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RIVAROXABAN IMPROVES MYOCARDIAL ISCHEMIA REPERFUSION INJURY COMPLICATIONS IN OBESE RATS

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ABSTRACT: Rivaroxaban is a direct inhibitor for Factor Xa that used orally for prevention and treatment of thromboembolic disorders through inhibition of thrombin synthesis. In this study we hypothesis that rivaroxaban pretreatment might play a role in reducing the complications of myocardial ischemic reperfusion injury (IRI) in a rat model of obesity. R- hirudin (a commercially available anticoagulant drug used previously in attenuating the harmful effects of myocardial IRI) was used as a positive control in this study. To achieve this hypothesis, male Wistar albino rats were randomly assigned into 4 groups (n = 6 per group): control subjected to IRI, obese subjected to IRI, obese rats pretreated with r- hirudin (1.8 mg/kg body weight) then subjected to IRI, obese pretreated with Rivaroxaban (3 mg/kg body weight/day) then subjected to IRI. Obesity was induced by feeding rats high fat diet. Myocardial ischemia was induced by left anterior descending artery ligation (LAD). The obese rats subjected to IRI showed significant increases in the inflammatory markers (myocardial angiotensin II, tumor necrosis factor alpha (TNF- α), interleukin 8 (IL-8) contents and monocyte chemoattractant protein 1 (MCP-1) gene expression) compared with control rats subjected to IRI. A significant increase in serum creatine kinase MB (CK-MB) activity was also observed in obese rats subjected to IRI compared to control rats subjected to IRI. Rivaroxaban pretreatment to obese rats that subjected to IRI showed a significant decrease in the inflammatory markers likely through inhibition of thrombin synthesis; a mediator of myocardial ischemia reperfusion injury. Histological examination and measuring the percentage of infraction of cardiac tissue showed a significant improvement in the rivaroxaban pretreated rats compared to obese rats subjected to IRI.

INTRODUCTION: Myocardial ischemia is an imbalance between the oxygen supply and the oxygen demand in the myocardium.[1] Although cardiac reperfusion is a defense mechanism against myocardial infarction to increase oxygen supply, cardiac reperfusion would result in cardiac injury called myocardial ischemia-reperfusion injury (IRI). [2, 3]

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Myocardial Injury due to IRI results in cardiac contractile dysfunction, arrhythmias, and irreversible myocytes damage.[4] Several studies have demonstrated that obese patients are twice as likely to die of ischemic heart disease compared to normal weight patients [5, 6], as obesity might alter myocardial metabolism leading to compromised cardiac function and tolerance to ischemia.[7]

Since thrombin plays a role in myocardial damage and impaired hemodynamic recovery during IRI; considerable attentions had been directed to study the effect of new therapeutic interventions that reduce thrombin synthesis. Inhibiting factor Xa, which is an essential enzyme in thrombin synthesis

pathway, may be a useful tool in the management of myocardial IRI.[8]

Rivaroxaban is a direct factor Xa inhibitor used orally for prevention and treatment of thromboembolic disorders [9], it had potent antithrombotic effects by inhibiting thrombin generation through the inhibition of factor Xa generated via either the intrinsic or the extrinsic coagulation pathways.[10] However, little is known about its effect in decreasing the complications that might be associated with the reperfusion of ischemic heart. Therefore, the present study was designed to show the effect of Rivaroxaban pretreatment, a new factor Xa inhibitor in a rat model of obesity and myocardial IRI. R- hirudin, anticoagulant drug known to inhibit thrombin was used in this study as a positive control.

MATERIALS AND METHODS:

Experimental Animals:

Male wistar albino rats (n=80), weighing 150±10g, were purchased from The Egyptian Organization for Biological Products and Vaccines, Cairo, Egypt. The rats were housed in stainless steel wire-bottomed cages. The rats were exposed to 12 hours light/dark cycle in the animal care facility at Faculty of Pharmacy, Zagazig University, Egypt. The room temperature was maintained automatically at 25 $^{\circ}$C ± 2°C and humidity at 65% to 69%. The rats were fed rodent chow and allowed free access to drinking water. All the experimental procedures were conducted in accordance with the guidelines of the National Institutes of Health for animal research. The experimental protocol was approved by the Institutional Laboratory Animal Care and Use Committee of Faculty of Pharmacy, Zagazig University, Egypt. Every effort was made to reduce the number of animals and their suffering.

