Myocardial Protection with Remote Ischemic Preconditioning in Congenital Heart Surgery: Does It Deliver What is Expected?

Running Title: Remote Ischemic Preconditioning Abstract

Abstract

Aim: The discovery of the protective effects of antioxidant agents on organ functions enabled this system to be tested artificially. The impact of remote ischemic preconditioning on surgical clinical outcomes in patients with congenital heart defects is unclear. This study investigated the early consequences of ischemic preconditioning on cardiac protection.

Materials and Methods: A prospective review of all patients who underwent complex congenital heart surgery procedures at a single center was performed. The antioxidant enzymatic analysis was performed on blood samples taken from randomly grouped patients.

Results: The patients' surgical median age was 19.1 months (3.7- 57.7) in the ischemic preconditioning group (group 1) and 16.7 months (7.8- 35.9) in the control group (group 2). The patients' median follow-up period was 58.3 months (54.3- 62.1) in group 1 and 37.1 months (34.8- 41.7) in group 2. Early mortality was in 4 (4.4%) patients. There was no late mortality. There was a significant difference between the groups regarding Superoxide Dismutase, Malondialdehyde, and cardiac markers levels ($p< 005$).

Conclusion: The effects of ischemic preconditioning on cardiac protection have not been proven yet. Especially in congenital cardiac patients, chronic stimuli such as hypoxia and cyanosis or drugs used before surgery may affect the study's results. Although there is no significant difference in mortality in these patients, a positive effect on the length of hospital stay is promising.

Key Words: Congenital heart defect, Ischemic preconditioning, myocardial protection

Konjenital Kalp Cerrahisinde Uzaktan Iskemik Önkoşullama ile Miyokardiyal Koruma: Bekleneni Sağlıyor mu?

Kısa Başlık: Uzaktan İskemik Ön Koşullandırma

Özet

Amaç: Antioksidan ajanların organ fonksiyonları üzerindeki koruyucu etkilerinin keşfedilmesi, bu sistemin yapay olarak test edilmesine olanak sağlamıştır. Bu ajanların endojen olarak vasküler yataktan salıverilmesini tetikleyecek geçici bir uzuv iskemisi oluşturmak suretiyle kalp, karaciğer gibi uzak dokuları iskemiye hazırlama prosedürü zamanla artarak denenmiştir. Ancak konjenital kalp defekti olan hastalarda uzaktan iskemik önkoşullamanın cerrahi klinik sonuçlar üzerindeki etkisi henüz belirsizdir. Bu çalışmada iskemik önkoşullamanın kardiyak koruma üzerindeki erken sonuçlarını araştırdık.

Gereç ve Yöntem: Tek merkezde kompleks konjenital kalp cerrahisi uygulanan çalışma hastalarının prospektif incelemesi yapıldı. Rastgele gruplandırılmış hastalarda ameliyathanede anestezi hazırlığı aşamasında yapay olarak beşer dakikalık aralıklı bacak iskemisi oluşturulup sonrasında alınan kan örneklerinde antioksidan enzimatik analiz yapıldı.

Bulgular: Çalışmaya dahil edilen toplam 67 hastanın ortanca cerrahi yaşı iskemik önkoşullama grubunda (grup 1; n=45) 19,1 ay (3,7-57,7), kontrol grubunda (grup 2; n=22) 16,7 ay (7,8-35,9) idi. Hastaların ortanca takip süresi grup 1'de 58,3 ay (54,3-62,1), grup 2'de 37,1 ay (34,8-41,7) idi. Erken mortalite 4 (%4,4) hastada görüldü. Geç mortalite görülmedi. Süperoksit Dismutaz, Malondialdehit ve kardiyak belirteçlerin düzeyleri açısından gruplar arasında anlamlı fark vardı (p< 005). Hastanede kalış süreleri grup 1'de 8 gün (6-14), grup 2'de 10 gün (8-22) idi (p=0,02).

