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Effects of rivaroxaban on myocardial ischemia-reperfusion injury in rats

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Abstract

Myocardial infarction and further ischemia-reperfusion injury is a life-threatening conditions in humans. In this study, the effects of rivaroxaban, an anticoagulant agent, were aimed to be studied in a myocardial ischemia-reperfusion (I/R) injury model in rats. Male Wistar-albino rats were allocated into three groups; Rivaroxaban (n=15), control (n=15) and sham (n=10). Myocardial ischemia (30 minutes) and then reperfusion (120 minutes) were surgically performed in the rivaroxaban given (3mg/kg/day by gavage for 10 days before surgical procedures) and the control groups. Electrocardiography changes, blood pressure and heart rate were recorded before ischemia, and during the periods of ischemia and the reperfusion. Hemodynamic and blood parameters were recorded. Necrotic tissue in the myocardium was determined by the triphenyl tetrazolium chloride dye method. The extent of myocardial necrosis and risk area was calculated using a computer-assisted image program. In the rivaroxaban administered group, the size of necrotic area in the myocardium decreased significantly, however, mean heart rate and mean arterial blood pressure did not change. K+ and CK levels in serum, which are indicative of tissue necrosis, were significantly lower in the rivaroxaban group compared to the control group ($p<0.05$). Rivaroxaban use, compared to the control group, effectively reduced the extent of myocardial injury as assessed by less necrotic myocardial tissue in rats. This protective effect of rivaroxaban may be attributed to its less thrombus formation in the coronary arteries during ischemia and less acidosis during tissue damage.

Keywords: Rivaroxaban, ischemia-reperfusion injury, myocardium, rats

Introduction

Despite important improvements, atherosclerotic cardiovascular disease continues to be an important cause of morbidity and mortality in the world [1]. The term "atherosclerotic cardiovascular disease" comprises a group of disorders of the heart and blood vessels and hence various physiopathology and consequences arise. Atherogenesis emerges as a result of the interactions between arterial intimal cells, blood components and messenger molecules [2]. Ischemic degeneration as a consequence of atherosclerosis is an important endpoint of this disease and clinicians primarily focus on bringing the blood flow as quickly as possible to the affected ischemic tissue [3]. However, regaining blood flow causes additional damage to the tissue, a phenomenon known as ischemia-reperfusion (I/R) injury [4]. The faith of I/R injury can be deadly in tissues such as the myocardium and hence it needs to be handled carefully.

Myocardial I/R injury is a complicated phenomenon where various factors cause the injury of myocardial tissue [5]. The coagulation cascade contributes significantly to cardiac injury after myocardial infarction [6]. In the process of coagulation cascade, factor Xa starts the final common pathway resulting in thrombin formation [7]. Both free factor Xa and prothrombinase complex can be inhibited by Rivaroxaban [8]. Used primarily as an anticoagulant agent, rivaroxaban was also shown to induce secondary prevention of cardiovascular events after I/R injury in mice model [9]. Decreased inflammation and less thrombus formation within the coronary arteries have been proposed [10]. However, the mechanism behind the effects of rivaroxaban in I/R injury is still largely unknown.

In this study, effects of rivaroxaban in experimental myocardial I/R model in rats were investigated. Special attention was given to myocardial degeneration, hemodynamics and blood biochemical

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parameters to assess the protective potential of rivaroxaban in myocardial I/R injury.

Material and Methods

Experimental Animals and Groups

Ethical permission was obtained from Inonu University Experimental Animals Ethics Committee (2013/A-43 ethics committee approval) and the study was funded by Scientific Research Project Unit of Inonu University (Project No: 2013-190). Total of 40 Wistar-Albino male rats (10 to 12-week-old and weighing 250-300 gr) were obtained from Inonu University Experimental Animals Research Center. The animals were fed with standard rat pellets and kept in standardized conditions (rooms with 12 hours light and dark cycle, 21±2ºC constant heat, air-conditioned and constant moisture). The rats were divided into three groups; Control group (n=15): Myocardial I/R procedure was applied, Rivaroxaban group (n=15): Following 10 days of rivaroxaban administration myocardial I/R procedure was performed, and Sham group (n=10): Only surgical procedure was done.

