Does pantoprazole protect against reperfusion injury following myocardial ischemia in rats?


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Abstract. – BACKGROUND: Myocardial ischemia is inadequate perfusion due to reduced blood flow. Sudden onset of reperfusion could result with damage to the myocytes that have not been affected during ischemia called ischemia reperfusion (I/R) injury. Extracellular accumulation of H⁺ ions resulting in tissue acidosis is one of the underlying mechanisms. Inhibition of myocardial H⁺/K⁺-ATPase, namely proton pump, may lead to intracellular acidification via decreasing the extracellular H⁺ transport.

AIM: The aim of this study is to investigate the effects of a proton pump inhibitor pantoprazole in intact rat I/R models.

MATERIALS AND METHODS: A total of 30 adult male Wistar albino rats weighing 200-300 g were studied. Rats were allocated into four groups: sham (n=6), ischemia (n=8), control (n=8), and pantoprazole (n=8). Left anterior descending coronary artery was occluded for 30 minutes and then reperfused for two hours. Pantoprazole was administered via jugular vein at the dose of 9 mg/kg starting from 30 minutes before ischemia, to the first 30 minutes of reperfusion. Haemodynamic parameters were recorded and serum CK-MB levels were measured. After reperfusion, heart was removed for the measurement of myocardial infarct size. Myocardial infarct area was measured using triphenyltetrazolium chloride (TTC) staining technique. Myocardial infarct size were expressed as the percentage of the total left ventricular weight.

RESULTS: Compared with other groups, plasma concentrations of CK-MB at the end of ischemia and reperfusion and myocardial infarct size were significantly lower in pantoprazole group (p < 0.008).

CONCLUSIONS: Pantoprazole preconditioning induces delayed cardioprotection in intact rat I/R model, which may be triggered via H⁺/K⁺-ATPase ion channels.

Key Words: Myocardial reperfusion injury, Pantoprazole, Myocardial ischemic Preconditioning, Proton pump inhibitors, Myocardial ischemia

Introduction

Ischemia is the inadequate tissue or organ perfusion due to arterial blood flow being partially or totally occluded due to thrombus, atherosclerotic plaque, vasoconstriction or inflammation. It leads first hypoxia then cell injury and death through negatively affecting oxidative respiration. Renewal of this blood flow and oxygenation of the ischemic tissue or organs is called reperfusion. Myocardial reperfusion injury is the inflicted damage of myocytes that have not been dead during ischemia through the metabolic, functional and structural catastrophic events triggered by renewal of the perfusion. Oxidative stress, intracellular and mitochondrial Ca²⁺ overload, complement activation, and the accumulation of inflammatory cells in the infarcted myocardial tissue are some of the mechanisms that were accused for this reperfusion injury.

Ischemia stimulates the intracellular accumulation of Na⁺, H⁺ and Ca²⁺ ions resulting in tissue acidosis. In contrast, reperfusion causes sudden changes in ion flow and the rapid normalization of pH may lead increase in the tissue toxicity paradoxically. Swift intracellular acidosis during myocardial ischemia activates the pH-regulating ion carriers and Na⁺/H⁺ exchangers resulting with extracellular proton accumulation. However, with the washing off the extracellular fluid, they reactivate during reperfusion. The inhibition of Na⁺/H⁺ exchangers, decrease first Na⁺, then Ca²⁺ flow into the cell and prevent intracellular Ca²⁺ accumulation. Furthermore, it decreases the outflow of H⁺ from the myocardial cells.

Generally known as “proton pump”, H⁺/K⁺-ATPase is an ion pump greatly expressed in different tissues and has distinct biological effects. While their main function is acid secretion in gastric parietal cells via actively taking (changing with
H\(^+\) ions) K\(^+\), H\(^+\)/K\(^+\)-ATPases were also described smooth muscle cells and cardiac myocytes. Considering the fact that myocardial tissues have H\(^+\)/K\(^+\)-ATPase, specific inhibitors of H\(^+\)/K\(^+\)-ATPase might change the mechanical and electrical properties of the myocardium may be expected. Inhibition of myocardial H\(^+\)/K\(^+\)-ATPase may lead intracellular acidification via decreasing the extracellular H\(^+\) transport and membrane depolarization through intracellular K\(^+\) import. The aim of this study is to reveal the preventive efficacy of specific H\(^+\)/K\(^+\)-ATPase inhibitor, pantoprazole on ischemia reperfusion injury with biochemical, hemodynamic and histopathologic data.