Sonuç: İskemik önkoşullamanın kardiyak korumaya etkisi henüz kanıtlanmamıştır. Özellikle doğuştan kalp hastalarında hipoksi, siyanoz gibi kronik uyaranlar ya da ameliyat öncesinde kullanılan ilaçlar çalışmanın sonuçlarını etkileyebilmektedir. Bu hastalarda mortalite açısından anlamlı bir fark olmasa da hastanede kalış süresine olumlu etki umut vericidir.

Anahtar Kelimeler: Konjenital kalp defekti, iskemik önkoşullama, miyokardiyal koruma

INTRODUCTION

Complex congenital heart diseases may predispose to forming many free radicals in the body due to their pathophysiology and exposure time. Free radical production may increase due to many factors, such as exposure to low saturation, the emergence of cyanosis, loss of pulmonary tissue compliance, and increased susceptibility to infection. During the operation, exposure to the patient's blood to extracorporeal circulation can trigger a similar process and create multi-organ involvement. Studies have reported low cardiac output syndrome (LCOS) incidence after open-heart surgery in newborn and infantile patients is around 25%- 40% .[1,2,3,4] This ratio's importance is the prolonged postoperative intensive care period and the additional complications it will cause. Other complex and high-mortality interventions may also be required, such as extracorporeal membrane oxygenation (ECMO) support .[5,6] In this study, we discuss the potential of remote ischemic preconditioning (RIPC) in protecting against the ischemic period during pediatric cardiac surgery.

Oxidative stress, characterized by an imbalance between oxidants and antioxidants in favor of oxidants, leads to impairment of physiological function.[7] Cell death and tissue damage are inevitable when tissue is exposed to ischemic conditions. However, this can be reversed depending on the recirculation time. Despite the recirculation here, there is a different concept called "reperfusion damage" paradoxically. The theory is that a mechanism that minimizes ischemia-reperfusion injury and is naturally found in the body in "remote ischemic preconditioning technique (RIPC)" could be pre-activated.[8,9] This natural mechanism is located in the vascular endothelium. Myocardial protection using the ischemic preconditioning method was first described in an animal experiment in 1986. According to this study, a few short ischemic periods created on the myocardium significantly reduce the infarction's severity after normal blood flow.[7] After coronary surgery, plasma isolated from the heart reperfused venous coronary sinus blood sample showed a 2-3-fold increase in protein carbonyls from ELISA measurement.[10]

The World Health Organization (WHO) defines a biomarker as any substance, structure, or process measured in the body or its products, affecting or predicting outcome/disease incidence.[11]

Phospholipid hydroperoxide glutathione peroxidase (PLGSH-PX) protects the membrane against peroxidation when vitamin E, an essential membrane-bound antioxidant, is

insufficient. The body's low molecular weight thiols (cysteine, glutathione -GSH-) are very sensitive to oxidation.

Superoxide Dismutase (SOD) biological effects occur through different mechanisms. The best-known is its role in redox signaling, NO signalization, and its effects on mitochondria. The physiological function of SOD is to protect cells that metabolize oxygen against the harmful effects of superoxide free radicals (O2 ·−), such as lipid peroxidation. SOD also plays a role in the intracellular killing of phagocyted bacteria. SOD activity is higher in tissues with increased oxygen utilization and increases with tissue pO_2 increase. Inadequate removal of superoxide anion results in oxidative stress. Studies show that extracellular SOD3 (Cu- Zn SOD) is essential in oxidative stress-related pathophysiology, including hypertension, heart failure, ischemia-reperfusion, and lung injury.[12,13,14]

MATERIALS and METHODS

Ethical statement: This study was planned as a single-center pediatric cardiovascular surgery clinic in Istanbul Medipol University Hospital in 2015. University Ethics Committee approval was obtained (March 21, 2014 /Number: 10840098-53). Our study was designed in accordance with the Declaration of Helsinki. After prospective data collection, these data were confirmed with Data Processing Center data. Post-discharge mid-term follow-up data of the patients were obtained from the national data recording system and hospital database.