Drug Administration

Rivaroxaban was given at a dose of 3mg/kg/day by gavage in 12-hour intervals for 10 days before the surgical procedures. The animals that completed their drug doses at the end of the 10^{th} day were included in the experiment.

Surgical Procedure

The animals were anesthetized by administering 1.2-1.4g/kg urethane (Sigma-Aldrich, Saint Luis, MO, USA) intraperitoneally. Tracheal cannulation was performed for artificial respiration. A cannula was placed in the carotid artery and the blood pressure, heart rate and ECG were recorded with the help of a transducer and a recorder (Bipoca MP-100 Data system).

Left thoracotomy was performed. After the thorax was opened, due to the removal of the negative pressure inside, respiration with positive pressure was started by giving room air with 1.5 ml/100 gr volume and 60 beat/min through a ventilation device (Harvard Animal Rodent Ventilator). This procedure aimed to maintain respiration and protection of pCO_2 and pO_2 values. Using a 10 mm, round-tipped needle, a 6/0 silk suture was performed underneath the left anterior descending coronary artery, which was followed by a 20-minute waiting period for stabilization. Based on the assessment criteria identified by the Lambeth Conventions guidelines [11], the animals were excluded from the study when arrhythmia was experienced or mean blood pressure decreased below 70 mm Hg before occlusion due to these procedures. 15 rats were excluded from the study because they could not fulfil the evaluation criteria set by the Lambeth Conventions guidelines during the ischemia or reperfusion period.

Tips of the suture that was put were transformed through a small plastic tube of 1mm diameter and 1cm height. At the end of the 20-minute stabilization period, ischemia (occlusion) was performed when the rope placed underneath the left anterior descending coronary artery was clamped with the help of a plastic tube to close it. This was followed by reperfusion when it was opened again. The period for the measurements of necrotic area included 30min ischemia and 120min reperfusion. At the end of the experiment, euthanasia was performed by taking blood from the animal's inferior vena cava.

Assessment of Hemodynamic and Biochemical Parameters

Following the experimental procedures, ECG, blood pressure and heart rate were recorded. Mean arterial blood pressure and heart rates were assessed. Blood samples were drawn and centrifugated for biochemical analysis. At the end of the experiment, prothrombin time (PT) in plasma, Activated Partial Thromboplastin Time (aPTT), glucose, total protein, albumin, creatine kinase (CK), Na^+, Cl^-, K^+, Mg^{++}, and Ca^{++} values were detected.

Assessment of Myocardial Necrosis

At the end of the experiment, the hearts were mounted on the Langendorff device. They were perfused with physiological saline solution for washing the blood remaining in the coronary artery. The silk suture around the coronary artery was clamped again. 2ml infusion perfusate was given from 1-10µmol diameter, 0.5% fluorescent particle (Duke Scientific Corp., Palo Alto, CA, USA) suspension. The area that did not have the fluorescent particles was identified as the area at risk. The hearts were taken from the Langendorff device, weighed, and frozen. It was then sliced at a thickness of 2 mm and incubated in a buffer (pH 7.4) including 1% triphenyl tetrazolium chloride (TTC) at 37ºC for 15min. TTC test differentiates metabolically active and inactive tissues as the live areas in the tissue are stained in dark red colour since they contain active enzymes and co-factors while the necrotic area is not stained [12]. After the staining, the heart slices were placed between two plate glasses with a 2mm distance between each other. Necrotic area borders (TTC negative tissue) (Figure 1) and area at risk (the area that did not have fluorescent particles under ultraviolet light) (Figure 2) were copied on transparent acetate. Infarct areas and the area at risk were measured using the computer-supported Image Tool 2.0 (Version 2.0 alpha 2 December 1997 Script Language Reference Manual) program. The volumes calculated by multiplying areas with slice thicknesses were obtained by adding all the slices for each heart. The infarct amount was expressed as the percentage of the area at risk.