Induction of obesity:

In order to induce obesity, 70 rats were randomly chosen to switch their diet to high fat diet for the next four months.[11] The rats which showed ≥ 30% increase in their body weight were considered obese and used for this study.

Development of myocardial IRI in the rats: Rats (control and obese) were anesthetized with pentobarbital (35mg/kg body weight, I.P) (NembutalR, Dainippon-Sumitomo Pharmaceutical Co., Ltd., Osaka, Japan).[12] First an incision was made at midline skin. This followed by left thoracotomy to reach the heart in 4th intercostal space. 6-0 10 mm prolen suture with a traumatic needle was placed around the left anterior descending artery (LAD). The threads of the suture were tightened to obtain a complete occlusion to develop ischemia for 30 minutes. Then the snare was loosened to obtain reperfusion. The rats were connected to a ventilator for one hour with reperfusion.[13]After one hour reperfusion; rats were sacrificed by spinal dislocation for serum and tissue collection.

The experimental models:

The obese and control animals were subjected to IRI. The obese animals were treated with r-Hirudin or Rivaroxaban. That results in four experimental models as explained below (n=6):

Control IRI rats: In which control rats were subjected to IRI.

Obese IRI rats: In which obese rats were subjected to IRI.

Obese IRI treated with r- hirudin rats: Obese rats were treated with r- hirudin (1.8 mg/kg body weight) (Thrombex$_x$®, Rhein-Minapharm**,** Germany). Before induction of ischemia, the rats were injected with a bolus dose of r- hirudin (12% of total dose) for ten minutes before induction of IRI ischemia. Then an infusion dose, the rest of total dose was given after induction of IRI.[14]

Obese IRI treated with rivaroxaban rats:
Obese rats pretreated with rivaroxaban (Xarelto ®, Bayer HealthCare, Germany) (3mg/kg body weight/day orally) for eight days.[15] Then the rats were subjected to IRI.

Serum collection:

The rats were sacrificed by spinal dislocation and the blood samples were collected. The blood samples were then centrifuged at 4500rpm for 20 minutes for serum separation. The serum was freshly used for the determination of CK-MB activity using commercially available kits (Spectrum diagnostics, Germany).

Histopathological analysis:
The rats were sacrificed and the hearts were collected for histopathological analysis. The hearts were kept in 4% paraformaldhyde. The hearts were then dehydrated with a series of ascending grade ethanol from 75 to 100%. The hearts were then placed in xylol and embedded in paraffin. Cross sections of about 4µm thickness were prepared and placed on slides. The slides were stained with hematoxylin and eosin stain to study the histological structure of heart.[16]

Determination of cardiac infarct size:
Hearts were cut into slices 2mm thickness and stained with Triphenyltetrazolium chloride (TTC) solution (Oxford laboratories, India) and incubated at 37°C for 20 min. The slices were then fixed in 4% neutral buffered paraformaldhyde for 20min. Infracted areas appeared as a pale discoloration. The infracted area was measured using Image J Software. The total myocardial area (TMA) was measured followed by measurement of the infracted area (IA). The infarction percentage was determined by dividing the infracted area (IA/heart) over the total myocardial area (TMA/heart).[17]

Determination of cardiac Angiotensin II, TNF-α and IL-8 contents: The rats were sacrificed and the hearts were excised instantly, rinsed with cold normal saline and quickly frozen in liquid nitrogen for 5 minutes and then stored at -20ºC. Angiotensin II, TNF-α and IL-8 contents were measured with a commercial ELISA kits (Cusabio Biotech Co., Ltd, China) following the instructions of the manufacturer protocols.

Quantitative RT-PCR:
RT –PCR was used to detect mRNA expression of MCP-1. **RNA extraction:** Total RNA was extracted from heart tissue using Total RNA Isolation system (Promega, Madison, WI, USA). The extracted RNA was quantified using a spectrophotometer at 260 nm.

