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Effects of ischemia and omeprazole preconditioning on functional recovery of isolated rat heart

Efeitos da isquemia e pré-condicionamento com omeprazol na recuperação funcional do coração isolado de rato

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Abstract

Objective: The aim of this study was to compare protective effects of ischemic and potential protective effects of pharmacological preconditioning with omeprazole on isolated rat heart subjected to ischemia/reperfusion.

Methods: The hearts of male Wistar albino rats were excised and perfused on a Langendorff apparatus. In control group (CG) after stabilization period, hearts were subjected to global ischemia (perfusion was totally stopped) for 20 minutes and 30 minutes of reperfusion. Hearts of group II (IPC) were submitted to ischemic preconditioning lasting 5 minutes before 20 minutes of ischemia and 30 minutes of reperfusion. In third group (OPC) hearts first underwent preconditioning lasting 5 minutes with 100 μ M omeprazole, and then submitted 20 minutes of ischemia and 30 minutes of reperfusion.

Results: Administration of omeprazole before ischemia induction had protective effect on myocardium function recovery especially regarding to values of systolic left ventricular pressure and dp/dt max. Also our findings are that values of coronary flow did not change between OPC and IPC groups in last point of reperfusion.

Conclusion: Based on our results it seems that ischemic preconditioning could be used as first window of protection after ischemic injury especially because all investigated parameters showed continuous trend of recovery of myocardial function.

On the other hand, preconditioning with omeprazole induced sudden trend of recovery with positive myocardium protection, although less effective than results obtained with ischemic preconditioning not withstand, we must consider that omeprazole may be used in many clinical circumstances where direct coronary clamping for ischemic preconditioning is not possible.

Descriptors: Coronary circulation. Ischemic Preconditioning, Myocardial. Omeprazole.

Resumo

Objetivo: O objetivo deste estudo foi comparar os efeitos protetores de efeitos protetores isquêmicos e potenciais de pré-condicionamento farmacológico com omeprazol no coração isolado de rato submetido à isquemia/reperfusão.

Métodos: Os corações de ratos albinos Wistar machos foram excisados e perfundidos em um aparelho de Langendorff. No grupo controle (grupo I), após o período de estabilização, os corações foram submetidos à isquemia global (a perfusão foi totalmente interrompida) por 20 minutos e 30 minutos de reperfusão. Corações do grupo II (IPC) foram submetidos a pré-condicionamento isquêmico com duração de 5 minutos antes de 20 minutos de isquemia e 30 minutos de reperfusão. No terceiro grupo (OPC), corações foram submetidos a pré-condiciona-

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Abbreviations, acronyms & symbols	
CF	Coronary flow
DLVP	Diastolic left ventricular pressure
HCl	Hydrochloride acid
HR	Heart rate
ICP	Ischemic preconditioning
PPI	Proton pump inhibitors
ROS	Reactive oxygen species
SLVP	Systolic left ventricular pressure

mento com duração de 5 minutos com 100 μ M de omeprazol, e, então, submetidos a 20 minutos de isquemia e 30 minutos de reperfusão.

Resultados: A administração de omeprazol antes da indução da isquemia teve efeito protetor sobre a recuperação funcional do miocárdio especialmente em relação aos valores de pressão

INTRODUCTION

Myocardial preconditioning represents exposure of myocardium to sublethal stimulus in order to protect it from a subsequent normal lethal stress^[1]. Myocardium can be preconditioned by two basic techniques such as ischemic and pharmacological preconditioning. Ischemic preconditioning (ICP) is a concept introduced by Murry et al. in 1986 by using canine models. He showed that single or multiple brief periods of myocardial ischemia that produce reversible myocyte injury can limit the size of the infarct and the degree of reperfusion injury after a subsequent and more prolonged period of myocardial ischemia^[2].

These protective effects of ICP on heart can be consequence of reduction in reactive oxygen species generation, delay in ATP depletion, reduction of: infarct size, apoptosis and neutrophil accumulation, as well as improvement of endothelial function and reduction of intracellular Ca^{++} overload^[3-5]. Two different time frames have been reported for pre-conditioning, early or "classical preconditioning" phase and late or "second window" phase. Duration of first phase, which involves the activation of different membrane receptors, is from several seconds and to 3h and for second phase from 12-72 h which represents changes in gene expression leading to production cardioprotective stress proteins^[4].

Ischemia is characterized by an absolute or relative decrease in the blood supply of tissue or organ due to blockage of blood vessels. Blood vessel can be occluded by thrombus, atherosclerotic plaque, vasoconstriction or inflammation. During myocardial ischemia absence of oxygen and metabolic substrates to cardiomyocyte can cause functional, structural and metabolic diseases. As a consequence, cell switches metabolism to anaerobic, resulting in accumulation of lactate and generation of acidosis. Hypoxic conditions lead to diminished intracellular concentrations of ATP (adenosine triphosphate)

sistólica ventricular esquerda e dp/dt max. Também os nossos achados são de que os valores de fluxo coronário não se alteraram entre os grupos OPC e IPC no último ponto de reperfusão.

