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EXPERIMENTAL STUDY

Iloprost and vitamin C attenuates acute myocardial injury induced by suprarenal aortic ischemia-reperfusion in rabbits

Iriz E[1], Iriz A[2], Take G[3], Ozgul H[1], Oktar L[1], Demirtas H[1], Helvacioglu F[3], Arslan M[4]

Gazi University, School of Medicine, Department of Cardiovascular Surgery, Ankara, Turkey.
marslan36@yahoo.com

ABSTRACT

OBJECTIVE: The aim of this study was to evaluate antioxidant and cytoprotective effects of iloprost and Vitamin C in a distant organ after abdominal aorta ischemia–reperfusion injury.

MATERIAL AND METHODS: Twenty-eight New Zealand rabbits weighing 2,400–2,800 g were used for this study. The rabbits were divided into four equal groups. These groups are control group, sham group, iloprost group, and iloprost + vitamin C group. Suprarenal aorta was occluded with a vascular clamp. Following 30 minutes of ischemia, the vascular clamp was removed. Rabbits in group 3 received 10 ng/kg/min iloprost and those in group 4 received 10 ng/kg/min iloprost and 10 mg/kg vitamin C. At the end of the reperfusion period, the rabbits were sacrificed by a high intraperitoneal dose of xylazine + ketamine injection. Myocardial tissue samples were taken for electron microscopic analysis. We evaluated SOD, MDA and catalase in myocardial tissue samples.

RESULTS: Iloprost and iloprost + vitamin C groups significantly reduced the oxidative stress markers in tissue samples ($p < 0.05$) and significantly decreased the myofibrillar injury and mitochondrial morphology changes in the myocardial tissue as shown with electron microscopy ($p < 0.05$). Myocardial edema was significantly alleviated by iloprost and iloprost + vitamin C administration ($p < 0.05$).

CONCLUSIONS: This study clearly showed that myocardial injury and edema occurred after ischemia–reperfusion of abdominal aorta and that groups administered with iloprost and iloprost + vitamin C showed an attenuation of ischemia–reperfusion injury in distant organs *(Tab. 3, Fig. 4, Ref. 30)*. Text in PDF *www.elis.sk.*

KEY WORDS**:** ischemia–reperfusion, remote, myocardium, iloprost, electron microscopy.

Introduction

During most thoraco-abdominal aorta surgeries, cases of major vascular ischemia–reperfusion (IR) develop. After the ischemic events taking place in the related tissues, these cases may cause ischemia–reperfusion injuries in the distant organs as well (1–3). These problems which develop after ischemia–reperfusion may cause high morbidity and mortality after surgical procedures (4). Organs, in which blood flow is high, such as lungs, kidneys, heart and liver, are the most affected distant organs (2).

Free radicals, systemic vasoconstrictors and active neutrophils, which develop from oxygen in tissues during the reperfusion period that follows ischemia, are freed (5). The active neutrophils stick to the endothelium and excrete reactive oxygen species (ROS) and cytokines, which thus cause damage in the distant organs and local tissues (2). The excretion of ROS activates leucocytes and causes lipid peroxidation, failures in cell calcium metabolism and water loss in the cell (6). Enzymes such as superoxide dismutase, catalase, and glutathione peroxidase which are endogenic antioxidant enzymes reduce the cell damage caused by the detrimental effects of ROS. The measurement of these enzymes after IR indicates the intensity of oxidative stress (7). It is used as an indicator of malondialdehyde lipid peroxidation and clinical cell damage (8).

It has been shown that antioxidants and vasodilators reduce the tissue damage after ischemia-reperfusion (2). Iloprost prostacyclin (PgI_2) is a stable analogue with long-term action (9). It shows cell-protective effects of prostacyclin through several mechanisms such as inhibition of leucocyte activation, reduction in thrombocyte aggregation and vasodilatation (10, 11). Vitamin C is a well-known antioxidant agent. This molecule shows its effect by reducing the microvascular permeability and preventing the clustering of neutrophils (12).

The aim of this study was to evaluate the degree of edema and damage developed in the myocardium as a result of distant organ damage, as well as to analyze the effects of iloprost and vitamin C on this damage by evaluating the changes in the myocardial tissue by means of electron microscopy and by assessing the levels of antioxidant enzymes.