In the prospectively designed study, we based on the period from admission to the operating room to the postoperative follow-up period. Some cases were reoperations (staged surgery) with a history of palliative surgery. ECMO support for patients with respiratory or circulatory problems in the preoperative period, early postoperative bleeding, and reoperation due to pericardial tamponade were described as exclusion criteria from the study.

Technique and procedure application: The patients were divided into the RIPC group (group 1) and the control group (group 2). Patients were randomly distributed into groups. All patients have complex congenital cardiac anomalies. There were cyanotic and acyanotic patients in both groups. In the RPIC group, an age-appropriate size cuff was attached to one lower limb. In this application, the cuff was inflated with a pressure of 150 mmHg and kept for 5 minutes, and reperfusion waited for 5 minutes after the cuff was deflated. This cycle

was repeated three times. After completing the last process, a blood sample was taken at the 5th minute (Figure 1).

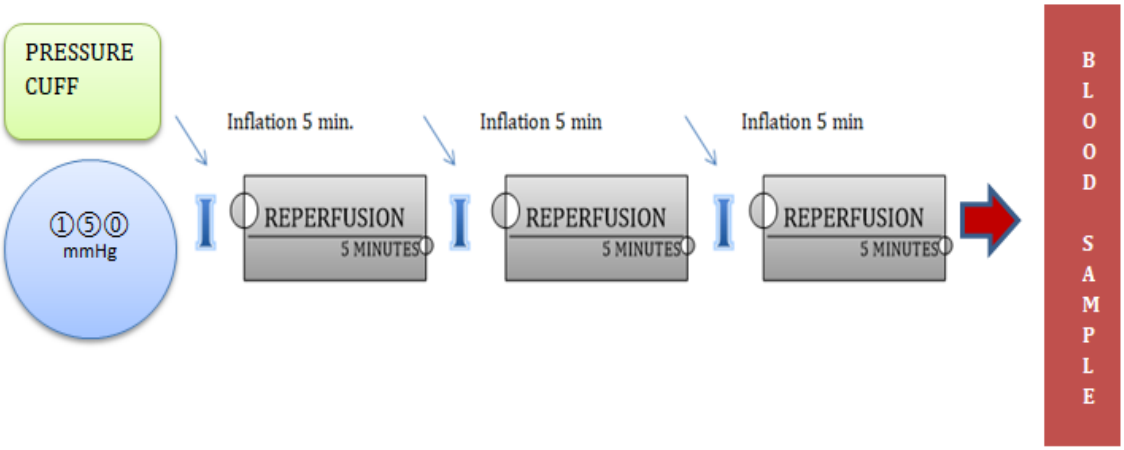

Figure 1. Remote ischemic preconditioning (RIPC) application method

The surgical procedure, anesthesia management, surgical team, and postoperative care were similar for both groups. Arterial blood pressure, central venous pressure, electrocardiographic monitoring, rectal temperature, and near-infrared spectroscopy (NIRS) (renal and cerebral) were followed during the operation. Non-pulsatile cardiopulmonary bypass was performed after standard aortic arterial and bicaval venous cannulation. Myocardial protection was provided with antegrade blood cardioplegia. Routine heparin administration and activated clotting time (ACT) follow-ups were performed. Protamine was used to neutralize the effect of heparin.

Patients with low cardiopulmonary performance data were taken to ECMO support immediately or within 24 hours after surgery. Patients who received ECMO support due to respiratory problems in the preoperative period were excluded from the study. Cardiac performance evaluation was performed by bedside echocardiography, vital sign monitoring, and arterial and mixed venous blood gas monitoring. The patients with low cardiopulmonary performance data were placed on ECMO support immediately after surgery or 24 hours later. Cardiac performance assessment was performed by bedside echocardiography, vital signs monitoring, and arterial and mixed venous blood gas monitoring.

Inotrope scoring (I.S.) and vasoactive inotrope scoring (V.I.S.) were calculated according to the data in the first 24 hours in patients taken to the intensive care unit after surgery.[15] These

values were median and [inter quartile range; IQR]. The formulas used in this score calculation are shown in "information box 1".

