Research Article
Effects of Ticagrelor on Skeletal Muscle Viability, Asymmetric Dimethylarginine, Malondialdehyde and Glutathione in Ischemia-reperfusion Model

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Abstract

Background and Objective: Although many agents have successful clinical results in experimental studies in order to prevent ischemia-reperfusion (I-R) injury, only a few of them have come into use clinically. In this study, the effects of ticagrelor-a new antiplatelet agent on skeletal muscle viability and blood asymmetric dimethylarginine, malondialdehyde and glutathione levels after experimental ischemia-reperfusion injury of lower limb have been analyzed. **Materials and Methods:** In this study, 21 male rats have been used. To induce lower limbischemia surgically at the ischemia-reperfusion group (n = 7) and ticagrelor group (n = 7) it clamped Infrarenal Abdominal Aorta (IAA) for 2 h after that were moved the clamp for reperfusion for 2 h. At the end of reperfusion, muscle tissue and blood samples of the rats were taken by sacrificing and excising their soleus muscles. Histopathological search was made in tissue samples and ADMA, MDA, GSH levels were studied in blood and muscle tissue. **Results:** The ADMA and MDA levels in blood and muscle tissue were more increased on I-R group than control group. This increase was less in ticagrelor administered group. Blood GSH levels were significantly more increased in the I-R group than control group. Compared to I-R group it was significantly decreased in ticagrelor group. Increase of muscle tissue GSH level was more significant in I-R group than in control group. In ticagrelor group muscle tissue GSH level was decreased significantly compared to I-R group. **Conclusion:** It was observed that ticagrelor reduces oxidant level and injury of skeletal muscle at lower limb ischemia-reper fusion injury. It is observed that ticagrelor is protective against ischemia-reperfusion injury.

INTRODUCTION

Microvascular dysfunction due to ischemia-reperfusion (I-R) injury is a potentially serious problem that may occur during various medical and surgical interventions, such as thrombolytic therapy, coronary angioplasty and cardiopulmonary bypass[1]. Oxidized metabolites are formed by the return of the blood flow to normal after ischemia enters into the bloodstream and spreads through the body, thus leading to distant organ damage. It has been suggested that oxygen free radicals are more likely to have a harmful effect, which can kill some cells that are susceptible to damage during ischemia.

The skeletal muscle plays an important role in the development of ischemia-reperfusion injury, because it has a large mass and is one of the tissues most susceptible to ischemic injury[2]. The inflammatory response, which begins with reperfusion in ischemic tissues, increases local damage. Degradation products released from the damaged tissues activate the coagulation process and cause microvascular injury and thrombosis, resulting in widespread muscle damage. Therefore, some studies have shown that high-dose heparin may reduce the permeability changes, improve the collateral flow and decrease the level of ischemic demarcation[3-5]. It is known that there is a significant increase in the mortality rate due to renal failure in the period following ischemia-reperfusion injury in extremities. Here, myoglobin or other toxic factors released from ischemic tissues are considered to play an important role[6]. The lung, liver, central nervous system, gastrointestinal system and myocardial dysfunctions may also be found. Some experimental studies have revealed that antioxidants, antithromboxanes, antileukotrienes and antiplatelet activating factors may be used to prevent systemic effects of reperfusion[7-9].

Asymmetric dimethylarginine (ADMA) impairs endothelium-dependent vasorelaxation and increases blood pressure by reducing NO levels via NOS inhibition[10]. Elevated ADMA levels significantly contribute to the increased risk of CVD. It is also known that ADMA increases and worsens oxidative stress and triggers inflammation[11,12].

Ticagrelor is a cyclo-pentyl-triazolo-pyrimidine (CPTP) and a reversible oral P2Y12 receptor antagonist[13]. Both thienopyridines and CPTPs act by blocking the P2Y12 receptor (one of two adenosine diphosphate (ADP) receptors on platelets) to inhibit ADP-induced platelet activation and aggregation[14].

It has been reported in the literature that antithrombositer agents may be effective in preventing the development of ischemia reperfusion injury. But according to our knowledge, it is not known whether ticagrelor, a new antiplatelet agent, has a protective effect in ischemia reperfusion injury. The aim of this study was to investigate the effects of ticagrelor (a new antiplatelet agent) on skeletal muscle viability and the levels of asymmetric dimethylarginine (ADMA), malondialdehyde (MDA) and glutathione (GSH) following lower extremity experimental ischemia-reperfusion in a rat model.