Conclusão: Com base nos nossos resultados, o pré-condicionamento isquêmico poderia ser usado como primeira janela de proteção após a lesão isquêmica, especialmente porque todos os parâmetros analisados apresentam tendência contínua de recuperação da função do miocárdio. Por outro lado, o pré-condicionamento induzido com omeprazol apresenta tendência repentina de recuperação com proteção miocárdio positiva, embora menos efetiva da obtida com o pré-condicionamento isquêmico. Devemos considerar que o omeprazol pode ser usado em muitas circunstâncias clínicas em que o pinçamento coronariano direto para pré-condicionamento isquêmico não é possível.

Descritores: Circulação Coronária. Precondicionamento Isquêmico Miocárdico. Omeprazol

and CP (creatine phosphate) which results in decreased activity of ATP reliant ion pumps including Na^+/K^+ ATP-ase pump and exacerbation of contractile function. Inactivation of Na^+/K^+ ATP-ase contributes to intracellular Na^+ overloading. Lower intracellular pH induces the Na^+/H^+ exchanger to extrude H^+ and results in intracellular accumulation of Na^+ , which leads to activation of the $2Na^+/Ca^{++}$ exchanger in order to extrude Na^+ and accumulate intracellular Ca^{++} ^[6-9]. All these facts and generation of reactive oxygen species (ROS) can lead to cell death induced by ischemic episodes^[10].

Furthermore, reperfusion is restoration of blood flow after an ischemic episode and it may result in paradoxical cardiomyocyte dysfunction caused by ROS, intracellular and mitochondrial Ca^{++} overload and accumulation of inflammatory cells. This phenomenon is called "reperfusion injury", where prompt changes in intracellular ions and normalization of pH can occur cell death and greater damage than it can be induced by pre-reperfusion ischemia^[6,8,11].

Besides ischemic preconditioning, which represents an adaptive response triggered by a brief ischemia applied before a prolonged coronary occlusion, the same response can be induced with pharmacological agents^[3-5]. Proton pump inhibitors (PPI) have been one of most important advances in the field of gastroenterology in past 15 years. These medications showed significant progress in acid-related diseases over other acid reducing medications. Most commonly used PPI are omeprazole, lansoprazole and pantoprazole^[12,13]. Omeprazole was first introduced into clinical practice and it is commonly used for treatment of gastroesophageal reflux and erosive esophagitis in children. The main mechanism of action of these drugs is suppression of acid secretion by binding to H^+/K^+ ATP-ase known as "proton pump" or "acid pump"^[14,15]. Proton pump is enzymatic pump expressed in different tissues like parietal cells where hydrochloride acid (HCl) is secreted. The main physiological effect of this pump is H^+ exchange for K^+ ions.

Due to existence of proton pump in myocardial tissue, which was first proven by Nagashima et al.^[16], mechanical and electrical properties can be changed by using PPI^[6]. Recently proton pump inhibitors had showed protective effects in treatment of myocardial ischemia in patients with coronary artery disease and gastroesophageal reflux^[17].

Regarding all above presented data, the aim of this study was to compare protective effects of ischemic and potential protective effects of pharmacological preconditioning with omeprazole on isolated rat heart subjected to ischemia/reperfusion.

METHODS

Preparation of isolated rat hearts

The hearts of male Wistar albino rats (n=36, 12 in each experimental group, body mass 180–200 g) were excised and perfused on a Langendorff apparatus (Experimetria Ltd, 1062 Budapest, Hungary). After a short-term ketamine/xylasin narcosis, animals were killed by cervical dislocation (Schedule 1 of the Animals/Scientific Procedures, Act 1986, UK), and premedicated with heparin as an anticoagulant. After emergency thoracotomy and rapid cardiac arrest by superfusion with ice-cold isotonic saline, rapidly excised, the aortas were cannulated and retrogradely perfused under a constant perfusion pressure (CPP).

The composition of the non-recirculating Krebs-Henseleit perfusate was as follows (mM): NaCl 118, KCl 4.7, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 2.5, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.7, NaHCO_3 25, KH_2PO_4 1.2, glucose 11, pyruvate 2, equilibrated with 95 % O_2 plus 5% CO_2 and warmed to 37 °C (pH 7.4). Immediately after the restoration of normal heart rhythm, through the created entrance to the left atrium of the heart and damaged mitral valve, the sensor (transducer BS473-0184, Experimetria Ltd, Budapest, Hungary) was inserted into the left ventricle for continuous monitoring of cardiac function (Figure 1).