Material and method

In the study conducted in Gazi University Experimental Research Center (GÜDAM), with the approval of Gazi University

[1]Gazi University, School of Medicine, Department of Cardiovascular Surgery, Ankara, Turkey, [2]Gazi University, School of Medicine, Department of Otorhinolaryngology, Ankara, Turkey, [3]Gazi University, School of Medicine, Department of Histology, Ankara, Turkey, and [4]Gazi University, School of Medicine, Department of Anesthesiology and Reanimation, Ankara, Turkey

Address for correspondence: M. Arslan, Dr, Gazi University Medical Faculty, Department of Anaesthesiology and Reanimation, 06510 Ankara, Turkey.
Phone: +90.312.2026739

Faculty of Medicine Animal Ethics Committee, a total of 28 white New Zealand rabbits weighing 2,400–2,800 g have been used. Prior to the experiment, the rabbits were fed with standard rabbit diet and water *ad libitum* and kept in cages under controlled room temperature and 12/12-hour day/night cycles for at least 10 days. The rabbits in all the groups were anesthetized with an injection of 40 mg/kg ketamine (Ketalar®) and 5mg/kg xylazine, intramuscular. After the ketamine + xylazine anesthesia, the test subjects' skin on abdominal and left femoral areas was shaved, cleaned with betadine solution, and covered with sterile cloths. The left femoral areas were opened with percutaneous intervention or vertical incision, and the femoral artery was cannulated. After the ear artery and vein had been cannulated, the arterial distal and aortic pressures were constantly monitored with a transducer. Twenty minutes prior to surgical incision, the iloprost and iloprost + ascorbic acid groups were given an IV infusion of iluprost in dosage of 5 ng/kg/min. The median starting from under the xiphoid was explored with 8–9 centimeters of abdominal incision by being located on top of the abdominal aorta renal arteries. The animals were anticoagulated with 100 U/kg heparin 5 minutes before aortic clamping. Their suprarenal aorta was clamped with metal bulldog clamps. The absence of pulse in the distal aorta was checked by palpation. At the same time, it was observed that the distal aorta pressure pulse was lost on the invasive monitor. The iloprost dosage was increased to 10 ng/kg/min. In this manner, ischemia was induced by applying a 30-minute aortic occlusion. After this procedure, the bulldog clamp was removed. Iloprost was continuously administered at constant dosage of 10 ng/kg/min during 4 hours after declamping. In the group 4, the ascorbic acid was first given in a dosage of 10 mg/kg 20 minutes prior to occlusion and was continuously administered during 4 hours of reperfusion in the same dosage. After 4 hours of reperfusion, the animals were sacrificed by a high intraperitoneal dose of xylazine + ketamine injection.

The test animals were separated into four equal groups;

Group 1 (n = 7) is the sham group, which completely represents the normal, where only medical premedication and surgical incisions were performed and myocardial biochemical and histopathological evaluations were done after sacrifice;

Group 2 (n = 7) is the ischemia-reperfusion (IR) control group, which was given 0.9 % of saline at a rate of 20ml/hour through the ear vein under room temperature from the periphery, and ischemia was induced by a 3-minute aortic occlusion;

Group 3 (n = 7) is the iloprost group. Twenty minutes prior to aortic occlusion, iloprost was given at a rate of 5 ng/kg/min through the ear vein. During the ischemic process as well during four hours of reperfusion iloprost was continuously administered at a rate of 10 ng/kg/min;

Tab. 1. The averages of biochemical measurement levels within groups.

	MDA (nmol/mg protein)	SOD (U/mg protein)	Catalase (k/mg protein)
Group 1	0.11±0.08	2.12±0.77	5.99±2.23
Group 2	0.35±0.05	4.82±1.13	14.11±2.01
Group 3	0.14±0.07	2.78±0.55	8.12±1.71
Group 4	0.12±0.04	2.34±0.59	7.14±1.41

Group 4 (n = 7) is the iloprost + ascorbic acid group. Twenty minutes prior to aortic occlusion, iloprost was given at a rate of 5 ng/kg/min through the ear vein; ascorbic acid was given in a dose of 10 mg/kg. During the ischemic process as well as during four hours of reperfusion, iloprost was given continuously in an infusion at a rate of 10 ng/kg/min and ascorbic acid at a dose of 10 mg/kg.