MATERIALS AND METHODS

This study was conducted after it was approved by the Local Ethics Committee (Decision Date: 12/03/2014 and Decision No.: 2014/7/65). In this study, 21 male Sprague-Dawley rats (8-10 weeks old, 250±20 g) were used. The rats were randomly divided into 3 groups (n = 7). They were placed in special cages, with ambient temperatures of 24-26°C (constant temperature and ventilated rooms) and a 12:12 h light-dark cycle, under standard conditions before and during the experiment. They were fed a standard rat pellet diet and provided drinking water. Animal treatment and maintenance were carried out in accordance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources (NIH publication No. 85-23, revised 1985).

The rats in Group 3 were treated with 20 mg kg^{-1} Ticagrelor (BRILINTA, AstraZeneca) twice daily by oral gavage for 1 week before experimental ischemia-reperfusion injury.

Preparation of subjects and operation technique: Anesthesia was induced by the intramuscular administration of ketamine hydrochloride (Ketalar, Pfizer, Groton, CT) at a dose of 30 mg kg^{-1} and xylazine hydrochloride (Rompun; Bayer, Leverkusen, Germany) at a dose of 3 mg kg^{-1}. The rats were monitored during anesthesia without the need for respiratory support. The operation was performed in a supine position under a heating lamp to prevent possible hypothermia. The antiseptic solution was used to prepare the skin before the operation. The laparotomy was performed through midline abdominal incision. In the peritoneal cavity, 10 mL of warm saline was instilled to protect the fluid balance. The abdominal aorta was reached by pulling the intestines to the left side with a wet gauze. Before aortic clamping, 150 U kg^{-1} heparin (Nevparin, Mustafa Nevzat Pharmaceuticals) was administered intravenously via the tail vein for 2 min. A non-traumatic microvascular clamp

was inserted into the Infrarenal Abdominal Aorta (IAA) to create a laboratory model of lower limb ischemia in the rats. The absence of blood flow in the distal part of the clamp was confirmed by a hand-held Doppler. The abdominal incision was kept closed to minimize heat and fluid loss. Ischemia was observed for 2 h. The abdomen was opened again following occlusion. After the microvascular clamp in the infrarenal abdominal aorta was removed, reperfusion was conducted for 2 h. During the ischemia-reperfusion process with aortic clamping, the absence of blood flow in the distal aorta during clamping and the presence of blood flow in the distal aorta after the removal of the clamp were confirmed by a hand-held Doppler. After all rats were sacrificed under anesthesia, following the experiment, the necessary blood and tissue samples were taken[15,16].

Experimental model: The rats were randomly divided into 3 groups (n = 7):

- **Group 1 (Sham control group) (n = 7):** Basal oxidant and antioxidant parameters were obtained
- **Group 2 (Ischemia-reperfusion group) (n = 7):** After the abdominal aorta was clamped by the surgical method, ischemia was applied for 2 h. The microvascular clamp in the infrarenal abdominal aorta was removed to provide reperfusion. After lower limb reperfusion was conducted for 2 h, the blood and tissue samples taken from the sacrificed rats were examined biochemically and histopathologically
- **Group 3 (Ticagrelor+ischemia-reperfusion group) (n = 7):** Ticagrelor was administered at 20 mg kg^{-1} twice daily by oral gavage for 1 week before experimental ischemia-reperfusion injury. The microvascular clamp in the infrarenal abdominal aorta was removed to provide reperfusion. After lower limb reperfusion was conducted for 2 h, the blood and tissue samples taken from the sacrificed rats were examined biochemically and histopathologically

Histopathological techniques: The obtained muscle tissue samples were delivered to the Department of Medical Pathology of the Fırat University, Faculty of Medicine for histopathological examination. All prepared slides were evaluated microscopically using the Olympus BX-51 microscope of the same pathologist, who was unaware of the experimental groups. They were photographed with an Olympus DP-71 digital camera.

At least two different sections were examined in each sample. The skeletal muscle viability of all slides containing muscle tissue samples were scored semiquantitatively, according to the criteria for necrosis, loss of muscular striae, muscle fiber separation, edema and inflammation.

Biochemical analysis: The obtained blood samples were taken into a normal biochemical tube, to analyze the ADMA and MDA levels and into an EDTA tube, to analyze the GSH level. The ADMA, MDA and GSH levels were examined in the plasma, serum and whole blood, respectively. The results were expressed as μmol L^{-1}.

The tissue samples removed from a deep freezer on the analysis day were divided into equal amounts (100 mg). Tissue homogenates were prepared and injected into the device. The AMDA, MDA and GSH levels were calculated by measuring the peak area and were expressed as μmol g^{-1}.