Physiological assay and experimental protocol

All study groups underwent 30 min perfusion at CPP of 70 cm H_2O . In control group (CG) after stabilization period,

hearts were subjected to global ischemia (perfusion was totally stopped) for 20 minutes and 30 minutes of reperfusion. Twelve hearts of group II (IPC) were submitted to ischemic preconditioning lasting 5 minutes before 20 minutes of ischemia and 30 minutes of reperfusion. In third group (OPC) hearts first underwent preconditioning lasting 5 minutes with 100 μM omeprazole, then submitted 20 minutes of ischemia and 30 minutes of reperfusion. In control group after 20 minutes of global ischemia during period of reperfusion (30 minutes) all cardiodynamic parameters and coronary flow were measured in intervals of 5 minute (RP1-RP7). In IPC group, after short period of ischemia (5 minutes) during period of reperfusion (10 minutes), all cardiodynamic parameters and coronary flow were measured in intervals of 1 minute (PR1-PR10), while during second period of ischemia (20 minutes)/reperfusion (30 minutes) cardiodynamic parameters and coronary flow were measured in intervals of 5 minute (RP1-RP7).

In OPC group (after 5 minutes preconditioning with omeprazole), during period of reperfusion all cardiodynamic parameters and coronary flow were measured during second period of ischemia (20 minutes)/reperfusion (30 minutes) in intervals of 5 minute (RP1-RP7). When the flow was considered stable (three measurements of the same values), coronary flow was recorded. Only the properly performed experiments were included in the study (i.e., the groups of the hearts in which the CPP/CF relationship was studied twice in the absence of any drug).

After placing the sensor in the left ventricle, the following parameters of myocardial function have been continuously registered:

1. Maximum rate of pressure development in the left ventricle (dp/dt max)
2. Minimum rate of pressure development in the left ventricle (dp/dt min)
3. Systolic left ventricular pressure (SLVP)
4. Diastolic left ventricular pressure (DLVP)
5. Heart rate (HR)

Coronary flow (CF) was measured flowmetrically.

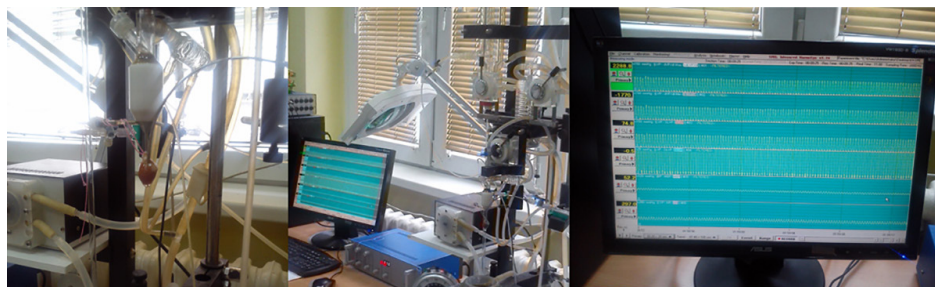


Fig. 1 - Immediately after the restoration of normal heart rhythm, through the created entrance to the left atrium of the heart and damaged mitral valve, the sensor was inserted into the left ventricle for continuous monitoring of cardiac function.

Drugs

All drugs were purchased from Sigma-Aldrich Chemie GmbH, Germany.

Statistical analysis

For statistical analysis we examined three measured points, first point was stabilization, second was the first minute of reperfusion and third was the 30 minute of reperfusion. Values are expressed as mean \pm SE. Statistical analysis was performed by ANOVA test. *P* values lower than 0.05 were considered to be significant.

The experimental protocol was approved by the Faculty of Medical Sciences Ethics Committee for the welfare of experimental animals, University of Kragujevac, number

01-12149 and by Ministry of Agriculture, Forestry and Water Management, Authority for Veterinary of Serbia number 323-07-09426/2013-05.

RESULTS

Maximum Rate of Left Ventricular Pressure Development (dp/dt max)

There were no significant differences among groups in the values of point of stabilisation and first minute of reperfusion.

