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Original Article

Effects of ischemic preconditioning and iloprost on myocardial ischemia-reperfusion damage in rats

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Abstract: This study investigates the effects of cardiac ischemic preconditioning and iloprost on reperfusion damage in rats with myocardial ischemia/reperfusion. 38 male Wistar Albino rats used in this study were divided into 5 groups. The control group (Group 1) (n=6), ischemia/reperfusion (IR) group (Group 2) (n=8), cardiac ischemic preconditioning (CIP) group (Group 3) (n=8), iloprost (ILO) group (Group 4) (n=8), and cardiac ischemic preconditioning + iloprost (CIP+ILO) group (Group 5) (n=8). Pre-ischemia, 15 minutes post-ischemia, 45 minutes post-reperfusion, mean blood pressure (MBP), and heart rates (HR) were recorded. The rate-pressure product (RPP) was calculated. Post-reperfusion plasma creatine kinase-MB (CK-MB), lactate dehydrogenase (LDH), troponin (cTn) vlaues, and infarct size/area at risk (IS/AAR) were calculated from myocardial tissue samples. Arrhythmia and ST segment elevations were evaluated during the ischemia and reperfusion stages. Although the MBP, HR, RPP values, biochemical parameters of CK-MB and LDH levels, IS/AAR rates, ST segment elevation values were found to be similar in CIP and CIP+ILO groups and the IR and ILO groups ($p>0.05$), CIP-containing group values had a positively meaningful difference ($p<0.05$) compared with the IR and ILO group. While mild-moderate findings of damage were observed in Group 3 and Group 5, severely findings of damage were releaved in Group 2 and Group 4. The arrhythmia score of the ILO group was meaningfully lower ($F: 41.4$, $p<0.001$) than the IR group. We can conclude that the effects of myocardial reperfusion damage can be reduced by cardiac ischemic preconditioning, intravenous iloprost reduced the incidence of ventricular arrhythmia associated with reperfusion, and its use with CIP caused no additional changes.

Keywords: Injury, ischemia-reperfusion, ischemic preconditioning, myocardial, iloprost

Introduction

Ischemia/reperfusion (IR) damage occurs in the myocardium as a result of sudden reperfusion of the ischemic myocardial tissue. Reperfusion damage is a factor that has negative impact on the morbidity and mortality of cardiac surgical and coronary interventions as it causes myocardial injury and cardiac dysfunction. Reperfusion damage can pose a serious problem particularly in coronary cardiac surgery. The cardiac ischemic preconditioning (CIP) method has been initially described by Murry in 1986 in order to protect myocardial tissue from reperfusion damage, and many studies have been made on the subject [1-3]. CIP is the application of intermittent and short-term ischemia on the tissue before the long ischemia in order to increase the resistance of the myocardial tissue to extended ischemia. Reperfusion after short ischemic periods increases the resistance of the myocardium to extended ischemia. In addition to its anti-ischemic effect, CIP shows a protective effect and anti-arrhythmic effect against reperfusion in coronary endothelial cells [4].

A prostaglandin analogue used mainly in the treatment of peripheral artery disease and pulmonary hypertension, iloprost is effective in vasodilatation and platelet aggregation inhibition, with fibrinolytic effects and protecting effects against ischemia-reperfusion damage in many organs as well as apoptosis-preventing

Figure 1. Illustration of the experimental protocols. Hearts in all groups were subjected to 15 min of ischemia followed by 60 min reperfusion. IR: ischemia-reperfusion; CIP: cardiac ischemic preconditioning; ILO: iloprost.

effects and protecting effects against reperfusion damage in myocardial tissue [5-7]. It has been observed that iloprost was effective in protecting the myocardium when placed in cardioplegia solution [8]. This experimental study investigates the effects of CIP and iloprost on reperfusion damage in rats receiving myocardial IR.