Information Box 1. Inotrope score (I.S.) and vasoactive inotrope score calculate
I.S. = Dopamine (mcg/kg/min) + Dobutamine (mcg/kg/min) + 100 x Epinefrin (mcg/kg/min)
V.I.S. = I.S. + 10 x Milrinone (mcg/kg/min) + 10.000 x Vasopressin (units/kg/min) + 100 x Norepinefrin (mcg/kg/dose)

I.S., inotrope score; V.I.S., vasoactive inotrope score; mcg, microgram; kg, kilogram; min, minute.

Serum sample analysis method: Serum samples were collected from each patient, and the control was centrifuged for 20 minutes. Then, samples were stored at -82˚C until analysis. The study's samples were thawed the same day and taken to the Bezmialem University, Istanbul, research laboratory. One hundred thirty samples from 42 patients and 23 controls were divided into the clamping and second groups. We measured SOD (LOD: 0.5 U/mL, interassay CV 3.3%), Glutathione Peroxidase activities (GPX), and Malondialdehyde (MDA) (LOD: 31.2 ng/mL, CV< 10%) levels in patient and control sera using ELISA kits manufactured by ELABSCIENCE Biotechnology Co. LTD (USA, 14780 Memorial Drive, Suite 216, Houston, Texas 77079) in one day. MDA was measured using a competitive ELISA kit. The microtiter plate was pre-coated with an antigen specific to MDA. We added samples to the wells. MDA in the sample competes with a fixed amount of MDA in the solid phase to bind biotinylated detection antibodies specific to MDA. After washing, HRP (Horse Radish Peroxidase) conjugated Avidin is added to each microplate well and incubated. After washing, TMB substrate is added and terminated by a stop solution. The color change is measured photometrically at 450 nm. A standard curve was formed using data from the measurement. For Glutathione Peroxidase (GPX) activity, change in NADPH absorbance was measured kinetically at 340 nm. For Superoxide Dismutase (SOD) activity, the inhibition activity of SOD was determined by detecting the change in absorbance of WST-1 (Water-soluble tetrazolium) at 450nm. Later, creatine kinase muscle-brain (CK-MB) (mass

immunoassay, CV:3.1%), Total creatine kinase (CK) (enzymatic, CV:0.5%), and Troponin I (TnI) (CMIA, CV:2%) levels were measured using Abbott reagents on Abbott Architect ci4100 (ABBOTT DIAGNOSTICS 5440 Patrick Henry Dr. Santa Clara, California 95054) in one run. QC procedures are run daily, and QC results were within ±2SD.

Statistical Analysis

IBM SPSS Statistics Software 21 (SPSS Inc., Chicago, Illinois, United States) was used for statistical analysis. Variables were examined using visual (histogram, probability plots) and analytical methods (Kolmogorov-Smirnov/Shapiro-Wilk test) to determine whether they were typically dispersed. Descriptive analyses were presented using medians and IQR for non-normally distributed and ordered variables. Since the number of patients for enzymatic activity measurement did not show a normal distribution, nonparametric tests were performed to compare these parameters and sequential variables. Inter-group correlation analysis was performed using Spearman's correlation test. The Rho (ρ) value indicated correlation severity. The Mann-Whitney U test was used for intragroup comparisons. A p-value of less than 0.05 was considered to show a statistically significant result.

RESULTS

Patients: Between April 2015 and December 2018, 67 pediatric patients with complex cardiac anomalies who underwent the first surgery and reoperation were included in the study (group 1, n=45; group 2, n=22). The demographic characteristics, cardiac diagnoses, surgical procedures, and intraoperative parameters of the patients are listed in Table 1.