The Shimadzu HPLC device was used for ADMA, MDA and GSH measurements.

EUREKA test kits were used for the ADMA assay in plasma. The IMMUCHROM test kits were used for the MDA assay in serum and for the GSH assay in whole blood. Measurements were conducted by the High-Performance Liquid Chromatography (HPLC) method[17-19].

Statistical analysis: Biochemical values were expressed as the mean with Standard Error (SE). Statistical analyzes and graphs were made using SPSS 21.0 and Sigma Plot 8.0 programs, respectively. The bi-directional variance analysis and chi-square test were used for statistical analysis. The Tukey's test was used for *post-hoc* calculation of the bi-directional variance analysis. In all analyses, p<0.05 was considered statistically significant.

In the experimental groups, histopathologic scoring was performed, according to histopathologic features, such as necrosis, loss of muscular striae, muscle fiber separation, edema and inflammation. All data analyses were performed with SPSS 21.0 and Sigma Plot 8.0 package programs. Continuous independent variables with normal distribution were analyzed by the Mann-Whitney U-test. A p-value of <0.05 was considered statistically significant.

RESULTS

ADMA, MDA and GSH values obtained from blood samples: The ADMA value was significantly higher statistically in the I-R group than in the control group (p = 0.001). There was no significant increase in the ticagrelor group, compared to the control group (p = 0.08).

The MDA value was significantly higher statistically in the I-R group than in the control group (p = 0.001). There was no significant increase in the ticagrelor group, compared to the control group (p = 0.09).

The GSH-T value was significantly higher statistically in the I-R group than in the control group (p = 0.001). The GSH-T value was significantly lower statistically in the ticagrelor group than in the I-R group (p = 0.03). The GSH-T value was significantly higher statistically in the ticagrelor group than in the control group (p = 0.007).

The GSH-R value was significantly higher statistically in the I-R group than in the control group (p = 0.001). The GSH-R value was significantly lower statistically in the ticagrelor group than in the I-R group (p = 0.01). The GSH-R value was significantly higher statistically in ticagrelor group than in the control group (p = 0.006).

The ADMA, MDA and GSH levels obtained from blood samples are shown in Table 1.

ADMA, MDA and GSH values obtained from muscle samples: The ADMA value was significantly higher statistically in the I-R group than in the control group (p = 0.001). The ADMA value in the ticagrelor group and the control group were similar (p = 0.99).

The MDA value was significantly higher statistically in the I-R group than in the control group (p = 0.001). The MDA value was higher in the ticagrelor group than in the control group. However, there was no significant increase in the ticagrelor group, compared to the control group (p = 0.2).

The GSH-T value was significantly higher statistically in the I-R group than in the control group (p = 0.001). The GSH-T value was significantly lower statistically in the ticagrelor group than in the I-R group (p = 0.001). However, there was no significant increase in the ticagrelor group, compared to the control group (p = 0.6).

The GSH-R value was significantly higher statistically in the I-R group than in the control group (p = 0.001). The GSH-R value was significantly lower statistically in the ticagrelor group than in the I-R group (p = 0.001). However, there was no significant increase in the ticagrelor group, compared to the control group (p = 0.94).

The ADMA, MDA and GSH levels obtained from muscle samples are shown in Table 2.

Histopathological evaluation: According to the histopathological results, necrosis was not observed in any group.

In the statistical analysis performed to determine the loss of muscular striae, the loss of muscular striae was significantly higher statistically in the I-R group than in the control group (p = 0.001). Similarly, the loss of muscular striae was significantly higher statistically in the ticagrelor group than in the control group (p = 0.007). However, the loss of muscular striae was significantly lower statistically in the ticagrelor group than in the I-R group (p<0.01).

In the statistical analysis performed to determine the muscle fiber separation, the muscle fiber separation was significantly higher statistically in the I-R group than in the control group (p = 0.001). Similarly, the muscle fiber separation was significantly higher statistically in the ticagrelor group than in the control group (p = 0.023). However, the muscle fiber separation was significantly lower statistically in the ticagrelor group than in the I-R group (p = 0.01).

In the statistical analysis performed to determine the edema, the edema was significantly higher statistically in the I-R group than in the control group (p = 0.002). Similarly, the edema was significantly higher statistically in the ticagrelor group than in the control group (p = 0.037). However, the edema was significantly lower statistically in the ticagrelor group than in the I-R group (p = 0.03).