Methods

Animals

A total of 38 male Wistar Albino rats of 8 weeks of age, weighing 285-350 grams, were included in this study. Before the experiment at the Animal Laboratory of Bezmialem Vakif University, the rats were fed ad libitum by regular rat chow and water for 1 week to achieve adaptation. Six hours before the surgical procedure, they were starved but were allowed to drink water freely. The effects of all intervention and surgical procedures to be carried out on the subjects on the hemodynamic, biochemical and pathologic data in the entire study were neutralized using sham-controlled group. All of the experimental protocols were performed according to the guidelines for the ethical treatment of experimental animals. The study was approved by the ethical committee.

Anesthesia and monitoring

The subjects were anesthetized by intraperitoneal administration of 35 mg/kg ketamine (Ketalar®, Eczacıbası, Istanbul, Turkey) and 5 mg/kg Xylazine (Rompun®, Bayer, Istanbul, Turkey). The neck and the anterior chest wall were shaved and the surgical area was stained using a 10% povidone-iodine (Isosol, Merkez Laboratuvarı A.S.) solution. Tracheostomy was opened by neck incision and intubation was performed. The subjects were then mechanical animal respirator at a respiratory rate of 60/min, with 100% oxygen support, and 15 ml/tidal volume. The carotid artery was catheterized for continuous pressure monitoring, and the jugular vein was catheterized for the intravenous (IV) administration of SF with a 24 G catheter.

Surgical procedure

Anterolateral thoracotomy was performed from the fourth left intercostal space to expose the heart. The surgical manipulation area was extended using a mini thoracic retractor and the heart was exposed by incising the pericardium. A 9/0 6,4 mm atraumatic needle prolene suture was advanced intramyocardially from the proximal of the left anterior descending (LAD) branch of the left main coronary artery extending intraventricularly. Snare was delivered and ischemia was induced in the LAD region. At the end of each ischemia period, the snare was loosened and the ischemic region was reperfused. To reduce insensible loss during this time, thoracotomy incision was approximated with temporary prolene suture.

Experimental protocols

The test animals were divided into 5 groups (**Figure 1**). Group 1: control group (n=6), thoracotomy was performed and the LAD branch was seen; Group 2: I/R group (n=8), test animals received only myocardial ischemia for 15 minutes and reperfusion for 45 minutes; Group 3: CIP group (n=8), following myocardial ischemia reperfusion to test animals in 3-minute intervals for 3 periods, ischemia was performed for 15 minutes and reperfusion for 45 minutes; Group 4: ILO group (n=8), during ischemia and reperfusion, an infusion of 2.0 ng/kg/min iloprost was administered to test animals, and then they received 15 minutes of myocardial ischemia and 45 minutes of reperfusion; Group 5: CIP+ILO group (n=8), during ischemia and reperfusion, an infusion of 2.0 ng/kg/min iloprost was administered to test animals, and

then they received 15 minutes of ischemia and 45 minutes of reperfusion following 3 minutes of myocardial ischemia reperfusion in 3 periods.

Hemodynamic functions

Mean blood pressure (MBP), heart rate (HR), ST-segment elevations were recorded before ischemia, 15 minutes after ischemia, and 45 minutes after reperfusion. The rate-pressure product (HR×SBP, heart rate pressure product (RPP)) was calculated.

Ischemia-induced ventricular arrhythmias were counted during the occlusion period and determined in accordance with the Lambeth Conventions. Ventricular ectopic beats (VEBs), ventricular tachycardia (VT), ventricular fibrillation (VF), multipart forms of VEBs such as bigeminy, couplet and salvos were counted at separate episodes. The incidence, time of occurrence and duration of arrhythmias were used to identify arrhythmias severity according to the following scoring system [30]; 0: 0-49 VEBs, 1: 50-499 VEBs, 2: >499 VEBs and/or 1 episode of spontaneously reverting VT or VF, 3: >1 episode of VT or VF or both with a total duration <60 s, 4: VT or VF or both 60-120 s total duration, 5: VT or VF or both >120 s duration, 6: fatal VF starting at >15 min after occlusion, 7: fatal VF starting between 4 and 14 min 59 s, 8: fatal VF starting between 1 and 3 min 59 s, and 9: fatal VF starting <1 min after occlusion [9].