Biochemical evaluation

The myocardium tissue samples were taken and the tests were performed in the following manner:

Malondialdehyde

MDA level, an indicator of free radical generation, was measured by the double heating method of Draper and Hadley (13). The principle of the method lies in spectrophotometric measurement of color produced by the reaction of thiobarbituric acid with malondialdehyde. For this purpose, 2.5 ml of 100 g/L trichloroacetic acid solution was added to 0.5 ml supernatant in each centrifuge tube. The tubes were placed in boiling water bath for 15 min and then cooled in tap water. The tubes were centrifuged at 1,000 g for 10 min, and 2 ml of the supernatant was added to 1 ml of 6.7 g /L thiobarbituric acid solution, and was placed in a boiling water bath for 15 min. The solution was then cooled in tap water and its absorbance was measured with a spectrophotometer (UV-1601; Shimadzu, Kyoto, Japan) at 532 nm. The concentration of MDA was calculated by the absorbance coefficient of the malondialdehyde-thiobarbituric acid complex (absorbance coefficient € = $1.56x10^5$ $cm^{-1}.M^{-1}$) and expressed as nanomoles per milligram of protein (nmol/mg protein).

Superoxide dismutase

Superoxide dismutase activity was measured by the method of Spitz and Oberley (14) and Woolliams (15). The determination of superoxide dismutase activity was based on the reaction of xanthine with xanthine oxidase to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazoliumchloride to form a red formazan dye. The superoxide

Tab. 2. Score table of myocard electron microscope findings.

Myocyte	Scoring (point)
Normal	0
Nucleus contraction	1
Sparce chromatine	2
Nucleus fragmantation	3
Extinction of myofibrils	
None	0
Mild	1
Intermediate	2
Severe	3
Mitochondrial morphology	
Normal	0
Clear crista	1
Obliteration of crista	2
Unshaped mitochondria	3
Collagen fiber	
Normal	0
Mild	1
Intermediate	2
Severe	3

Tab. 3. Average score of heart electron microscope findings.

Groups	Myocyte -nucleus	Myocyte -edema	Myofibrile obliteration	Mytochondrial morphology
1	0	0.16	0.5	0
2	1.5	2.16	2.1	1.66
3	0.6	1.16	2	1.5
4	0.5	0.33	0.66	0

dismutase activity is then determined as the degree of inhibition of this reaction. Results are expressed as units per milligram of protein (U/mg protein).

Catalase

Catalase activity was measured by the method of Aebi (16). The assay is based on the determination of the rate constant (s^{-1},k) of hydrogen peroxide decomposition. The rate constant was calculated with the formula $k = (2.3/\Delta t)(a/b) \log (A_1/A^2)$ where A1 and A2 are the absorbance values of hydrogen peroxide at times t1 (0th second) and t2 (15th second), while a is the dilution factor, and b is the protein content of the supernatant. Results are expressed as catalytic activity per milligram of protein (k/mg protein).

Histopathological evaluation

Electron microscopic studies

Tissues of all groups were fixed in phosphate buffer containing 2.5 % glutaraldehyde (Sigma-Aldrich Co.) for 2–3 h, post-fixed in 1 % osmium tetra oxide (Sigma-Aldrich Co.) and dehydrated in a series of graded alcohols (50, 60, 70, 80, 90, 96 and 100 % ethanol). After passing through propylene oxide (Sigma-Aldrich Co.), the specimens were embedded in Araldite CY 212 (Ciba-Geigy), (2-dodecen-1-yl) succinic anhydride (Sigma-Aldrich Co.), benzyldimethyl amine (Poly Sciences Inc.) and dibutylphtalate (Sigma-Aldrich Co.). The semi-thin sections were stained with toluidine blue (Sigma-Aldrich Co.), and examined with a photomicroscope (Leica DM4000, Germany). After the selection of appropriate specimens, thin sections were cut and stained with uranyl acetate (Pro Sci Tech) and lead citrate (Sigma-Aldrich Co.). They were examined by means of an electron microscope (Carl Zeiss EM 900, Germany) (17).

Statistics

Statistical analyses were carried out using SPSS version 20.0 statistical software package (SPSS Inc., Chicago, IL). The results were expressed as mean ± standard deviation (SD). p values < 0.05 were considered statistically significant in all statistical analyses.

Kolmogorov–Smirnov test was used to determine the normality of the distribution of parameters. Kruskal-Wallis test was used to evaluate the difference among the independent groups with normal distribution. In case of a difference between the groups, the comparison was made using Mann-Whitney U test.

Results

Biochemical analysis

The average measurements of MDA, SOD, and catalase enzyme levels, which have undergone biochemical changes, are shown per each group in Table 1. According to these findings, after the aortic ischemia–reperfusion procedure, the measurement values of group 2 have shown a statistically significant increase in comparison to group 1 ($p < 0.05$). A statistically significant decrease was observed in enzyme levels of groups 3 and 4, in comparison to group 2 ($p < 0.05$). No statistically significant difference was determined between the enzyme levels of groups 3 and 4 ($p > 0.05$).