In control and IPC group there were no significant difference between periods of stabilisation and first minute of reperfusion, however there were high statistical significant increase ($P < 0.01^{**}$) of values of dp/dt max between these

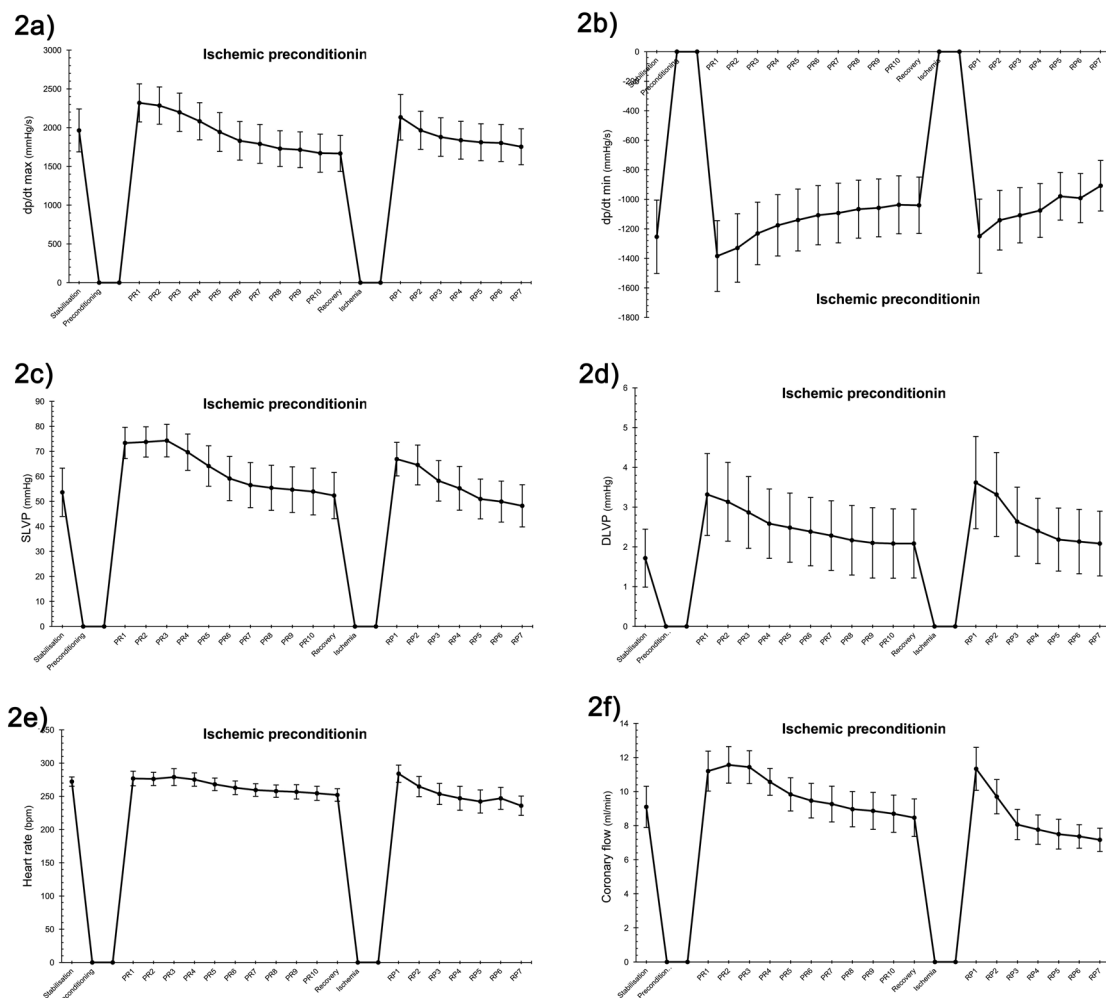


Fig. 2 - The influence of ischemic preconditioning (5 min ischemia/10 min reperfusion) on cardiodynamic parameters of the isolated rat heart during subsequent ischemia (20 minutes)/reperfusion (30 minutes): 2a) dp/dtmax, 2b) dp/dtmin, 2c) SLVP, 2d) DLVP, 2e) HR, 2f) CF.

points in OPC group. Trend of values in period of reperfusion was the same in all investigated groups. Furthermore, there were statistical significant changes between control group and IPC group in values of the last point of reperfusion ($P<0.05^*$) but in comparison with mentioned groups, OPC group has statistical different values ($P<0.01^{**}$) which were very similar with values before omeprazole administration (Figures 2a, 3a and 4a).

Minimum Rate of Left Ventricular Pressure Development (dp/dt min)

There were no significant differences among groups in the values of point of stabilisation and first minute of reperfusion.

In control, IPC and OPC groups there were no significant difference between periods of stabilisation and first minute of reperfusion. Trend of values in period of reperfusion was the same in OPC, IPC and control group without any statistical difference. Furthermore, there were changes between control group and OPC group in values of the last point of reperfusion ($P<0.01^{**}$) in comparison between control group with IPC group values were statistical different ($P<0.05^*$) at the end; in comparison OPC group with IPC group values were statistical different ($P<0.01^{**}$) but in OPC group values were similar with values before omeprazole administration (Figures 2b, 3b and 4b).