Statistical analysis

Statistical analysis was performed using the IBM SPSS version 21.0 software (IBM Corp., Armonk, NY, USA). Kolmogorov-Smirnov test was performed for testing the normality of the group data. Data were summarized as means ± standard deviation. Homogeneity of the variances was performed using Levene's test. Comparison of the variables with normal distribution was performed using one-way variance analysis and t-test in independent samples. Multiple comparisons were done using Tukey test as the variances were homogenous. Statistical significance was taken $p<0.05$.

Results

Mean heart rates (MHR) (Table 1) and mean arterial blood pressure (MAP) (Table 2) were monitored and recorded before ischemia, during ischemia (10^{th}, 20^{th} and 30^{th} minutes) and reperfusion (30^{th}, 60^{th} and 120^{th} minutes) periods. No significant differences were

noted in MHR among the groups in all periods. MAP did not show any differences among the three groups before and during ischemia periods, however significant differences were observed at the 30th and 120th minutes of reperfusion between control and sham groups. MAP was also significantly different between rivaroxaban and sham groups at reperfusion 30th minute ($p<0.05$). No difference was present between rivaroxaban and control group at all periods recorded.

Table 1. Mean heart rate (MHR) of rats in control, rivaroxaban and sham groups. Measurements were performed before ischemia and during periods of ischemia (10th, 20th and 30th minutes) and reperfusion (30th, 60th and 120th minutes)

Heart Rate (beat/min)	Control	Rivaroxaban	Sham	P value
Before ischemia	**386.3±77.1**	**435±73.4**	**385±81**	**0.154**
Ischemia 10th minute	391.1±101.4	373.5±82.4	350.6±109.8	0.596
Ischemia 20th minute	388.±90.2	352.3±102.3	312.1±106.1	0.183
Ischemia 30th minute	328.4±107.4	358±118	361.8±69.3	0.659
Reperfusion 30th minute	325.8±127.5	392.6±81.4	305.9±82.3	0.083
Reperfusion 60th minute	364.±86.2	360.1±61.2	324.3±96	0.441
Reperfusion 120th minute	371.±84.1	363.4±92.2	317.3±61.1	0.258

Significant differences were detected in the sizes of necrotic area and necrotic/risk area as well as the rate of necrotic/total heart area between the control and rivaroxaban groups ($p<0.05$). They were all significantly lower in the rivaroxaban group as compared to the control (Table 3). In terms of the risk area in the myocardium, no significant difference was found between the control and the rivaroxaban group.

Table 2. Mean arterial blood pressure (MAP) of rats in control, rivaroxaban and sham groups. Measurements were performed before ischemia and during periods of ischemia (10th, 20th and 30th minutes) and reperfusion (30th, 60th and 120th minutes)

Blood pressure (mm/Hg)	Control	Rivaroxaban	Sham	P value
Before ischemia	82.3±12	85.3±15.6	80.5±10,93	0.663
Ischemia 10th minute	68.6±10.4	76.3±23	77.5±109	0.314
Ischemia 20th minute	68.6±16.8	65.4±18.4	78.5±7.4	0.131
Ischemia 30th minute	66.9±16.5	63.7±20.4	80.1±11.5	0.065
Reperfusion 30th minute	67.9±12.2[a]	65.9±9.6[b]	80.6±9.4	0.004
Reperfusion 60th minute	66.3±14.7	68.8±13	76.6±11.1	0.051
Reperfusion 120th minute	57±14.9[a]	69.7±13.7	78.1±11.2	0.002

[a] Difference between control and sham groups is significant ($p<0.05$); [b] Difference between rivaroxaban and sham groups is significant ($p<0.05$); Data were given as mean ±standard deviation

The Rivaroxaban group had a significantly higher international normalized ratio (INR) value in comparison to the sham group. Similarly, the group that was given rivaroxaban had a higher aPTT value in comparison to the control and sham groups (Table 4).