**Materials and Methods**

In this study, 30 male, 8 weeks old Wistar albino type rats (mean weight 200-300 g, body temperature 37.5°C, respiratory rate 100-150 per minute, systolic blood pressure 80-130 mm Hg, mean blood volume of 1/20 of body weight and pulse rate 200-360 per minute) raised in the Experimental Animal Laboratory of Bezmialem Foundation University Medical Faculty were used. We preferred rats as our experimental animal because it is much used in myocardial ischemia reperfusion models and has small collateral circulation in its myocardium. The rats were cared for in a clean environment at room temperature, under standard laboratory conditions in cages with five rats each and fed with pellet feed. Twelve hours before the surgical procedure, feeding stopped except free drink water. Because they will be sacrificed following the experiment, they did not need postoperative care. Study protocol is consistent with the international care and use of laboratory animals\(^{17}\) and is accepted by the Ethics Committee for Experimental Animals of Bezmialem Foundation University Medical Faculty. All rats fed for two weeks before the experiment to let them to adapt the environment.

**Experimental Groups**

Rats were randomly divided into four groups. All of them underwent thoracotomy. The properties of the study groups were as follows:

**Group 1 (six rats):** Sham group; LAD (left anterior descending) coronary artery did not occluded with a stitch passing myocardium following the intervention and surgical procedures.

**Group 2 (eight rats):** ‘Only ischemia’ group; myocardial ischemia had constructed following the intervention and surgical procedures; however, reperfusion did not permitted.

**Group 3 (eight rats):** Control group; LAD coronary artery had occluded following the surgical procedure. Intravenous saline infusion had given starting 30 min before ischemia, continuing during 30 minutes of ischemia and in the first 30 min of reperfusion. Total reperfusion time was 120 min.

**Group 4 (eight rats):** Pantoprazole group. LAD coronary artery had occluded following the surgical procedure. 9 mg/kg (the dose leading to 300 M of serum concentration as shown in in previous studies) intravenous pantoprazole (Pantpas\(^{®}\) 40 mg IV injectable vial, Bayer Türk Kimya San. Tic. Ltd. ti.) had given starting 30 minutes before ischemia, continuing during 30 minutes of ischemia and in the first 30 minutes of reperfusion\(^{14}\). After 120 min reperfusion, rats were sacrificed by cervical dislocation.

The effects of all interventions and surgical procedures on examined hemodynamic, biochemical and pathologic data during the study had neutralized in the sham group.

**Anesthesia and Monitoring**

Rats were anesthetized with 60 mg/kg ketamine (Ketalar\(^{®}\) vial 100 mg/ml Alfasan International Holland) and 5 mg/kg xylazine (Alfazyne\(^{®}\) 20 mg/ml Alfasan International Holland), and the same doses were repeated if necessary. The neck and the anterior wall of chest were shaved; then surgical area were painted with povidone-iodine solution 10 per cent (Isosol Solution, Merkez Laboratuare A.S.). Tracheostomy were performed with a neck incision, and intubated. Then endotracheal tube were connected to the mechanical animal ventilator with respiratory rate of 60 per minute, 100 per cent oxygen support and 15 ml/tidal volume.

Carotid artery and jugular vein were catheterised with 24 G Branula for continuous pressure monitoring and saline infusion/pantoprazole injections, respectively. 15 minutes following the intubation, 0.2 ml arterial blood were drawn for blood gas determinations. Heart rate (HR) and mean arterial blood pressure (MABP) were monitored continuously with ECG-pressure monitor until the end of the experiment.