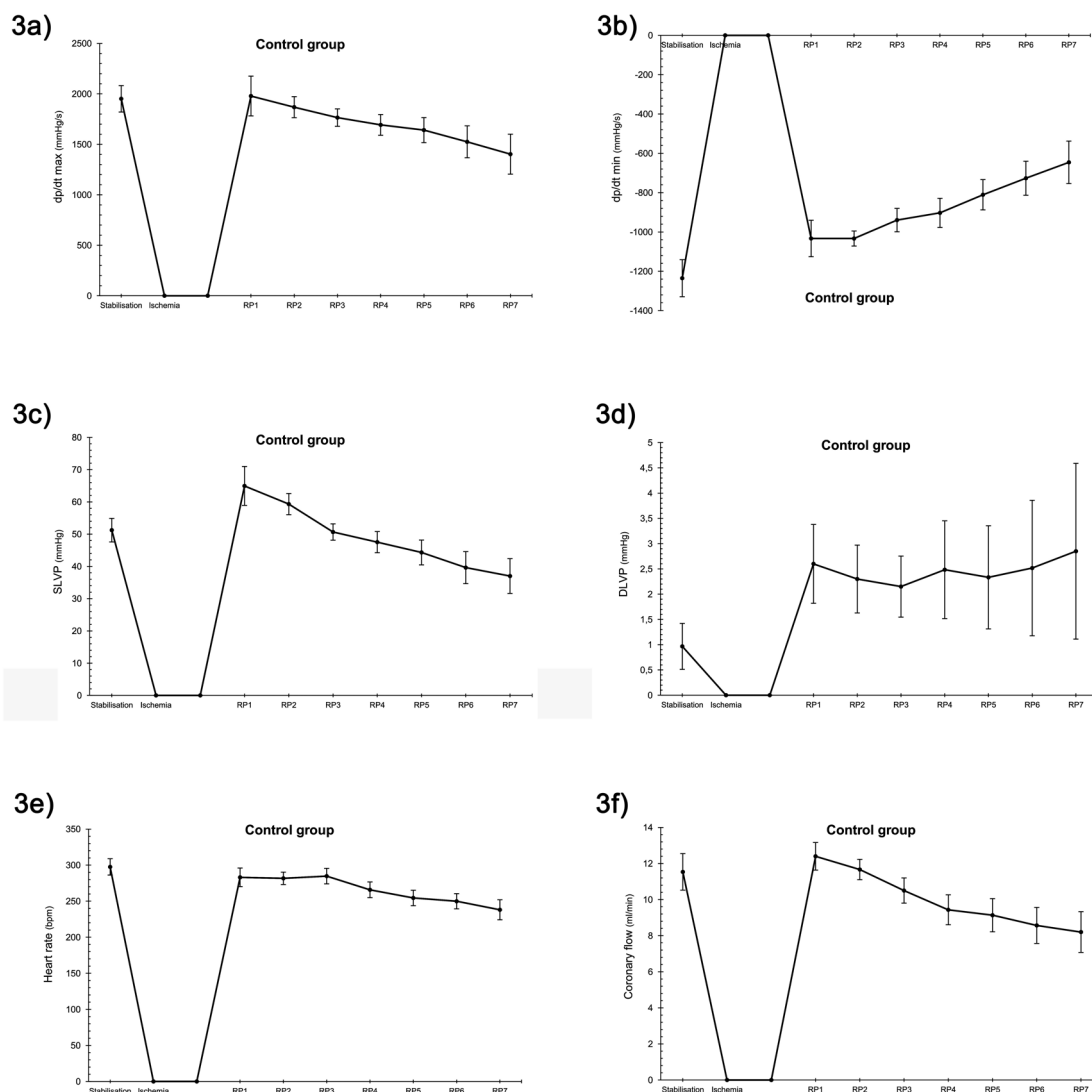


Fig. 3 - The influence of ischemia (20 minutes)/reperfusion (30 minutes) on cardiodynamic parameters of the isolated rat heart: 3a) dp/dtmax, 3b) dp/dtmin, 3c) SLVP, 3d) DLVP, 3e) HR, 3f) CF.

Systolic Blood Pressure in the Left Ventricle (SLVP)

There were no significant differences among groups in the values of point of stabilisation and first minute of reperfusion.

In control and IPC group there were no significant difference between periods of stabilisation and first minute of reperfusion, however there were high statistical significant increase ($P<0.01^{**}$) of values of SLVP between these points in OPC group. Trend of values in period of reperfusion was the same in OPC and control group on the other hand trend in IPC group was statistical significantly lower than in mentioned groups ($P<0.05^{*}$). Furthermore, there were statistical significant changes between control group and IPC group in values of the last point of reperfusion ($P<0.05^{*}$) but in comparison with

mentioned groups, OPC group has statistical different values ($P<0.01^{**}$) which were very similar with values before omeprazole administration (Figures 2c, 3c and 4c).