Histopathological evaluation

The impact of ischemia and reperfusion on heart muscle cells manifested in mitochondrial increase and lipid droplets due to the reflection of excessive need for energy as a result of distinct myofibrillar extinction (Figs 1 and 2). Intracytoplasmic edema was distinct around the nucleus. In the groups where iloprost was applied, no positive structural change has been observed in terms of extinction of myofibrils after ischemia and reperfusion, but it is noteworthy that the edema decreased. As distinct from the ischemia and reperfusion group, it has been determined that the mitochondria completely filled the areas, where the myofibrillar organization was corrupted (Fig. 3). In the experiment group where the effect of iloprost and ascorbic acid on ischemia and reperfu-

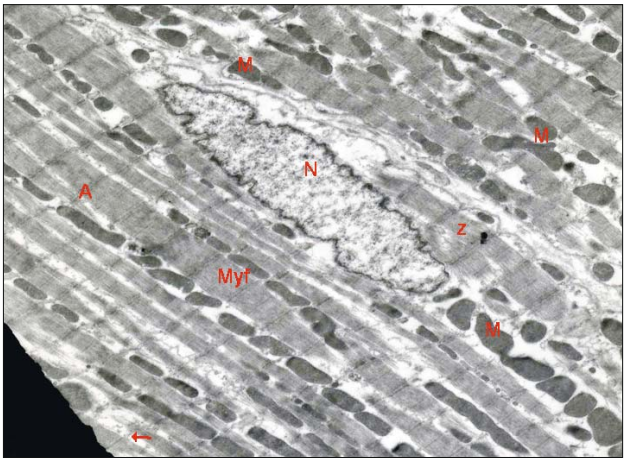

Fig. 1. Sham control group.

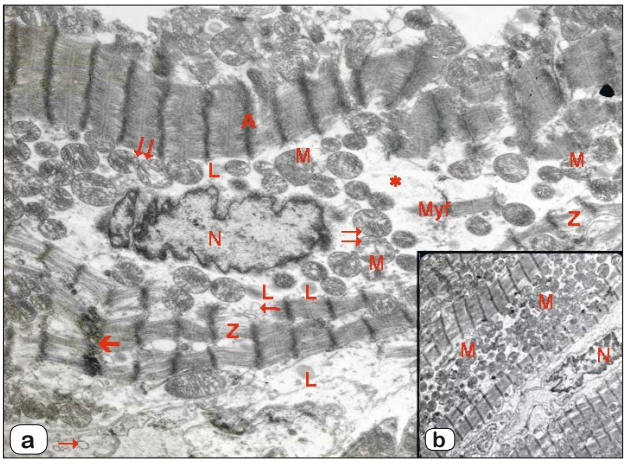

Fig. 2a–2b. Ischemia and reperfusion group.

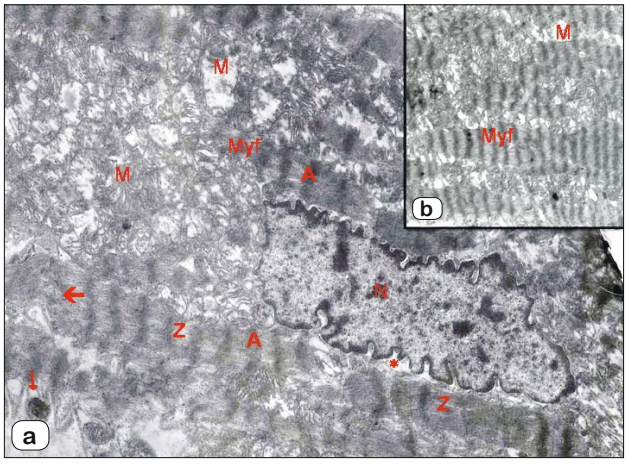

Fig. 3a–3b. Group administered with iloprost with ischemia and reperfusion.

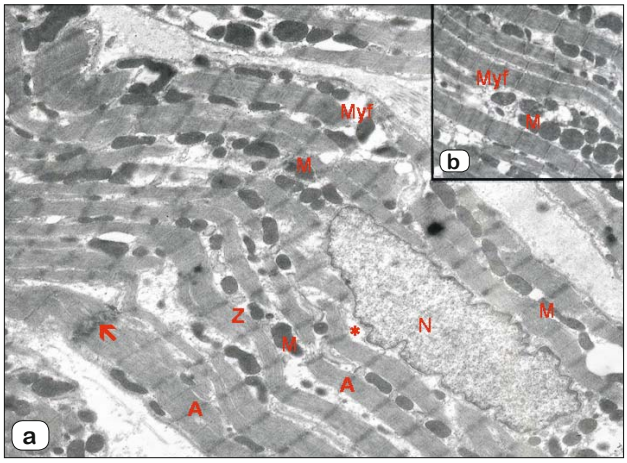

Fig. 4a–4b. Group, which was administered with iloprost and ascorbic acid with ischemia and reperfusion; heart muscle electron microscope pictures.

sion was evaluated together, it has been observed that myofibril organization and mitochondrial distribution showed similarities with the sham control group (Fig. 4).