Histopathological examination of heart

Histopathological changes of heart tissues were investigated using light microscope. Heart tissues of control group rats were observed in normal structure (**Figure 2A**). Damage of the heart tissue was evaluated according to the following condition; contraction band necrosis, coagulation necrosis with cytoplasmic eosinophilia, cytoplasmic vacuolization in cardiac muscle cells, inflammatory cell infiltration, edema, disorganization and degeneration in myocardial fibers, loss of nuclei. The damages were divided to mild, moderate and severe for histopathologically (**Figure 2B-F**).

Cardiac area at risk and infarct size determination

The coronary artery was re-occluded in order to identify the AAR. Evans blue dye (2 ml, 2%) was then injected through the lateral tail jugular vein. The heart was harvested 2 min. later, excised; atria and the roots of the great vessels were removed. Remaining tissues were frozen for 24 h. Then they were cut into 2 mm slices. All pieces were incubated in 1% solution of 2,3,5-triphenyltetrazolium chloride at 37°C for 15 min. to visualize the infarct area. Then, they were fixed for 2 days in 10% formalin to enhance the contrast. The non-ischemic area, AAR and infarcted area were colored blue, brick red and pale, respectively. Sections were scanned to determine normal area, AAR and IS by calculating pixels occupying each area using Adobe PhotoShop software. Total AAR and IS were expressed as the percentage of total ventricle and AAR, respectively.

Biochemical analysis

Blood samples were collected and centrifuged after each test. Plasma samples were stored at -70°C until an analysis was performed. The CK-MB isoenzyme, LDH levels were analyzed. The quantity of cardiac cTnI was determined by an enzyme-linked immunoassay (ELISA) according to the manufacturer's protocol.

Statistical analysis

SPSS 13.0 software (SPSS, Chicago, IL, USA) was used for statistical analysis. Data were given in mean and standard deviation. One-way Anova test was used for independent group comparisons, and repeated Anova test was used for repeated intragroup comparisons. Tukey's test was used after one-way Anova, and Bonferroni's method was used after repeated Anova as the post-hoc test. $p<0.05$ (95% confidence interval) was considered to be statistically meaningful.

Results

Hemodynamic data

There was no meaningful difference between the groups in HR values ($F=2.66$, $p=0.067$; $F=0.22$, $p=0.87$; $F=1.26$, $p=0.30$; $F=0.45$, $p=0.71$) during basal MBP, HR, RPP and ischemia. A meaningful difference was found between the IR, ILO, CIP and CIP+ILO groups between MBP, HR, RPP measured during reperfusion, and MBP and RPP values measured during ischemia ($F=38.19$, $p<0.001$; $F=19.17$, $p<0.001$; $F=59.56$, $p=0.001$; $F=7.031$, $p=$