Diastolic Blood Pressure in the Left Ventricle (DLVP)

There were no significant differences among groups in the values of point of stabilisation and first minute of reperfusion.

In control and IPC group there were no significant difference between periods of stabilisation at the first minute of reperfusion, however there were high statistical significant increase ($P<0.05^{*}$) of values of DLVP between these points in OPC group. Trend of values in period of reperfusion was different among groups, in

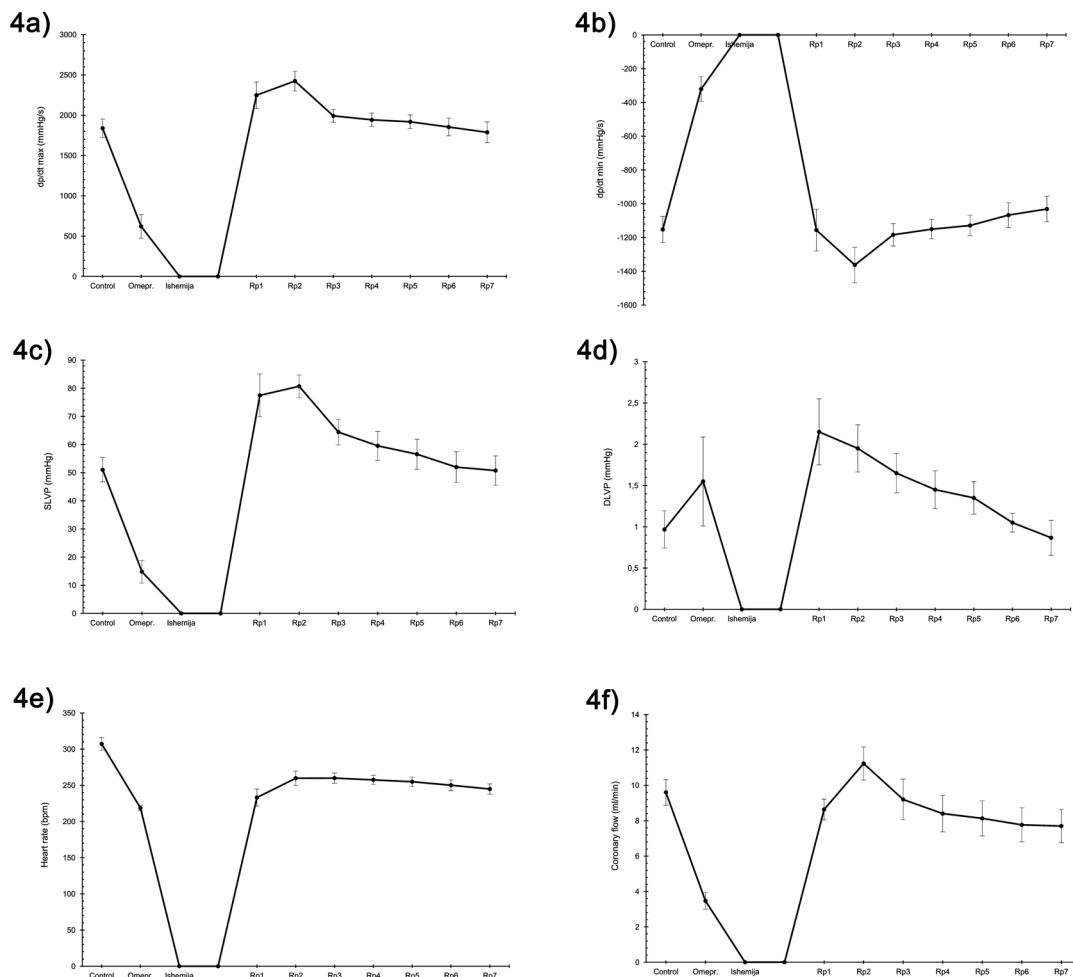


Fig. 4 - The influence of preconditioning with omeprazole (5 minutes) on cardiodynamic parameters of the isolated rat heart during subsequent ischemia (20 minutes)/reperfusion (30 minutes): 4a) dp/dtmax, 4b) dp/dtmin, 4c) SLVP, 4d) DLVP, 4e) HR, 4f) CF.

control group values of DLVP in period of reperfusion increased compared with period before ischemia. In IPC and OPC groups values of DLVP in period of reperfusion decreased compared with period before preconditioning. Furthermore, there were statistical significant changes between control group and IPC group. Compared with values in control group, IPC and OPC groups values were statistical different in last point of reperfusion ($P<0.05^*$) (Figures 2d, 3d and 4d).