Table 1. Demographic and operative data

	RIPC (n=45)	CTRL (n=22)	p-Value
Preoperative data			
Age (month) median (IQR)	19.1 (3.7-57.7)	16.7 (7.8- 35.9)	0.98
Gender (F/M)	20/25	8/14	0.53
STAT Category 1	23	10	
STAT Category 2	9	7	

STAT Category 3	7	3	
STAT Category 4	6	2	
STAT Category 5	-	-	
Cyanosis	30	13	0.54
Syndromic appearance	11	7	0.61
Postoperative data			
Re-do surgery	13	7	0.8
Postoperative ECMO	3	4	0.35
ICU stay (day) median (IQR)	2 (2-5)	3 (2-5)	0.33
Hospital stays (day) median (IQR)	8 (6-14)	10 (8-22)	0.02
Early mortality	1	3	0.26
Follow-up after discharge (month)	58.3 (54.3-62.1)	37.1 (34.8-41.7)	<0.01
From induction to CPB time (min.)	59 (53-69)	60.5 (48-69)	0.95
ACC time (min.)	59 (42-79)	65 (41.2-83.5)	0.54
CPB time (min.)	71 (55-99)	80 (66-102.2)	0.35
Reperfusion time (min.)	11 (10-20)	15 (10-18.7)	0.28
VIS	5.5 (1.7-8.2)	5.9 (4-8.4)	0.78
Arrhythmia after CPB	4	2	0.49

RIPC, remote ischemic preconditioning; CTRL, control; IQR, interquartile range; ECMO, extracorporeal membrane oxygenation; ICU, intensive care unit; ACC, aortic cross-clamp; CPB, cardiopulmonary bypass; VIS, vasoactive inotrope score.

The operative risk score of the patients was made according to the Society of Thoracic Surgery - European Society of Cardio-Thoracic Surgery (STS-EACTS [STAT]) classification system (Category 1 operations with the lowest risk of death and category 5, procedures with the highest risk of death).[16] This classification system is shown in Table 2.

Table 2. Common types of surgery within each STAT Category

STAT Category 1	Atrial septal defect repair, ventricular septal defect repair, coarctation repair, subaortic stenosis resection, pulmonary valve replacement, conduit replacement
STAT Category 2	Tetralogy of Fallot repair, Fontan operation, Ross operation
STAT Category 3	Hemi-Fontan operation, arterial switch operation, complete atrioventricular septal defect repair
STAT Category 4	Aortic arch repair, arterial switch operation with ventricular septal defect closure, heart transplant, aortopulmonary shunt, total anomalous pulmonary venous return repair, truncus arteriosus repair
STAT Category 5	Norwood (stage I) operation, hybrid stage 1 procedure, double switch operation, truncus arteriosus with interrupted aortic arch repair

The patients' surgical median age was 19.1 months (IQR= 3.7- 57.7 months) in Group 1 and 16.7 months (IQR= 7.8- 35.9 months) in Group 2. The median time between ischemic preconditioning and initiating cardiopulmonary bypass was 59 minutes (IQR= 53- 69 min) in group 1 and 60.5 minutes in group 2 (IQR= 48- 69 min) (p=0.95).

When the SOD analysis was compared according to the groups, the preoperative blood sample result was significantly higher in favor of group 1 (p= 0.015). No statistically significant difference was detected in the blood samples analyzed after surgery (p= 0.21).

MDA levels did not reveal a significant difference between groups in preoperative blood samples ($p = 0.14$). However, in the tests performed in the postoperative period, the difference was statistically significantly higher in favor of group 1 ($p= 0.03$).

A significant increase in GPX activity was detected in preoperative blood samples in favor of group 1 ($p < 0.04$). No statistically significant difference was detected in the blood samples analyzed after surgery ($p= 0.24$).

Our study used TnI, total CK, and CK-MB as postoperative cardiac protection markers. Troponin I level did not show a statistically significant difference between the groups in the preoperative period ($p= 0.46$). It was significantly lower in favor of group 1 in the postoperative period ($p<0.01$).

CKMB values did not show a statistically significant difference between the groups in the preoperative period ($p = 0.27$). It was significantly lower in favor of group 1 in the postoperative period ($p<0.01$).

