) (Figure 1 and Figure 2). One-way analysis of variance (ANOVA) and Tukey's multiple comparison test were used for the statistical analysis of the results. $P<0.05$ was considered significant.

Figure 1: Comparison of anastomotic bursting pressure between the groups

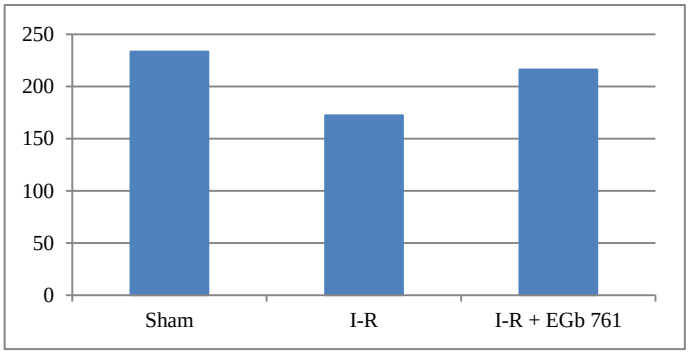

Figure 2: Comparison of anastomotic hydroxyproline level between the groups

Results

The bursting pressures and hydroxyproline levels of the materials obtained from the subjects are shown in Table 1. The one-way ANOVA was performed for both parameters. A statistically significant difference was found between the three groups (P<0.001). Tukey's multiple comparison test was conducted to determine which group caused the significant difference.

Table 1: Bursting pressures (mm HG) and hydroxyproline (µmol/g) levels of the groups

Subject number	Sham group		I-R group		I-R + EGb 761 group	
	HP	BP	HP	BP	HP	BP
1	5.56	240	4.18	132	5.34	194
2	6.11	280	4.67	192	5.22	200
3	6.47	210	4.56	108	5.89	300
4	6.91	260	3.78	168	5.56	232
5	6.23	198	4.89	140	6.45	262
6	5.11	190	4.18	240	6.89	248
7	6.78	190	4.33	110	6.23	220
8	6.34	300	5.11	236	5.56	194
9	6.56	240	3.56	182	5.34	206
10	7.56	222	3.80	152	7.34	168
11	5.89	200	4.16	202	5.34	180
12	7.34	270	4.22	206	5.40	190

I-R: ischemia-reperfusion, HP: hydroxyproline, BP: bursting pressure

The mean anastomotic bursting pressures of the three groups are shown in Table 2, and their mean anastomotic hydroxyproline levels are given in Table 3. According to the results of ANOVA and Tukey's multiple comparison test in the intergroup evaluations of anastomotic bursting pressures, there was a significant difference between the sham group and the I-R group and between the I-R group and the I-R + EGb 761 group (P<0.001 and P<0.01 respectively) while no statistically significant difference was observed between the sham group and the I-R + EGB 761 group in terms of bursting pressure (P>0.05). Similarly, the statistical analysis of the hydroxyproline level measurements performed in the samples taken from the anastomosis line revealed was a statistically significant difference between the sham group and the I-R group and between the I-R group and the I-R + EGb 761 group (P<0.001 and P<0.01, respectively); however, the values of the sham group and the I-R + EGb 761 group were similar (P>0.05).

Table 2: Mean values of anastomotic bursting pressure

Groups	Median (SD) mm Hg	*P*-value
Sham	233.333 (37.60)	<0.001
I-R	172.333 (44.97)	
I-R+ EGb 761	216.166 (38.21)	

I-R: ischemia-reperfusion

Table 3: Mean values of anastomotic hydroxyproline levels

Groups	Median (SD) µmol/g
Sham	6.405 (0.70)
I-R	4.28 (0.46)
I-R + EGb 761	5.88 (0.69)

I-R: ischemia-reperfusion

In light of these values, it was concluded that the anastomotic bursting pressure and anastomotic line hydroxyproline level values showed significant changes due to the I-R process, but they returned to normal with the use of EGb 761.

Discussion

LEI is an arterial pathology which can present with acute and chronic forms, both with high morbidity. Acute LEI, which is less common, continues to be one of the major circulatory disorders of the arterial system despite the progress in diagnostic and therapeutic modalities, and maintains its importance in clinical practice with its high morbidity and mortality rates [2]. Cardiac arrhythmias, advanced age, low cardiac output, severe heart valve diseases, recent myocardial infarction, and cardiac surgery are clinically defined risk factors for LEI [15]. In LEI, the contemporary approach aimed at reducing mortality is the continuation of aggressive diagnosis and treatment protocols [17].

The duration of ischemia directly affects the prognosis of the patient; therefore, early diagnosis and treatment are vital. However, the resulting injury is of biphasic nature and has an important share in reperfusion, as well as ischemia [2, 15, 18]. Parks and Granger showed that in the I-R process, the intestinal mucosa was damaged less in the ischemic period, and most of the injury actually occurred in the reperfusion period [19]. In addition, the idea that reperfusion is responsible for the injury seen in the intestinal models of I-R was supported by the significant reduction in mucosal damage with agents administered before ischemia [3, 5]. Especially after acute LEI, the sudden reversal of blood supply paradoxically results in systemic complications and unexpected mortality [20, 21]. It is assumed that lower extremity I-R (LEI-R) causes damage to the intestinal mucosa and permeability disorders through various mediators [22]. Although it is predicted that ischemic injury can be reduced with early diagnosis, reperfusion injury and its consequences are still inevitable in this approach. Therefore, molecular studies to prevent injury caused by reperfusion, regardless of local or remote ischemia, remain popular and are conducted extensively.