Heart Rate (HR)

There were no significant differences between IPC group and control group and also between OPC and control group, but there were significant difference between IPC and OPC at the point of stabilisation.

In control and IPC group there were no significant difference between periods of stabilisation at the first minute of reperfusion, however there were high statistical significant drop ($P<0.01^{**}$) of values of HR between these points in OPC group. Trend of values in period of reperfusion was the same in IPC and control group on the other hand trend in OPC group was the same as in mentioned groups but with statistical significant lower decrease. Furthermore, there were no changes between control group and OPC group in values of the last point of reperfusion but in comparison with IPC group those values were statistically different ($P<0.05^*$) (Figures 2e, 3e and 4e).

Coronary Flow (CF)

There were no significant differences among groups in the values of point of stabilisation and first minute of reperfusion.

In control and IPC group there were no significant difference between periods of stabilisation and first minute of reperfusion however there were high statistical significant increase ($P<0.01^{**}$) of values of CF between these points in

OPC group. Trend of values in period of reperfusion was the same in OPC, IPC and control group without any statistical difference. Furthermore, there were no changes among control, IPC and OPC group in last point of reperfusion (Figures 2f, 3f and 4f).

Summary table shows all cardiodynamic parameters compared between the groups (Table 1).

DISCUSSION

Acute myocardial infarction is leading cause of morbidity and mortality worldwide each year. Consequence of acute myocardial infarction, a diminished blood supply to the heart exceeds a critical threshold and overwhelms myocardial cellular repair mechanisms designed to maintain normal operating function and homeostasis^[18]. The treatment of acute myocardial infarction has been prospering in last few decades and new methods such as preconditioning, post-conditioning and pharmacological agents have been examined to protect the heart^[19,20].

Preconditioning (PC) involves reduction of necrotic tissue mass, improvement of cardiac contractile performance after ischemia and reperfusion, and reduction of arrhythmias. Although the ischemic preconditioning is not entirely clarified, recently parts of the signal transduction cascade of ischemic preconditioning have been identified. According to different species, the organism increases the production of several chemical mediators that trigger the cardio protection process^[21]. To avoid the problems which can cause ischemic preconditioning in clinical use, administration of pharmacological agents could be ideal solution. Pharmacological agents such as a cardio-selective β_1 -blocker and the adenosine triphosphate-sensitive potassium channel openers have been shown the ability to protect the heart but none has been widely accepted^[19].

Table 1. Summary table of statistically significant difference between groups in points of interest.

Groups	Dp/dt max [mmHg/s]	Dp/dt min [mmHg/s]	SLVP [mmHg]	DLVP [mmHg]	CF [ml/min]	Point of interest
CG vs. IPC	$P>0.05$	$P>0.05$	$P>0.05$	$P>0.05$	$P>0.05$	Stabilisation
	$P>0.05$	$P>0.05$	$P>0.05^*$	$P>0.05$	$P>0.05$	First point of reperfusion
	$P>0.05^*$	$P>0.05^*$	$P>0.05^*$	$P>0.05^*$	$P>0.05$	Last point of reperfusion
CG vs. OPC	$P>0.05$	$P>0.05$	$P>0.05$	$P>0.05$	$P>0.05$	Stabilisation
	$P>0.05$	$P>0.01^{**}$	$P>0.05$	$P>0.05$	$P>0.05$	First point of reperfusion
	$P>0.05^*$	$P>0.01^{**}$	$P>0.01^{**}$	$P>0.05^*$	$P>0.05$	Last point of reperfusion
IPC vs. OPC	$P>0.05$	$P>0.05$	$P>0.05$	$P>0.05$	$P>0.05$	Stabilisation
	$P>0.05$	$P>0.01^{**}$	$P>0.05^*$	$P>0.05$	$P>0.05$	First point of reperfusion
	$P>0.05^*$	$P>0.01^{**}$	$P>0.01^{**}$	$P>0.05$	$P>0.05$	Last point of reperfusion

*Statistically significant

**High statistically significant

CG=control group; IPC=ischemic preconditioning; OPC=preconditioning with omeprazole

Model of isolated rat heart is one the most convenient experimental tool for preclinical investigations of mammalian heart, and also very reliable for connection between animal and human studies. Generally, viewed morphology of the rat heart is very similar with human one^[22]. Namely, structure of the left ventricular, wall thickness and properties of the papillary muscles are almost the same as in the human heart^[22]. In addition, examination of nodal cells showed that they are very similar to human T cells, and begin with the functioning during the early embryogenesis^[23]. Moreover, both ventricular and atrial cardiomyocytes are showed to possess high percent of histological similarity^[24]. In this sense, we can assume that there are significant analogy between cardiac (patho) physiological events in rat and human heart.