Cl^- and Ca^{++} values were significantly lower in the rivaroxaban group in comparison to the control group. Ca^{++} value in the control group was significantly higher in comparison to the sham group.

In the control group, K^+, Mg^{++}, and CK values were significantly higher in comparison to the sham group. As to the rivaroxaban group, no significant increase was found in the K^+ and CK values in comparison to the sham group. However, Mg^{++} value in the rivaroxaban group was significantly higher in comparison to the sham group. The glucose, total protein, and albumin ratios indicated no significant differences.

Table 3. Assessment of necrosis in myocardium in control and rivaroxaban groups following ischemia/reperfusion injury in rats

Variables	Control	Rivaroxaban	P value
Total Heart Area (cm²)	3.1±0.4	3.4±0.4	0.072
Risk area (cm²)	1.7±0.1	1.7±0.1	0.367
Necrotic Area (cm²)	1.1±0.8	0.9±0.1	<0.0001
Necrotic/Risk Area	63.1±4.3	53.5±5.7	<0.0001
Necrotic/Total Heart Area	36±8	27.1±3.7	0.001

Table 4. Biochemical parameters of blood samples from control, rivaroxaban and sham groups.

Variables	Control	Rivaroxaban	Sham	P value
INR	1.2±0.5	1.5±0.4[c]	0.9±0.1	0.009
aPTT (sec)	40.7±9.6	65.2±23.6[c,b]	38.8±9.8	<0.001
Glucose (mg/dl)	285.2±159.8	427.6±190	342.4±102.5	0.064
Total protein (g/dL)	5.5±0.9	5.1±0.8	5.4±0.6	0.375
Albumin (g/dL)	0.7±0.1	0.7±0.1	0.7±0.1	0.289
CK (IU/L)	7003.2±3656[a]	6147.3±2872	3473.9±2448.98	0.026
Na (mmol/L)	137.9±4.4	135.2±3.4	137.4±2.5	0.125
Cl (mmol/L)	109.3±3.3	105.9±4.7[b]	107.5±1.5	0.04
K (mmol/L)	6.3±1.5[a]	5.5±1.3	5±0.7	0.032
Mg ((mg/dL)	5.5±1.6[a]	5.5±1.3[c]	3.1±0.4	<0.001
Ca (mg/dL)	11.72±1.65[a]	10.1±1.2[b]	9.5±0.3	<0.001

[a] Difference between control and sham groups is significant ($p<0.05$); [b]Difference between control and rivaroxaban groups is significant ($p<0.05$); [c]Difference between rivaroxaban and sham groups is significant ($p<0.05$); Data were given as mean ± standard deviation. INR (international normalized ratio), aPTT (Activated Partial Thromboplastin Time), CK (creatine kinase), Na (sodium), Cl (chloride), K (Potassium), Mg (magnesium), Ca (calcium)