LEI-R causes dysfunction in various organs, such as the liver, lung, and cardiovascular and hematopoietic systems [23]. Pulmonary injury is characterized by the accumulation of neutrophils in the lung tissue as a result of increased microvascular permeability and the activation of agents released from the tissue exposed to I-R. Some of the systemic consequences of LEI occur through neural pathways. Although the underlying mechanism is not yet fully known, metabolites such as PAF, bradykinin, lactic acid, and prostaglandins promote a cardiovascular response and stimulate abdominal sensory nerves. In two similar studies conducted on the I-R injuries of different organs, Grace [3] and Stammberger et al. [23] mentioned that reactive oxygen metabolites, especially hydrogen peroxide and hydroxyl radicals, activated abdominal visceral C fibers, and thus affected systemic vascular tone.

In addition to LEI due to acute embolization, procedures such as kidney transplantation and less frequently liver transplantation, aortic surgery, and traumatic vascular surgery may provide a basis for the development of LEI in clinical surgery practice. In all these clinical situations, especially in the presence of traumatic LEI and multiorgan injury, intestinal anastomoses may be required if there is intestinal injury in the abdomen. There are many studies in the literature suggesting that in such cases, systemic and local injury caused by I-R have negative effects on anastomotic perfusion and the released mediators increase anastomotic complications by causing adverse effects around the anastomosis. Therefore, researchers aim to examine anastomotic healing during the reperfusion period and compare the injury caused by the severity of the

systemic effect with that which occurs during reperfusion [2, 15, 24].

In their consecutive studies, Kologlu et al. [20, 25] investigated the effects of remote organ ischemia on colonic anastomosis and found that the group exposed to LEI-R had a significant decrease in bursting pressure and mean tissue hydroxyproline level compared to the control group. Similarly, the authors showed the negative effects of 60-minute segmental small intestine I-R, unilateral lower extremity reperfusion, and renal I-R on healing in anastomoses in the right colon. In light of all these data, it can be stated that I-R injury is a systemic phenomenon, and remote I-R has a significant negative effect on intestinal anastomotic healing.

LEI-R injury is difficult to examine in clinical settings; therefore, animal models are used in studies. However, the selected model and the depth and duration of ischemia are particularly important for evaluating the results. The prevention of reperfusion injury will also not be meaningful in the absence of a sufficiently deep ischemia. In our rat model, we induced deep LEI over 30 minutes and investigated the effects of reperfusion following ischemia on the anastomotic healing process based on bursting pressure and hydroxyproline level, which are defined as the two most important parameters indicating anastomotic healing in the literature. The anastomotic bursting pressure and hydroxyproline levels being significantly lower in the I-R group compared to the sham group suggests that LEI-R negatively affected the anastomotic healing process, which supports the literature data.

There are experimental studies in the literature to prevent remote organ injury caused by LEI-R. Although various pharmacological agents can be used in animal experiments to reverse the injury caused by free oxygen radicals, most are not applicable in clinical practice. PAF is one of the most effective proinflammatory agents released in I-R, and it is known to play an important role in I-R-related intestinal dysfunction and remote organ dysfunction. In pharmacological strategies to prevent I-R injury, considering these effects of PAF, many studies have shown that different PAF antagonists can be used to partially eliminate pathophysiological mechanisms that play a role in I-R-related local and remote organ injury. Wehrens et al. [16] and Ateş et al. [18] showed that the increase in PAF levels after intestinal I-R and the resulting leukocyte adhesion and extravasation can be prevented by PAF antagonists. Similarly, in a study conducted with lexipafant, a PAF antagonist, the rats exposed to intestinal I-R were found to have significantly increased mucosal endothelial and epithelial permeability, improvement in mucosal barrier function, and decreased bacterial translocation rates after lexipafant treatment [9].

In addition to PAF antagonists mentioned above, *Ginkgo biloba* extract EGb 761, a new PAF antagonist with vascular relaxation properties and free radical scavenging effects, has also been used in some I-R models. EGb 761 has the effect of reducing mucosal injury associated with small bowel ischemia. The use of EGb 761 in rats where I-R was formed by the occlusion of the superior mesenteric artery was reported to reduce mucosal injury and significantly increase free oxygen radical levels in the intestinal mucosa compared to the control group [10]. At the same time, through the effects of the downregulation of TLR4/NF-κB and inhibition of inflammatory response, EGb 761 was shown to reduce the rate of ventricular fibrillation, the most common cause of sudden cardiac death due to myocardial I-R [26].

In light of the literature data, we further investigated EGB 761, which has the potential to prevent or regress various pathophysiological mechanisms through its role in I-R injury, in terms of its effects on anastomotic healing.

Limitations

The most important strength of this study is that it is the first and most comprehensive study on the remote effects of lower I-R on intestinal anastomotic healing conducted in an animal model, offering an alternative comparable treatment modality. The sole and only limitation of the study is that the subjects of the study population have not been assessed about an already present ischemic pathology in their lower extremities. However, there is a need for further studies on this subject, including other remote I-R models of various organs or systems of human anatomy.

Conclusions

We evaluated changes in anastomotic healing based on bursting pressure and perianastomotic hydroxyproline level measurements to determine the strength of colonic anastomosis. When the results were evaluated, anastomotic bursting pressure and hydroxyproline values were significantly lower in the LEI-R-induced rats compared to the other groups. In the group in which EGb 761 was used, there was a significant improvement, reversing the negative effects of I-R injury. In brief, bursting pressure and hydroxyproline values in the sham group significantly decreased with I-R, and returned to normal with the use of EGb 761. These results were attributed to the effects of the ability of EGb 761 to partially recover intestinal, cardiovascular and pulmonary injury by preventing endothelial and epithelial injury caused by I-R. Based on these findings, we consider that EGb 761 may become a treatment modality in anastomotic healing, especially in cases negatively affected by remote I-R in the presence of multitrauma, which is difficult to treat and has its paradoxical course.

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