In that sense, data collected from these experimental studies could be of great interest in improving knowledge about ischemic and especially pharmacological form of preconditioning.

The present study aimed to compare potential protective effects of ischemic and pharmacological preconditioning with omeprazole on isolated rat heart subjected to ischemia/reperfusion. Considering the fact that myocardial tissues have $H^+/K^+-ATPase$ ^[19], we examined the effects of one of clinically most used $H^+/K^+-ATPase$ inhibitor (omeprazole) in isolated rat heart.

Proton pump inhibitors may be a particularly important in patients with intrinsic cardiac disease however their safety has not been well studied. Omeprazole was first introduced into clinical practice and it is commonly used for treatment of gastroesophageal reflux and erosive esophagitis but some studies pioneering demonstrated the protective effect of omeprazole on myocardial contractility in isolated rat hearts^[25].

Considering the fact that $H^+/K^+-ATPase$ exists in myocardial tissues it could be expected that specific proton pump inhibitors might change the mechanical and electrical properties of the myocardium and might cause intracellular acidification via decreasing the extracellular H^+ transport and membrane depolarization through intracellular K^+ import^[6,16].

Gomes et al.^[25] have shown that administration of omeprazole before ischemia induction had protective effect on myocardium function recovery and our results were very similar especially regarding to values of SLVP and dp/dt max. These results are coherent with findings in studies where only ischemic preconditioning was induced^[2].

On the other hand, in case of coronary flow (CF), in the present study there were no difference between OPC and IPC groups in last point of reperfusion (Figures 2a and 4a), which was very close to findings of Gomes et al.^[25].

It has been shown, that ischemic preconditioning can reduce the magnitude of ischemia/reperfusion injury via activation of K^+ adenosine triphosphate (ATP)-sensitive ($K(ATP)$) channels^[26]. Concerning this fact, Kersten et al.^[27] found that left-ventricular pressure and coronary flow, respectively,

were recovered to a greater extent after inducing ischemic preconditioning. In our study, results are very similar (Figures 2a, 2b, and 2f).

A study evaluating animal model of frog by Gautam et al.^[28], showed interesting results. They found that PPI in minimal used dose did not change heart rate, but when they increased doses twice, they noticed bradycardia. However, Gomes et al., on rat model did not show any effect on heart rate^[25]. In this investigation, we found high statistical significant drop of values of HR between period of stabilisation and first minute of reperfusion in OPC group (Figure 4e).

Birnbaum et al.^[29] showed that induction of ischemic preconditioning on isolated rabbit heart leads to slower heart rate then in control group as we also demonstrated (Figures 2e and 3e).

In our study, omeprazole decreased tension and complete inhibition of cardiac contractility (Figure 4d) and these results correlate with studies of other authors who investigated another PPI on similar models^[6,30,31]. In OPC group we remarked that values of every parameter was significantly increased in first minute of reperfusion compared to point of stabilisation (Figures 4a to 4f).

Based on findings by Murry et al.^[2], we investigated ischemic preconditioning using similar procedure and we also concluded that in IPC group there was recovery of all measured parameters.

Furthermore, based on our results it seems that ischemic preconditioning could be used as first window of protection after ischemic injury especially due to all investigated parameters showed continuous trend of recovery of myocardial function. On the other hand, after administration of omeprazole we noticed sudden trend of recovery with positive myocardium protection, although less effective than results obtained with ischemic preconditioning not withstand we must consider that omeprazole may be used in many clinical circumstances where direct coronary clamping for ischemic preconditioning is not possible.

CONCLUSION

Although there is lack of data regarding the events occurring during cellular adaptation to stressful stimuli, ischemic preconditioning may be powerful means in protection of ischemic myocardium, and the results of this investigation also confirmed the important preconditioning effect of the omeprazole in the protection against reperfusion lesions of the myocardium ischemia. However, current research on the different aspects of preconditioning seems to diverge more and the possibility of pharmacological manipulation of the pathways involved becomes reality. Therefore, this study is meant as another contribution for many other investigations, with emphasis on pharmacological preconditioning.

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Authors' roles & responsibilities	
NJ	Analysis and/or interpretation of data; statistical analysis; study design; operations and/or experiments conduct; writing of the manuscript or critical review of its content
AP	Analysis and/or interpretation of data; statistical analysis; final approval of the manuscript; operations and/or experiments conduct
IS	Conception and design; operations and/or experiments conduct; writing of the manuscript or critical review of its content
VZ	Final approval of the manuscript; study design; operations and/or experiments conduct; writing of the manuscript or critical review of its content
DD	Final approval of the manuscript; study design
VJ	Final approval of the manuscript; study design

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