Table 1: ADMA, MDA and GSH values in rat blood samples

Parameters	Control group (n = 7)	I-R group (n = 7)	Ticagrelor grubu (n = 7)
ADMA (μmol L^{-1})	0.29±0.0262	0.64±0.058* (p = 0.001)	0.42±0.039+ (p = 0.007)
MDA (μmol L^{-1})	0.57±0.0436	1.48±0.114* (p = 0.001)	0.82±0.053+ (p = 0.001)
GSH-T (μmol L^{-1})	1003.85±61.288	1468.85±47.515* (p = 0.001)	1263.57±47.818+,* (p = 0.033, p = 0.007)
GSH-R (μmol/ L^{-1})	1183.00±43.127	1757.14±45.862* (p = 0.001)	1377.85±23.193+,* (p = 0.001, p = 0.006)

*p<0.05 compared with control group, +: p<0.05 compared with I-R group

Table 2: ADMA, MDA and GSH values in rat muscle tissue specimens

Parameters	Control group (n = 7)	I-R group (n = 7)	Ticagrelor group (n = 7)
ADMA (μmol g^{-1})	0.08±0.078	0.40±0.044* (p = 0.001)	0.08±0.051+ (p = 0.001)
MDA (μmol g^{-1})	0.33±0.098	0.85±0.112* (p = 0.001)	0.42±0.119+ (p = 0.001)
GSH-T (μmol g^{-1})	530.14±131.117	815.71±47.908* (p = 0.001)	577.14±102.910+ (p = 0.001)
GSH-R (μmol g^{-1})	746.00±166.564	918.57±76.478* (p = 0.02)	765.71±49.280+ (p = 0.04)

*p<0.05 compared with control group, +: p<0.05 compared with I-R group

Table 3: Histopathological examination of rat muscle tissue

Parameters	Control group (n = 7)	I-R group (n = 7)	Ticagrelor group (n = 7)
Necrosis	0.00	0.00	0.00
Loss of muscle fibers	0.00	1.57±0.53* (p = 0.001)	0.71±0.48*,+ (p = 0.007, p = 0.01)
Splitting in muscle fibers	0.00	1.57±0.53* (p = 0.001)	0.57±0.53*,+ (p = 0.023, p = 0.01)
Edema	0.00	1.42±0.53* (p = 0.002)	0.71±0.48*,+ (p = 0.037, p = 0.03)
Inflammation	0.00	0.14±0.37	0.00

*$p<0.05$ compared with control group, +: $p<0.05$ compared with I-R group

Fig. 1: Light microscopic appearance of normal soleus. Ovary skeletal cells and peripheral oval nuclei (HE, X400)

Fig. 3: Ischemia-reperfusion group, streak in gloss and edema (HE, X200)

Fig. 2: Ischemia-reperfusion group; edema, loss of streaking and separation in muscle fibers, (HE, X400)

Fig. 4: Ticagrelor group; decrease in edema and separation in muscle fibers (HEX400)

There was no statistically significant difference between the three groups in terms of inflammation.

According to the histopathological results, the loss of muscular striae, muscle fiber separation and edema were significantly higher statistically in the ticagrelor and I-R groups than in the control group. However, the loss of muscular striae, muscle fiber separation and edema were significantly lower statistically in the ticagrelor group than in the I-R group ($p<0.05$). The histopathological data are shown in Table 3.

The slides prepared from rat muscle tissues were evaluated microscopically using an Olympus BX-51 microscope. The obtained images were photographed with an Olympus DP-71 digital camera (Fig. 1-4).

DISCUSSION

In this experimental study, investigating the effects of ticagrelor on skeletal muscle viability and the levels of ADMA,

MDA and GSH following lower extremity experimental ischemia-reperfusion in a rat model, the vascular occlusion technique was used by clamping the infrarenal abdominal aorta, with the help of a microvascular clamp, in order to create ischemia[20,21]. The authors advocated the tourniquet method, since it is non-invasive and easy to apply[22,23], although the tourniquet method was not preferred in this study, because it can cause venous and lymphatic occlusion as well as muscle and nerve damage due to prolonged mechanical compression and it does not create a clear ischemia condition.

The protective effect of ticagrelor against oxidative stress-related parameters such as MDA, ADMA, GSH and the development of ischemia-reperfusion injury was first investigated in this study.

An experimental study, rats in the drug group, before the day of the experiment for five days 50 mg/day ticlopidin, orogastric tube given twice a day by preconditioning was created. In the study, the right lower extremities of the rats were fixed with a tourniquet at the hip joint and only 4 h of ischemia in 1 group and 4 h of ischemia in the other groups followed by 2 h of reperfusion. As a result of the study, Enkaya *et al.*[24] found that lower extremity damage caused by lower extremity damage after 2 h of ischemia of the lower extremities and the antithrombotic agent, ticlopidine, prevented this. Kiris *et al.*[25] investigated renal injury in the infrarenal aortic ischemia reperfusion model. About 30 min of ischemia and 60 min of reperfusion with ischemia reperfusion injury in the distal organs proximal to the occlusion of MDA levels and antioxidant enzyme activity were determined by the increase.