**Surgical Procedure**

Left thoracotomy was made to reach the heart in 4th intercostal space. The surgical manipulation area was widened with mini-thorax retractor and
the heart was reached through a pericardial incision. The LAD branch of left main coronary artery on the interventricular septum was crossed intramyocardially before giving its diagonal side branch using 6-0 10 mm prolen suture with an atraumatic needle. At the beginning of the 15 minutes balancing period, 600 IU/kg heparin (Nevarpin® vial 5000 IU/ml, Mustafa Nevzat Ilac San A.S.) was given intravenously to prevent thrombus formation in the coronary artery. At the end of this period, the needles of the suture was passed through plejit and over it, the threads of the suture were tightened with the help of snare to prevent the LAD trauma and to obtain complete occlusion an ischemia. 0.5 ml blood will be drawn for cardiac enzyme (CK-MB and troponin I) measurement before the snare tightened. The blood loss during the procedure was replaced with lactated Ringer in volume, which is three times for the blood loss. Ischemic period was 30 minutes long; at the end of this, the snare was loosened to obtain reperfusion. Experimental animal were connected to the ventilator for two hours with reperfusion. During this period, the edges of thoracotomy incision was approximated with temporary prolen suture to decrease the insensible loss. At the end of second hour, the heart of the rat was taken out and put in an empty pathology case. This case was transferred to the laboratory of Pathology Section of B ezmialem University in a mixture of cold water-ice without losing time (in 3-4 minutes).

**Calculation of the Myocardial Infarction Size**

The hearts were kept in the freezer of the pathology laboratory in -30 °C for 30 minutes to make it semi-frozen. During this time, triphenyltetrazolium chloride (TTC) phosphate buffer solution 1 percent at pH 7.4 was prepared. TTC painting technique was used in calculation of the myocardial infarction size. While there was no change during gross examination of myocardial infarction area before twelve hours, the necrotic area in the tissue sections could be visible in 2-3 hours with this technique. Because viable cells had dehydrogenase activity, they react with TTC and the color of the tissue would be converted to red. Because a membrane injury was developed, dehydrogenase enzyme oozed to the extracellular space and its activity in the cell decreased. Because there was no enzymatic reaction with TTC, tissue stayed colorless. The heart was sliced in three mm-thick slices from apex to base in parallel to the atrioventricular sulcus. These slices were kept in saline for 15 minutes to remove the blood from tissue. Then the same slices were incubated in TTC phosphate buffer solution at 37.0°C for thirty minutes. For fixation of the color difference between slices, tissue specimens were kept in formol solution 10% and photographed (Figure 1A). The sizes of the infarcted areas were calculated in Bezmialem University Medical Faculty’s Section of Pathology with the computerized planimetry program. Myocardial infarction size were expressed as the percentage of the total left ventricular weight.

**Biochemical Measurements**

Venous blood samples have drawn before (baseline) and 30 minutes after the coronary artery occlusion; furthermore, 30 and 120 minutes following reperfusion to determine the levels of cardiac creatinine kinase enzyme (CK-MB). These samples have kept in room temperature for 15 minutes permitting coagulation then centrifuged in

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**Figure 1.** A. Gross photograph of triphenyltetrazolium chloride-stained rat heart slice. Pale areas denote infarcted tissues. B. Hematoxylin and eosin staining of the rat infarcted heart specimen demonstrated myocytolysis and edema (magnification × 600).
400 × g for twenty minutes. The serum was separated with the aid of a pipette, transferred to Eppendorf and kept in −80 °C until analyzing.

**Histopathologic Examination**

The slices obtained from apex to base to calculate the infarcted areas subsequently kept in formal 10% stained with hematoxylin-eosine and examined under the light microscope for myocardial edema, myocyte loss, focal hemorrhage and polymorphonuclear leucocyte (PNL) infiltration then scored (0, none; 1, mild; 2, moderate; 3, severe) (Figure 1B).