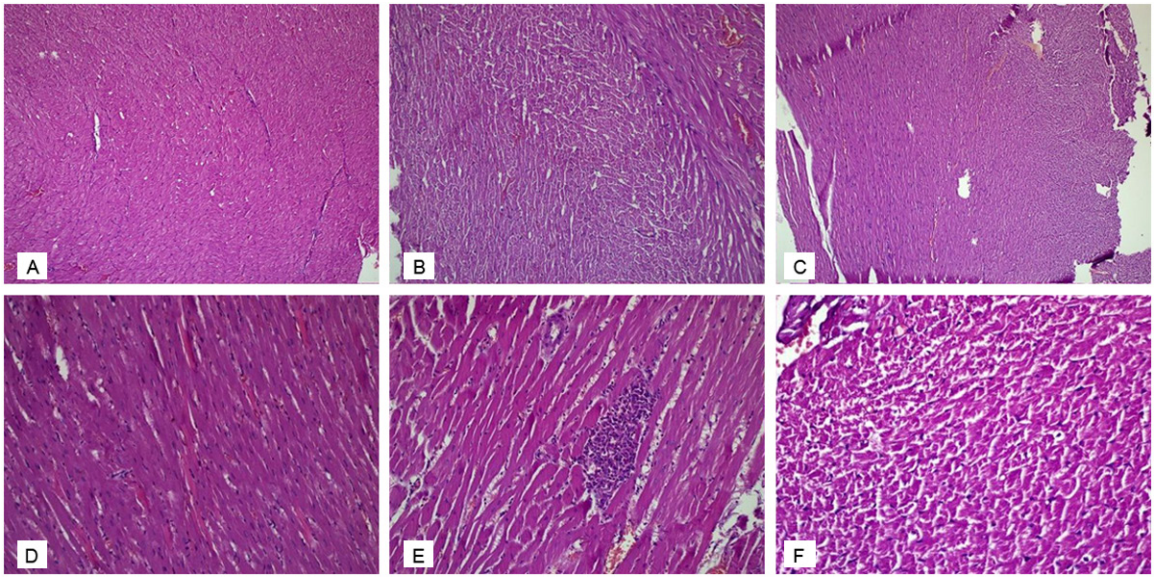

Figure 2. A. Shown is a microscopic view of normal heart muscle (H&E stain, x40). B. Focal affect: mild – moderate degenerative changes on the left side, relatively normal areas on the right side (H&E stain, x100). C. Focal affect: mild – moderate degenerative changes on the right side, relatively normal areas on the left side (H&E stain, x40). D. Contraction band necrosis (H&E stain, x200). E. Inflammatory cell infiltration (H&E stain, x200). F. Coagulation necrosis with cytoplasmic eosinophilia, cytoplasmic vacuolization in cardiac muscle cells, loss of nuclei, edema, disorganization and degeneration in myocardial fibers (H&E stain, x200).

0.001; F=4.787, p=0.00, respectively). While a comparison of the groups in pairs showed no meaningful differences for MBP values measured during ischemia between the IR and ILO groups and CIP and CIP+ILO groups (p=0.955; p=0.986, respectively), MBP was found to be significantly lower in the CIP and CIP+ILO groups compared with the IR and ILO groups (p=0.02, p=0.006 and p=0.44, p=0.13, respectively). RPP values calculated during ischemia were found to be significantly higher (p=0.021) only in the CIP group compared with the ILO group.

A meaningful decrease (F=67.9, p<0.001; F=7.04, p=0.02; F=19.8 p=0.002; F=11.6, p=0.009) was observed in MBP values compared with basal values in all groups. During the reperfusion process following ischemia, no meaningful difference (p=1.0, p=0.59, p=0.61, p=0.65) was observed in the MBP values of groups.

While a meaningful decrease (F=39.1, p<0.01; F=17.9, p=0.003; F=38.2, p<0.001; F=10.08, p=0.01) was observed in HR values during ischemia compared with basal values in all groups, a meaningful decrease was found (p<0.001; p=0.04) in HR values in IR and ILO groups during reperfusion, with an insignificant increase (p=0.30; p=0.34) in CIP and CIP+ILO groups.

While there was a meaningful decrease (F=73.4, p<0.001; F=25.5, p=0.001; F=536.3, p<0.001; F=6.3, p=0.03) in RPP values during ischemia compared with basal values in all groups, there was a meaningful decrease (p=0.008) in RPP values in the IR group during reperfusion, an insignificant decrease (p=1.0) in the ILO group, and an insignificant increase (p=0.30; p=0.34) in the CIP and CIP+ILO groups (**Table 1**).

A meaningful ST elevation was observed during ischemia, with a meaningful decrease (F=107.4, p=0.001; F=93.2, p=0.001; F=88.6, p=0.001; F=13.1, p=0.01) in ST elevation during reperfusion in all groups. A comparison of the groups in pairs showed that ST elevation levels of IR and ILO groups after 10 and 15 minutes of ischemia, and after 20 minutes of reperfusion were meaningfully higher (F=45.2, p<0.001; F=22.7, p<0.001; F=32.3, p<0.001) than the CIP and CIP+ILO groups. However, no meaningful difference was found after 10 and 15 minutes of ischemia and after 20 minutes of reperfusion between the IR and ILO groups (p=0.70; 0.97;