In cases where oxidative stress increases in the body, ADMA levels increase. The number of studies showing a close relationship between the oxidant/antioxidant system and ADMA has been increasing steadily[26]. Prolonged exposured to ADMA may increase atherogenesis and cause hypertensive damage to target organs. Prolonged NOS inhibition by ADMA may lead to left ventricular hypertrophy.

In a study on experimental animals, the protective effects of trapidil were investigated after abdominal aortic occlusion and reperfusion injury in the lung. As a result of histopathological and biochemical evaluations, MDA, PMN infiltration, interstitial edema and hemorrhagic score were significantly decreased in the group exposed trapidil[27]. It has been discussed that trapidil with its vasodilatator, NO release, inhibition of thrombocyte aggregation effects might have some potential to diminish the tissue injury[27]. In the current study, it was observed that, although ADMA and MDA levels were significantly increased in the ticagrelor and I-R groups, compared with those in the control group, the administration of ticagrelor reduced this increase by a statistically significant degree.

In our study, the GSH level was significantly higher in the I-R group than in the control group. The GSH level was higher in the ticagrelor group than in the control group, although the GSH level was lower in the ticagrelor group than in the I-R group. In the ischemia-reperfusion group, GSH increased compensatory, whereas in the ticagrelor treated group GSH may be reduced due to decreased oxidative stress. This result may confirmed that ticagrelor reduces oxidative stress in ischemia reperfusion injury.

In a study by creating an experimental ischemia-reperfusion model in rats, the effect of clopidogrel was investigated. The right lower extremity circulation was blocked for 6 h at the trochanter major level and then reperfused for 4 h. It has been observed that ischemia-reperfusion caused free radical production with a compensatory decrease in GSH levels and SOD activity and pretreatment with clopidogrel prevented these changes[28]. The MDA was reduced in our study similarly, on the other hand GSH was reduced in our study and this is the difference between two studies. These differences can be caused by differences in drugs, ischemia-reperfusion procedure or differences in sample tissues. In a study conducted in rabbits by applying the ischemia reperfusion model, the suprarenal aorta was clamped for 30 min. Oxidative stress-related parameters such as SOD, MDA and catalase, myocardial damage and edema were decreased in the iloprost treated group[29]. Iloprost, prostacyclin (PgI 2) is a stable analogue with long-term action[30]. It showed cell-protective effects of prostacyclin through several mechanisms such as inhibition of leucocyte activation, reduction in thrombocyte aggregation and vasodilatation[31].

In the histopathological evaluation of the groups, it was shown that there was a significant difference between the control, I-R and ticagrelor groups in terms of loss of muscular striae, muscle fiber separation and edema in the soleus muscle. This damage was found to be lower in the ticagrelor group, compared to the I-R group. The antiaggregant effect of ticagrelor may also reduce oxidative stress and related parameters by suppressing the inflammatory response. Thus, the development of tissue damage may be less.

CONCLUSION

As a result, the full understanding of the mechanism of ischemia-reperfusion injury will help to prevent damage quickly and properly. Our study has revealed that ticagrelor

decreases oxidant levels and skeletal muscle injury by a statistically significant degree in lower extremity ischemia-reperfusion injury. However, there is a need for new and more extensive experimental studies to fully elucidate the mechanisms that mediate this beneficial effect. If the results of large-scale studies to be performed on this subject support our study, it is thought to provide great benefits in daily clinical use.

SIGNIFICANCE STATEMENT

Today, many treatment strategies have been developed to prevent and reduce ischemia-reperfusion injury. Despite improvements in cardiovascular surgical techniques and early postoperative follow-up, ischemia-reperfusion injury, following aortic interventions, is a serious problem affecting postoperative morbidity and mortality. The most appropriate strategy for treatment is still a matter of debate. One of the important points leading to the progression of tissue damage depends on the inflammatory mediators that form due to coagulation. It has observed that ticagrelor reduces oxidant level and injury of skeletal muscle at lower limb ischemia-reperfusion injury. More extensive studies should be performed to scientifically determine ticagrelor's potential to reduce ischemia-reperfusion injury. It is thought that ticagrelor may have protective effects against ischemia-reperfusion injury as a clinical agent and may be included in the treatment strategies.

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