**Statistical Analysis**

SPSS 13.0 program (SPSS Inc., Chicago, IL, USA) used for statistical analyses. The results were expressed as mean ± standard deviation. To compare the groups, we performed Kruskal-Wallis analysis followed by Mann-Whitney U test. We also performed Friedman and Wilcoxon signed ranks tests to analyse intra-group differences. All the p-values were adjusted using the Bonferroni method of correction. Using this approach, Bonferroni-adjusted p-values < 0.008 were considered statistically significant (which corresponds to an initial, unadjusted p-value < 0.05).

**Results**

**Hemodynamic Parameters**

The hemodynamic indexes (HR and MABP) of the four groups during the experiment were demonstrated in Table I. There were no significant differences among groups at baseline before ischemia. Compared to baseline values, MABP and HR were decreased significantly in groups 2, 3, and 4 (p < 0.008). The MABP and HR decreased significantly in the group 2 compared to the other groups (p = 0.002) at the end of ischemia. During reperfusion (both at 30. and 120. minutes), MABP decreased significantly in groups 2, 3 and 4 compared to control group (group 1) (p < 0.008). Also, HR of the groups 2, 3 and 4 decreased significantly during ischemia and reperfusion compared to group 1 (p < 0.008). Compared to group 2, MABP was preserved in groups 3 and 4 during ischemia and reperfusion (p < 0.008) but there was not any significant difference between group 3 and 4. HR significantly decreased in group 2 compared to groups 3 and 4 at the end of reperfusion. Also HR was similar in groups 3 and 4 during the experiment.

| Table I. Summary of mean arterial blood pressure (MABP) and heart rate (HR) in each group. Values are given as mean ± standard deviation. |
|-------------------------------------------------|-----------------|-----------------|-----------------|
| **MABP (mmHg)** | **Baseline** | **End of ischemia** | **End of reperfusion 30’** | **End of reperfusion 120’** |
| Group 1 | 138.3 ± 6.0 | 141.7 ± 10.8 | 134.2 ± 3.8 | 136.7 ± 4.1 |
| Group 2 | 145.0 ± 3.8 | 112.5 ± 4.6 | 101.2 ± 5.2 | 84.4 ± 4.2 |
| Group 3 | 142.5 ± 6.0 | 129.4 ± 5.6 | 122.5 ± 4.6 | 116.2 ± 5.8 |
| Group 4 | 138.1 ± 5.3 | 128.7 ± 5.2 | 123.8 ± 5.2 | 119.4 ± 4.9 |
| **HR (bpm)** | **Baseline** | **End of ischemia** | **End of reperfusion 30’** | **End of reperfusion 120’** |
| Group 1 | 316.2 ± 9.1 | 340.0 ± 7.1 | 341.7 ± 8.7 | 333.3 ± 9.3 |
| Group 2 | 312.5 ± 8.0 | 300.0 ± 8.4 | 275.0 ± 6.5 | 246.2 ± 9.9 |
| Group 3 | 320.0 ± 10.0 | 304.4 ± 9.4 | 281.9 ± 10.7 | 277.5 ± 8.9 |
| Group 4 | 314.4 ± 6.2 | 293.8 ± 8.3 | 273.7 ± 6.9 | 271.9 ± 8.0 |
Histopathologic Examination

Only the severity of polymorphonuclear leukocyte (PNL) infiltration was decreased slightly in pantoprazole group among histopathological indexes of myocardial ischemia-reperfusion injury (1.6 ± 0.7, 1.4 ± 0.9, and 0.9 ± 0.5 for groups 2, 3 and 4 respectively; \( p > 0.008 \)).

Discussion

In the present study, the effect of pretreatment with pantoprazole was evaluated in a rat model of ischemia reperfusion injury. The main findings of this study were pretreatment with pantoprazole had a significant protective effect against myocardial ischemia reperfusion injury and significantly decreased myocardial infarct size. The important outcome of the study is that it provides new evidence for the cardioprotective effect of proton pump inhibitors.