Table 1. Hemodynamic datas

		Baseline	Ischemia	Reperfusion		
Group	n		**MBP**		F*	p*
IR	8	100±5	78±9	77±5	67.972	0.000
CIP	8	102±5	92±9	96±3	7.049	0.027
ILO	8	96±3	76±8	79±5	19.886	0.002
CIP+ILO	8	98±3	90±7	94±2	11.687	0.009
	F#	2.668	7.031	38.198		
	p#	0.067	0.001	<0.001		
Group	n		**HR**		F*	p*
IR	8	347±21	315±10	291±9	39.170	0.000
CIP	8	353±19	314±11	319±6	17.966	0.003
ILO	8	349±19	317±12	303±8	38.214	0.000
CIP+ILO	8	344±23	310±15	317±9	10.081	0.012
	F#	0.226	0.459	19.179		
	p#	0.877	0.713	<0.001		
Group	n		**RPP**		F*	p*
IR	8	34.798±3557	24.992±2585	22.425±1416	73.482	0.000
CIP	8	36.236±2855	28.971±3613	30.712±1454	25.553	0.001
ILO	8	33.659±1503	24.228±3159	24.027±1913	536.390	0.000
CIP+ILO	8	33.680±3784	28.272±2707	29.868±1203	6.382	0.033
	F#	1.268	4.787	59.567		
	p#	0.304	0.008	<0.001		

CIP: Cardiac Ischemic Preconditioning, HR: Heart Rate, IR: Ischemia-Reperfusion, ILO: Iloprost, MBP: Mean Blood Pressure, RPP: Rate Pressure Product. *Adjustment for multiple comparisons, Bonferroni, #Tukey HSD.

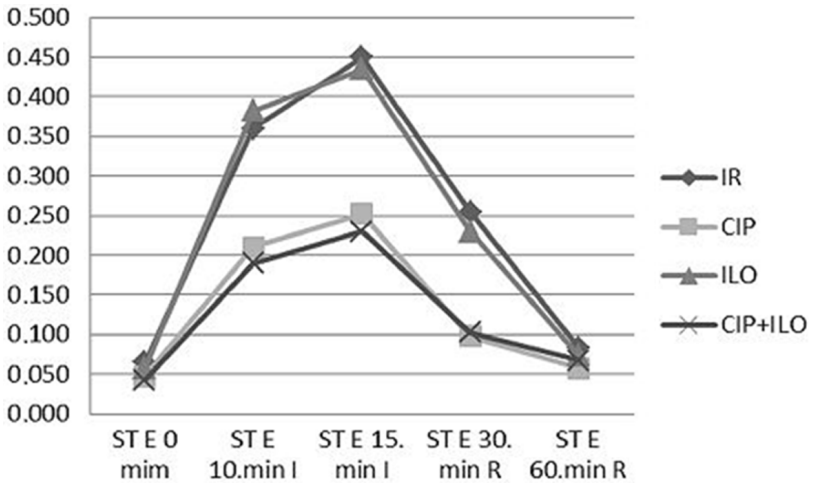

Figure 3. ST segment elevation. CIP: Cardiac Ischemic Preconditioning, IR: Ischemia-Reperfusion, ILO: Iloprost.

0.62) and CIP and CIP+ILO groups (p=0.77; p=0.91; p=0.99) (**Figure 3**).

Biochemical analysis

There was a meaningful increase (p<0.001) in CK-MB and LDH values compared with the control group. While there were no differences between the IR and ILO group in CK-MB and LDH values (p=0.99, p=0.52), there was a meaningfully higher (p<0.001) difference compared with the CIP and CIP+ILO groups. No meaningful difference (p=0.94, p=0.95) was found between the CK-MB and LDH values of CIP and CIP+ILO groups (**Figure 4A**).

A meaningful increase (p<0.05) was found in cTn values of all groups compared with the control group. While there was no meaningful difference (p=0.93) between the cTn values of the IR and ILO group, the cTn value of the CIP+ILO group was found to be meaningfully lower than the IR and ILO groups (p=0.006, p=0.042). There was no meaningful difference (p=0.14) between the cTn values of CIP and ILO groups (**Figure 4B**).