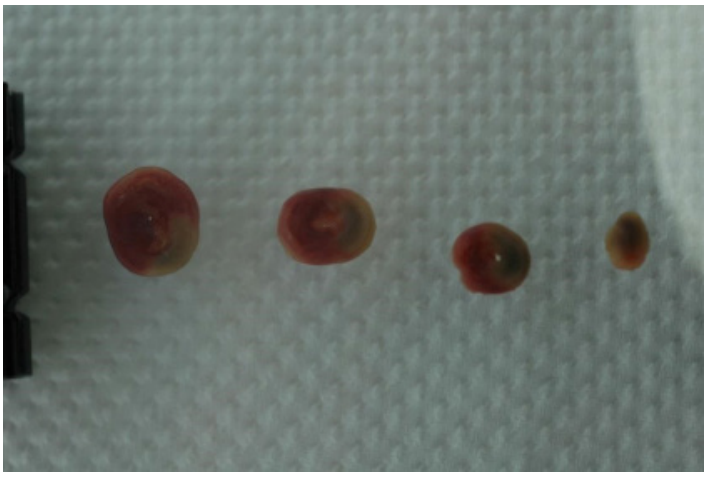

Figure 1. View of the necrotic field in the heart slices

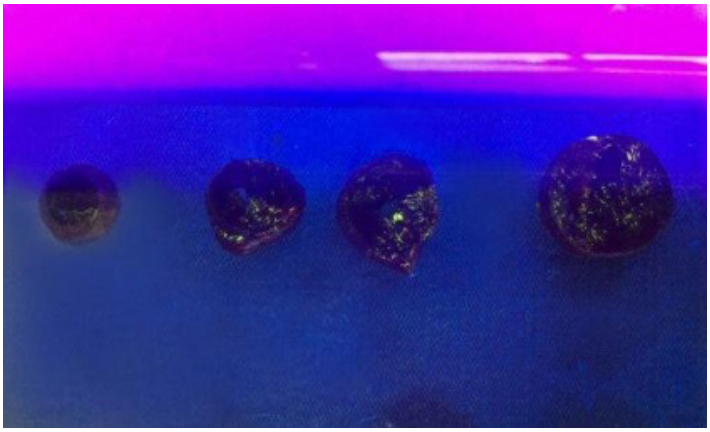

Figure 2. View of the risk area under fluorescent light

Discussion

Ischemia-reperfusion injury is mainly the result of oxidative injury and is mediated by toxic oxygen radicals, hence anti-oxidant agents are frequently investigated in preventing the injuries due to I/R. However other unknown factors contribute significantly to the process [13]. Cellular and molecular interactions during the formation of atherogenesis and following manipulations to regain the blood flow to prevent myocardial necrosis may have important consequences on the process. Cardiogenic shock following myocardial ischemia is one of the leading causes of hospital mortality [14]. In this context, rivaroxaban, which is a drug used primarily as an anticoagulant agent, was investigated in myocardial I/R injury model in rats. Rivaroxaban use was shown to significantly reduce the size of necrotic area as compared to the control group in this study. In addition, necrotic area/risk area and necrotic area/total heart area rates were also found to be lower as compared to the control group.

Myocardial functions deteriorate as a result of ischemic infarctions [15]. In the present study, no significant differences in MHR were seen between the three groups. There was a difference between the control and sham groups at the 30th and 120th minutes of MAP. Since these periods are ischemia and reperfusion periods, the effect of blood pressure in the control group in this way is an indication that the ischemia and reperfusion procedure were performed successfully. On the other hand, no differences in MAP were observed between the control and the rivaroxaban groups in all periods including during ischemia and reperfusion periods investigated in this study. These findings indicate that although myocardial degeneration occurred as a result of ischemia and the following reperfusion as shown by increased necrotic tissue, these effects were not strong enough to induce changes in MHR and MAP or the periods investigated here were not long enough to see differences.

Cellular homeostasis is disrupted during ischemia due to interruptions in cellular membrane ion channels resulting in increased Ca^{++} concentration inside the cells. Increased intracellular Ca^{++} concentration is an indication of cellular degeneration and near death. These events may lead to tissue degeneration and necrosis. Some blood biomarkers may also change due to increased cellular destruction. Muscle injury may cause increase in the amounts of myoglobin, urate, K^+, and phosphorus in the circulation [16]. This condition may lead to significant hyperkalemia when high amounts of tissue are damaged as in severe muscle damage or erythrocyte lysis [17]. In prolonged ischemia, ATP level and pH value decrease, and lactate accumulation and ion transportation mechanisms dependent on ATPase become unfunctional, which increases the intracellular and mitochondrial Ca^{++} level [18]. In the present investigation, a significant increase was found in the K^+ and CK values in the control group compared to the sham group, which indicates tissue necrosis as a result of ischemia/reperfusion injury. Rapid increase in K^+ following coronary artery occlusion was also previously shown [19]. No significant increase was found in the K^+ and CK values of the rivaroxaban group compared to the sham group indicating that rivaroxaban application reduced tissue degeneration as also supported by histopathological analysis.