CK levels did not show a statistically significant difference between the groups in the preoperative period ($p = 0.35$). It was significantly lower in favor of group 1 in the postoperative period ($p<0.01$).

Enzymatic and biomarker levels are listed in Table 3.

Table 3. Statistical analysis of enzyme activities according to groups

	RIPC group		Control group		P-value
ENZYME	Median	IQR	Median	IQR	
Superoxide Dismutase (SOD)					
Preoperative	17.8	12.3-21	14.3	14.1-17.2	.05
Postoperative	16.9	11.3-19.7	14.7	14.5-20.5	.021
Glutathione Peroxidase activities (GPX)					
Preoperative	71	27-153	58	30-132	.08
Postoperative	70	21-139.4	55.9	29.4-156	.24

Malondialdehyde (MDA)					
Preoperative	84.9	69-97.4	72.5	56-94.9	.14
Postoperative	61.4	38.2-61	47.2	44.7-69.3	.03
Creatine Kinase (CK)					
Preoperative	45	36-63	53	37.7-86.7	.35
Postoperative	202	53-692	693.5	275-1104	.01
Creatine Kinase, Muscle- Brain (CKMB)					
Preoperative	1.7	1.2-3.5	2	1.5-3.9	.27
Postoperative	4.9	1.4-59.9	109.2	30-170	<.01
Troponin I (TnI)					
Preoperative	9.1	3.2-107.6	13.25	3.3-22.5	.46
Postoperative	1222	9.2-13712	23048	4711-46080	<.01

No significant difference was found between the groups regarding postoperative arrhythmia incidence (p = 0.49).

ECMO support was provided to 7 patients (group 1 n=3, group 2 n=4) due to LCOS in the early postoperative period (p=0.35). Among the postoperative cardiac performance indicators of the patients, the first 24-hour VIS values were also recorded. There was no significant difference between the groups (p=0.78). Here, since the inotropic support of patients receiving ECMO support will decrease, the VIS values of these patients were excluded.

The median length of stay in the intensive care unit (ICU) was two days (IQR= 2-5 days) in the RIPC group. In the control group, this value median was three days (2-5 days). There was no significant difference between the groups regarding the duration of stay in the ICU (p=0.33). The hospital's median length of stay was 8 days (IQR= 6-14 days) in the RIPC group. In the control group, this value median was 10 days (8-22 days). According to the

groups, the patient's hospital stay duration showed a statistically significant difference (p= 0.02). This difference was in the direction of fewer hospital stays in Group 1.

The patients were followed up in 2 stages: the early postoperative period and after discharge. The early death rate was 4.4% (groups 1 n=1 and 2 n=2). Three of the ECMO-dependent 7 patients died (42%). 1 of them was from group 1 patients. No mortality was observed in the mid-term follow-ups. The median follow-up period of group 1 patients was 58.3 months (IQR= 54.3- 62.1 months). The median follow-up period of group 2 patients was 37.1 months (IQR= 34.8- 41.7 months). Among these patients, there were three patients whose long-term records could not be reached. Five patients applied again for the second or third-stage operation post-discharge period. These patients were planned staged operations (e.g., Fontan completion) independently of RIPC. There was no significant difference in reoperation (p> 0.05).

DISCUSSION

Theoretical: The ischemic preconditioning theory was based on antioxidant enzymatic pathways present in the body, particularly endothelium-derived.

Purpose of application: In this study, our theoretical expectation was to activate the antioxidant activity before contributing to the body in combating operative stress. Our ultimate goal was to see this benefit in the cardiac tissue at the maximum level and minimize the damage. However, oxidative stress markers alone may not give specific results. Therefore, using more than one indicator is an advantage for accurate analysis.[18]