Histopathological examination of heart

Heart tissues of control group rats were observed in normal structure (**Figure 2A**). While mild-moderate findings of damage were observed in Group 3 and Group 5 (**Figure 2B** and **2C**), severely findings of damage we-

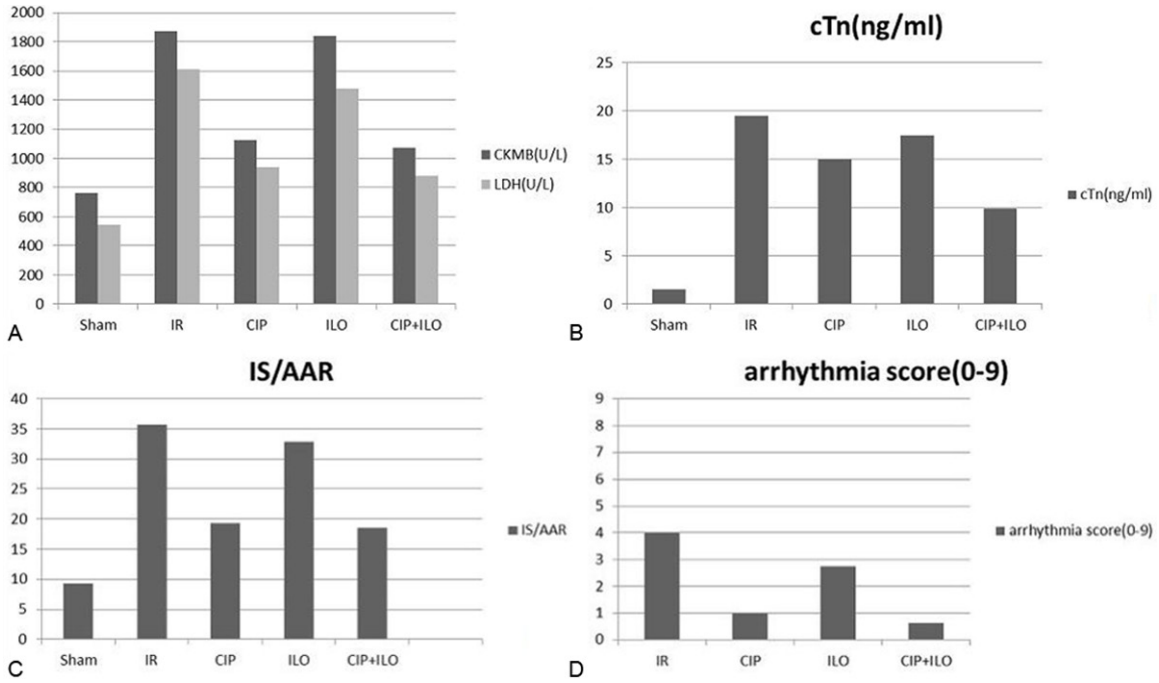

Figure 4. A. The creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) values. B. Troponin (cTn) levels. C. Infarct size/area at risk (IS/AAR) ratio. D. Arrhythmia score. CIP: Cardiac Ischemic Preconditioning, IR: Ischemia-Reperfusion, ILO: Iloprost.

re releaved in Group 2 and Group 4 (**Figure 2D-F**).

Infarct size/area at risk ratio

The IS/AAR rate was bigger ($p<0.001$) in all groups compared with the control group. While no difference ($p=0.53$) was observed between the IS/AAR rates of IR and ILO groups, the difference was meaningfully higher compared with the CIP and CIP+ILO groups ($F=65.3$, $p<0.001$). No meaningful increase ($p=0.99$) was found between the IS/AAR rates of CIP and CIP+ILO groups (**Figure 4C**).

Arrhythmia score

Arrhythmia score was highest in the IR group. While there was no meaningful difference ($p=0.73$) between the arrhythmia scores of CIP and CIP+ILO groups, the difference was meaningfully low ($F=41.4$, $p<0.001$) when compared with the IR and ILO groups. The arrhythmia score of the ILO group was meaningfully lower ($p=0.01$) than the IR group (**Figure 4D**).