It is known that a change in the blood pH level is inversely proportional to a change in the ionized calcium level [20]. Hence, after acidosis develops following tissue ischemia, there is a decrease in the connection of Ca^{++} to plasma proteins. As a result, there is an expected increase in the plasma ionized Ca^{++} level. In the current study, increase in Ca^{++} concentration in the control group compared to the sham group suggests that acidosis had also effects on the increase of these parameters in the control group. Ca^{++} concentration in the rivaroxaban group was found to be significantly lower compared to the control group. On the other hand, when the $HCO3^-$ concentration increases in plasma, there is a decrease in CI^- amount with the condition defined as CI^- shift [14]. Cl^- ratio was significantly lower in the rivaroxaban group compared to the control group and could be associated with the fact that it increases the $HCO3^-$ level in the rivaroxaban group. These changes in the amount of Ca^{++} and Cl^- support the view that acidosis in the rivaroxaban group was lower than that in the control group.

In terms of the Mg^{++} value, both the rivaroxaban group and the control group demonstrated a significant increase compared to the sham group in this study. Cardiac ischemia was previously reported to significantly decrease the renal blood flow and pressure at a ratio of approximately 40% [21]. Therefore, during coronary ischemia, the glomerular filtration rate might change depending on the renal perfusion impairment which might increase the plasma Mg^{++} value in both groups.

Rivaroxaban is a direct and selective inhibitor of factor Xa, which is the common end pathway of the coagulation cascade [22]. The use of rivaroxaban has therefore significant effects on coagulation parameters. In the current investigation, a significant increase was detected in the INR value in the rivaroxaban group as compared to the sham group. The aPTT value was also detected to be significantly higher in the rivaroxaban group as compared to both the control and the sham groups. Similar to our findings, Perzborn et al [23], reported that rivaroxaban prolonged aPTT and especially PT in dose-dependent plasma coagulation tests. However, PT and aPTT are not appropriate tests for measuring the pharmacodynamic effects of rivaroxaban. As a result of this prolongation in coagulation tests, the thrombosis amount formed within the coronary artery during ischemia is expected to be lower in the rivaroxaban group. Therefore, the affected infarct area was found to be more limited, and hence caused less reperfusion damage in the rivaroxaban group.

Although plasma pH was not measured in this study, plasma Ca^{++}

and Cl^- values in the rivaroxaban group were found to be lower as compared to the control group. This finding indicates that acidosis was less in the rivaroxaban group as compared to the control group. As a result, no increase was noted in the plasma K+ and CK values, which are the common indicators of tissue necrosis. Decreased tissue K+ and CK values as a result of anoxia in rabbit myocardium were shown previously and correlated with tissue degeneration [24]. Here, increased blood K+ and CK values might be related to the previous findings.

Conclusion

Rivaroxaban, a drug used as an anticoagulant agent, was investigated for its effect on myocardial ischemia-reperfusion models in rats and shown to have beneficial effects on preventing cardiac injury by decreasing the amount of necrosis. Therefore, it was concluded that rivaroxaban may be effectively used in patients with myocardial infarction. However, more advanced studies to be conducted in clinical settings are still in need to understand the whole effects of rivaroxaban.

Conflict of interests
The authors declare that there is no conflict of interest in the study.

Financial Disclosure
The authors declare that they have received no financial support for the study.

Ethical approval
Ethical permission was obtained from Inonu University Experimental Animals Ethics Committee (2013/A-43 ethics committee approval) and the study was funded by Scientific Research Project Unit of Inonu University (Project No: 2013-190).

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