The relationship between SOD reported in studies and ischemia-reperfusion was also found in our research. It was at a statistically significant level (p< 0.05). There is a broad spectrum of congenital heart diseases, from genetic anomalies to acquired anomaly types. In other words, the balance change between free oxygen radicals and the antioxidant system may cause chromosomal abnormalities and accompanying cardiac effects. The effect of pharmacological agents may disrupt this balance. Or, there may be antioxidant dysfunction due to a genetic disorder. Increased SOD3 activity was shown in the erythrocytes of patients with Down syndrome; in our study, there were patients with intracardiac defects associated with Down syndrome. In light of these facts, in our research covering many types of congenital cardiac malformations, it may not be possible for the antioxidant system preconditioning to have an equal effect on all patients with RIPC application. In this case,

this patient group's enzymatic activity is higher than others and will likely affect the results. The variety of metal cofactors (Cu, Zn, Mn) is critical in SOD activity and directly affects its biological role.[19] The specific activity of Cu-Zn SOD is high in the erythrocytes of Down syndrome patients.[20,21]

Mezzetti A. et al.[22] examined enzymatic activity and capacity in human artery, vein, and heart tissue (right atrium) samples. As a result, they found a high rate in every tissue. In our study, the presence of enzymes that were found spontaneously and showed high cardiac tissue activity in both groups may have affected the statistical difference. In our study, GSHPX levels did not show a statistically significant difference between the groups. So, it can be concluded that preoperative activity was already high in both groups.

The other biomarker with significant elevation was MDA. Malondialdehyde (MDA) measurement is the most commonly used test to assess the degree of lipid peroxidation. MDA was also a widely used biomarker associated with cardiovascular events.[17] However, our study's correlation with other cardiac markers was weak (rho< 0.3). As Spiteller,[23] reviewed the role of lipid peroxidation in various chronic diseases, lipid oxidation end products emerged as oxidative stress markers, with 4-HNE and MDA being among the most studied. However, since MDA is a product affected by heat, its plasma level may increase in its reactions.

Regarding cardiac biomarkers (TnI, CK, CKMB), the results were significantly lower in favor of the RIPC group. In other words, even under conditions where surgical modalities were similar, superiority was observed in terms of myocardial protection. Even though these results were not reflected in mortality at a statistically significant rate, they played an active role in reducing the length of hospital stay. Considering the secondary benefits of this situation, it will be of great importance in these high-risk patient groups.

Although there was no significant difference in the patients' duration in the ICU, the total hospital stay duration showed a substantial difference between the groups. This situation could be interpreted as a decrease in serous effusion or the reduction of the need for pharmacological support in RIPC patients because there was no difference between the groups regarding risk groups.

Studies have shown the role of free oxygen radicals in cardiovascular events. However, using antioxidant agents specifically as biomarkers has not been possible.

Limitation: One of the limiting factors in the study is the difficulty of obtaining a more significant number of enzymatic kits. It is essential in enzymatic processes that all samples taken are stored and analyzed under equal conditions. In addition, although the patients have a similar distribution in demographics, the possibility of not receiving reliable medication use and follow-up information from a sociocultural perspective may have affected the equality of conditions in the background. Again, considering the number of patients, efforts to create a more extensive series that can provide similar situations will also affect the study duration, design, and laboratory processes (separation, freezing, analysis, etc.).

CONCLUSION

The effects of pre/post-conditioning procedures by activating antioxidant systems on various organs have been published in the literature. However, no significant increase in survival was observed, especially in cardiac patients. More studies and different enzyme kits with an effective correlation rate could be investigated involving larger patient groups isolated from pharmacological agents or examining similar chromosomal anomalies as subgroups.

Ethics Approval

This study was planned as a single-center pediatric cardiovascular surgery clinic in Istanbul Medipol University Hospital in 2015. University Ethics Committee approval was obtained (March 21, 2014 /Number: 10840098-53).

Peer-review

Externally and internally peer-reviewed.

Authorship Contributions

Concept: A.A., E.C., Design: E.C., Data Collection or Processing: A.A., E.C., Analysis or Interpretation: T.A., E.C., Literature Search: A.A., E.C., Writing: A.A., T.A.

Conflict of Interest

The authors declared no conflict of interest.

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Informed Consent: The patients were sampled through a convenient sampling technique and enrolled after obtaining their written informed consent.

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