Discussion

Ischemia-reperfusion lads to an inflammatory reaction lading to irreversible tissue damage in parenchymal organs such as the heart. Membrane integrity is interrupted during ischemia, so calcium, phospholipid A2, polyunsaturated fatty acids and fatty acid radicals are formed. If oxygenation starts again during ischemia, the fatty acid radicals react with the oxygen, starting lipid peroxidation. This reaction increases membrane permeability and stimulates leucocyte chemotaxis, free oxygen radicals are released and proteolytic enzymes are activated. Activated leucocytes release various inflammatory mediators including cytokines, neutrophil proteases and reactive oxygen species. All such products cause endothelial cell damage which is considered to play a key role in tissue damage. Following the restoration of blood flow due to the free oxygen radicals that have formed, tissue damage and cellular necrosis occur during reperfusion [10]. Reperfusion damage after coronary ischemia-reperfusion can lead to necrosis in myocytes, coronary endothelial and microvascular dysfunction, myocardial stunning, and arrhythmia.

Applied in the form of intermittent and short-term ischemia before long ischemia, cardiac ischemic preconditioning has a strong protective effect against reperfusion damage of the myocardium. It is common knowledge that 3-4

times administration of ischemia reperfusion episodes carried out before long ischemia in CIP procedure is more effective than single administration. CIP is effective in two phases. The first one, the early phase, starts immediately after ischemia and last for about 1-2 hours. And the late phase occurs 12-24 hours later, showing protective effect for up to 72 hours [11]. Although the early phase is more effective than the late phase in reducing the infarct area, the late phase is more effective against reversible postischemic myocardial dysfunction (myocardial stunning). Experimental studies generally evaluate the effects of CIP in the early phase. As an example to the late phase, it has been reported that preinfarct angina, the clinical analog of CIP, positively contributes to left ventricular remodeling following complete revascularization, LV systolic and diastolic functions [12]. It has also been established that it has protective effects against noninvasive limb ischemic preconditioning in myocardial reperfusion damage [13].

We had a 3-episode CIP in our study and checked its effects in the early phase. No meaningful difference was seen between ST segment elevations and arrhythmia scores in all stages between CK-MB, LDH, cTn, IS/AAR rates in groups where iloprost was administered with CIP and CIP. So, it was seen that the iloprost support had no negative effect on CIP administration, and that it also had no additional benefits in protecting against reperfusion. This can be due to iloprost remaining in the shadow of CIP's very strong effect against reperfusion damage.

Iloprost is a protective agent for endothelial cells, a potent vasodilator, causes dilatation in arterial venules, reduces vascular permeability increased during microcirculation, platelet adhesion and aggregation, leucocyte migration following endothelial damage, and the production of free oxygen radicals [14, 15]. It has been reported that iloprost has a protective damage against reperfusion damage in lower extremities in lower extremity I/R studies as well as reducing potential renal injury in distant organ damage [10]. This study investigates the effects of intravenous iloprost administration during myocardial ischemia reperfusion. No differences were observed between the CK-MB, LDH, IS/AAR rates of IR and ILO groups and ST segment elevations in all phases. cTn values of the CIP+ILO group was found to be meaningfully lower compared with the CIP group (p=0.02, p=0.006). The arrhythmia score was observed to be meaningfully lower in the ILO group.

In our study, the CK-MB, LDH, cTn values were meaningfully higher in all groups compared with the control group. It was found that the CK-MB, LDH values of CIP and CIP+ILO groups were lower compared with the IR and ILO groups, IS/AAR rates were lower, and ST segment elevations were lower in all stages.

Our study found that the ILO group was not meaningfully different from the IR group for hemodynamic and laboratory values, but that the arrhythmia score was meaningfully lower in the ILO group.

Conclusion

We consider that cardiac ischemic preconditioning in myocardium strongly protects from the effects of I/R damage, iloprost administered during myocardial I/R reduces the incidence of ventricular arrhythmia secondary to I/R, and the use of iloprost with CIP contributes to the effects of CIP by reducing the incidence of arrhythmia and through reduction in plasma cTn values.

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Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

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