Ischemia is the inadequate tissue or organ perfusion due to arterial blood flow being partially or totally occluded due to thrombus, atherosclerotic plaque, vasconstriction or inflammation\(^1\). It leads initially hypoxia, then cell injury and death through negatively affecting oxidative respiration\(^2\). Renewal of this blood flow and oxygenation of the ischemic tissue or organs is called reperfusion. If the duration of ischemia is less than 20 minutes, and reperfusion obtained during this period, no structural or biochemical injury develops. However, if this period is longer than 45 minutes, tissue or organ injury following the ischemia is inevitable and is defined as reperfusion injury. Myocardial reperfusion injury is the inflicted damage of myocytes that have not been dead during ischemia through the metabolic, functional and structural catastrophic events triggered by renewal of the perfusion. Many mechanisms were accused for this reperfusion injury. Chiefly, the free oxygen radicals rapidly forming with the presentation of molecular oxygen then, the disturbed calcium ion balance in myocytes, the stimulation of neutrophil accumulation and of adhesion molecule forming by cytokines and interleukins released from endothelial cells or macrophages are some of these mechanisms\(^3\). This reperfusion injury may be greater than the damage caused by pre-reperfusion ischemia. Reperfusion injury is reversible or not according to the duration of the ischemic period.

Ischemia stimulates the intracellular accumulation of Na\(^+\), H\(^+\) and Ca\(^{2+}\) ions resulting in tissue acidosis. In contrast, reperfusion causes swift changes in ion flow and the rapid normalization of pH may lead increase in the tissue toxicity paradoxically\(^4\). Under normal conditions, hydrogen ions are necessary in the production of ATP for using in the Na\(^+\)/ATP-ase exchange pump\(^5\). Myocardial is-

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<th>Table II. Serum CK-MB levels of the study groups. Values are given as mean ± standard deviation.</th>
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<td><strong>CK-MB (IU/dL)</strong></td>
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CK-MB: myocardial creatine kinase.

![Figure 2](image_url)

**Figure 2.** The percentage of infarct size assessed following myocardial ischemia reperfusion. Values are expressed as mean ± standard deviation.
chemia is characterized with anaerobe metabolism and intra-cellular acidosis. Swift intracellular acidosis activates the pH-regulating ion carriers and Na+/H+ exchanger. Consequently, the protons which are essential for ATP production decrease and Na+ accumulates in the cell. Over-consumed ATP, contributes to the intracellular Na+ accumulation via inactivating Na+/K+-ATPase. Increasing intracellular Na+, leads first Ca2+ accumulation in the cell through activating the Na+/Ca2+-exchanger, then hyper contracture and cell death. Because all of these exchangers are self-limited, extracellular proton accumulation may occur. However, with the washing off the extracellular fluid, they reactivate during reperfusion. The inhibition of Na+/H+ exchangers, decrease first Na+, then Ca2+ flow into the cell and prevent intracellular Ca2+ accumulation. Furthermore, it decreases the outflow of H+ from the myocardial cells. Those protons may be consumed during ATP production.