Based on the evaluation of mitochondrial increase as a result of the corruption of the structural unity caused by ischemia, reperfusion, and increase in the need for energy in the heart muscle cells, it has been determined that the single application of iloprost has been insufficient for achieving structural unity in terms of mitochondrial increase. It has been determined that iloprost and ascorbic acid in combination were more efficient in attenuating structural degeneration observed after ischemia and reperfusion.

The scoring of the changes which took place in the myocardial tissue are shown in Table 2 while the myocardial electron misroscope findings' scoring averages in parallel with this scoring are shown in Table 3. As seen from this scoring table, in group 1 all four cell components (myocyte nucleus, myocyte edema, extinction of myofibril and mitochondrial morphology), are equal or close to 0, which is the normal cell score and in group 2, all four cell components (myocyte nucleus, myocyte edema, extinction of myofibril and mitochondrial morphology) are closer to score 2, which is the best score in comparison to score 3 which is the worst score in the scoring average in terms of indicating degeneration (Average 2). In group 3, however, it can be seen that the average of these values decrease significantly in myocyte edema and myocyte nucleus parameters while in group 4, all four scoring parameters are significantly lower compared to group 2 and their measured values show to be even similar to those in group 1.

Discussion

Aortic cross-clamp application is a routine process, performed during cardiovascular interventions. After thoraco-abdominal aorta surgery, tissue changes may be seen not only in the tissues remaining in ischemia, but also in distant organs such as lungs and heart, depending on post-ischemic conditions (18). After aortic clamping, the tissue reactions resulting from ischemia under the clamp may without doubt cause damage in distant organs through systematic inflammation reaction as the clamp is removed (19, 20). In the reperfusion period taking place in the tissues, significant increases are observed in microvascular permeability through an increase in inflammation in distant organs (3).

In many studies conducted in the past, it has been published that after aortic ischemia-reperfusion, damage occurred in distant organs such as kidneys (2, 21), lungs (3) and liver (22). However, although it was thought that distant organ damage may occur in the myocardium after ischemia–reperfusion of the abdominal aorta, only one study has been found in literature on this subject (23). Therefore, this study analyzed biochemical as well as electron microscopic aspects of distant organ damage in the myocardium after aortic ischemia–reperfusion.

During the ischemic period, the blood membranes cannot preserve their unity and this causes the release of calcium, phospholipid A2, fatty acid radicals and polyunsaturated fatty acids (2, 24). This reaction increases the membrane permeability, stimulates chemotaxis of leucocytes, and increases the release of proteolytic enzymes and oxygen-based free radicals (24). Various inflammatory mediators are secreted such as cytokines, protease, and reactive oxygen species (ROS) from active leucocytes. These molecules play a key role in tissue damage by affecting the surrounding and distant endothelial cells (24). In the reperfusion period, the molecules formed during the earlier mentioned ischemia, increase the damage in tissues even more (25, 26). During the reperfusion period, these toxic molecules are carried to distant organs by blood, where they cause respiratory insufficiency, heart dysfunctions and systemic toxic reactions which are life-threatening in organs such as lungs, heart, liver, and kidneys (27–30).

As stated in a single study dealing with aortic ischemia–reperfusion (23), the levels of myeloperoxidase and IL-6 on the myocardium levels have been determined to be high after 48 hours of reperfusion. In the latter study, the aorta clamping was performed in the infrarenal aorta, contrary to our study.

In our study, the tissue levels of MDA, SOD, and catalase enzyme have been observed to be significantly lower in groups 3 and 4, in comparison to group 2; in addition, in terms of changes

which took place in myocyte edema and mitochondria formation, parallel results were observed by means if electrone microscopic analysis . In the light of these findings, it has been determined that iloprost and especially a combination of iloprost and vitamin C have reduced the distant organ damage developed after suprarenal aortic ischemia–reperfusion in the myocardium.

As a result, a 30-minute ischemia in the suprarenal aorta and 4-hour reperfusion can cause distant organ ischemia–reperfusion damage in the myocardium tissue. Although iloprost showed to have protective properties, it was the combination of vitamin C with iloprost that showed to be much more beneficial.

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