Generally known as ‘proton pump’, H+/K+-ATPase is an ion pump greatly expressed in different tissues and has distinct biological effects. While their main function is acid secretion in gastric parietal cells via actively taking (changing with H+ ions) K+, H+/K+-ATPases were also described in other places such as renal and colonic tissues. These non-gastric H+/K+-ATPases contribute the absorption of potassium ions and luminal acidification. For these reasons, they are responsible for serum K+ homeostasis and acid-base balance. They have also been shown in smooth muscle cells. H+/K+-ATPase inhibitors decrease the absorption and amount of potassium ions in vascular smooth muscle cells and lower intracellular pH. Proton pump inhibitors (PPI), cause in vitro relaxation of airway smooth muscle cells of pigs and humans. It has been established that omeprazole, a specific inhibitor of H+/K+-ATPase, causes relaxation of the smooth muscle cells of human myometrium and inhibits spontaneous contractions. SCH 28080, another strong PPI, leads reversible relaxation in isolated pig and human arteries. An active, functional form of H+/K+-ATPase was located in rat atrial myocytes and identified as K+ dependent, sensitive to omeprazole and insensitive to ouabaine. In another report, the coupling of intracellular K+ import and extracellular H+ import in swine heart and ventricular myocytes perfused with Langendorff technique, which was energy-dependent and sensitive to omeprazole. Beisvag et al assessed myocardial H+/K+-ATPase and K+ transport in isolated rat cardiomyocytes functionally through with sequencing of the polymerase chain reaction products, immunochemical protein analysis and Western blot analysis. Considering the fact that myocardial tissues have H+/K+-ATPase, it could be expected that specific inhibitors of H+/K+-ATPase might change the mechanical and electrical properties of the myocardium. Inhibition of myocardial H+/K+-ATPase, may lead intracellular acidification via decreasing the extracellular H+ transport and membrane depolarization through intracellular K+ import. Positive inotropic and negative chronotropic effects omeprazole, and lansoprazole in isolated rat atria demonstrated previously. This concentration-dependent, reversible and repeatable effect of PPIs was independent of the mechanisms which increase the contractility such as inhibition of H+/K+-ATPases and type III phosphodiesterase, and adrenoreceptor activation. In contrast to the data obtained from in vitro studies in rats, omeprazole, lansoprazole and pantoprazole had no effects on pulse rate, blood pressure and ECG when administered intravenously (IV). Their in vivo cardiovascular ineffectiveness was explained with being highly protein-bound and quick elimination rates of the compounds. In the same study, omeprazole and lansoprazole did not show negative inotropic effect in isolated human atria.

Gomes et al evaluated the effects of omeprazole pretreatment on pulse rate, coronary blood flow, systolic blood pressure and also on dP/dt max as an indicator of myocardial contractility of the left ventricle in isolated rat hearts with ischemic perfusion injury. There were no differences in pulse rate and coronary blood flow between the animals pretreated and the animals not pretreated with omeprazole. However, the blood pressure and myocardial contractility were preserved in the omeprazole-pretreated rats. Investigators explained this positive effect with changes in transmembrane H+/K+ gradient, which is the first sign of myocardial ischemia reflected as T wave change in ECG. Similarly in our study, a moderate decline in blood pressure was investigated in pantoprazole group compared to control, but the difference did not reach statistical significance (Table I). In another work examining the in vivo preventive effect of lansoprazole against reperfusion injury; in rats with intestinal ischemia obtained with ligation of artery mesenterica superior and truncus celcius, the intraluminal hemoglobin and protein levels as an indicator of mucosal injury were preserved. The inhibition of lipid peroxidation and decreased development of intestinal inflammation were suggested as probable preventive mechanisms against ischemic reperfusion injury.
The results of the present study revealed that the treatment with a proton pump inhibitor prior to and during regional myocardial ischemia and reperfusion, could provide cardioprotection against reperfusion injury. Although we did not measure intracellular ion concentrations and pH of cardiomyocytes in our research. Limited infarct size and relatively less elevated serum CK-MB levels in rats pretreated with pantoprazole suggested us the potential role of H+/K+ pump inhibitors in ischemic myocardial preconditioning. It could be speculated that inhibition of the H+/K+-ATPase pump in rat myocytes with pantoprazole decreases extracellular H+ transport causing enhanced intracellular acidification. It is already well known that protons are essential for ATP production and play an important role in regulating intracellular sodium and calcium homeostasis. Pantoprazole could be demonstrated its beneficial effects by activating Na+/K+-ATPase and decreasing intracellular calcium overload. Furthermore, previously reported positive inotropic and negative chronotropic effects of proton pump inhibitors in isolated myocardium could contribute to a more favorable ischemia reperfusion injury.

Conclusions

The administration of pantoprazole before myocardial ischemia and reperfusion reduced the extent of myocardial injury by a mechanism probably involving inhibition of the cardiac H+/K+-ATPase pump.

Acknowledgements

The rats were kindly provided by Dr. Inan, Bezmialem Foundation University, Research Center, Istanbul, Turkey. The study was supported by funds from Bezmialem Foundation University, Scientific Investigation Projects Support Committee.

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