Reverse transcriptase-PCR procedure: The extracted RNA was converted into cDNA and amplified by PCR using real-time polymerase chain reaction (RT-PCR) kit (Stratagene, USA). 3µl of random primers was added to the 10 µl of RNA, which was denatured for 5min at 95°C in a thermal cycler (Robocycler, USA). The RNA primer mixture was cooled to 4°C. Then cDNA master mix was prepared according to the kit instructions and was added to each sample. The mixture was incubated in a programmed thermal cycler for 1h at 37°C followed by inactivation of the enzyme at 95°C for 10min and cooled at 4°C. The sequence of the primers used is listed in **Table 1**. GAPDH was used as a house-keeping gene

Quantitative real time PCR:
After normalization of gene-specific forward and reverse primer pairs, a real time- PCR reaction mixture was prepared (25µl SYBR Green Mix (2x) - 0.5µl cDNA - 2µl primer pair mix (5pmol/µl of reverse and forward primer) - 22.5µl RNA free H_2O). The reaction mixture was then subjected to PCR amplification as follows: (50°C for 2min, 1 cycle; 95°C for10 min,1 cycle; 95°C for 15 sec, 60°C for 30 sec, 72°C for 30 sec , 40 cycles; 72°C for10min, 1cycle) mRNA expression was calculated using delt- delt CT method.[18]

TABLE 1: INDICATES THE PRIMER SEQUENCES, ANNEALING TEMPERATURE AND PRODUCT SIZE FOR THE STUDIED GENES (MCP-1AND GAPDH).

Gene	Primer sequence	Annealing Temp.	Product size
MCP-1	Forward primer: 5′-AGC AGC AAG TGT CCC AAA G-3′ Reverse primer: 5′-TTG GGT TTG CTT GTC CAG G-3	58°C	120bp
GAPDH	Forward primer: 5′- GTCGGTGTGAACGGATTTG-3′ Reverse primer: 5′- AAGATGGTGATGGGCTTCC-3′	61°C	215bp

Statistical Analysis: All data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using Graph pad prism software version 5 (Graph Pad Software, Inc. La Jolla, CA).

The inter groups variation was measured by one-way analysis of variance (ANOVA) followed by Tukey post hoc. The minimal level of significance was identified at $p < 0.05$.

RESULTS:

High fat diet induced obesity in rats: To induce obesity, wistar albino rats were subjected to high fat diet (25% total fat including 11% unsaturated fat, 44% carbohydrate, 18% protein, and 13% fiber, ash and other ingredients). The high fat diet induces obesity in the rats indicated by a significant increase in the body weight of rats that fed by high fat diet (obese) compared with control rats that fed normal chow (control) (**Table 2).**

TABLE 2: HIGH FAT DIET INDUCED OBESITY IN RATS.

Group	Control	Obese
Body weight	191 ± 21.78	358.1 ± 33.7 #

Data are represented as means ± SD. # Significant at $p< 0.05$ (control n = 10, obese n = 40)

Determination of CK-MB activity in the serum:
The control and obese rats were subjected to IRI. The rats were then sacrificed and serum was extracted. A (Spectrum diagnostics, Germany) kit was used to determine the CK-MB activity in serum**.** As expected obese rats subjected to IRI showed a significant increase in serum CK –MB activity compared to the control rats subjected to IRI. Pretreatment with rivaroxaban significantly reduced the elevated serum CK- MB activity while r- hirudin showed a non significant reduction in serum CK-MB activity compared with obese rats subjected to IRI. That indicated the effect of rivaroxaban pretreatment on decreasing the severity of IRI which was manifested by reducing CK-MB activity.

TABLE 3: EFFECT OF RIVAROXABAN AND R- HIRUDIN PRETREATMENT ON SERUM CK-MB ACTIVITY IN RATS SUBJECTED TO IRI

Groups	Control IRI	Obese IRI	r-hirudin pretreated obese IRI	Rivaroxaban pretreated obese IRI
CK-MB activity (U/L)	1118 ± 251.3	1456 ± 230.2#	1422 ± 229.6	566.6 ± 122.9 *

Data are represented as means ± SD, n= 6 per group, # Significant at $p< 0.05$ versus control IRI, * Significant at $p< 0.05$ versus obese IRI.

Determination of cardiac Angiotensin II, TNF-α, IL-8 contents:
A commercially available Eliza kits were used to determine the Angiotensin II, TNF- α, IL-8 contents in heart tissue. As expected the induction of IRI in obese rats developed a significant increase in cardiac Angiotensin II, TNF- α, IL-8 contents. Pretreatment either with rivaroxaban or r-hirudin significantly reduced Angiotensin II, TNF- α, IL-8 levels in obese rats compared with obese IRI (**Fig.1**).

Determination of mRNA gene expression of MCP-1:
RT-PCR was used to determine the MCP-1 gene expression. As shown in **Fig. 2** obese IRI showed a significant increase in myocardial MCP-1 gene expression compared with control IRI. Pre-treatment with rivaroxaban significantly reduced MCP-1 gene expression in obese rats with IRI compared with obese IRI.

Pre-treatment with r-hirudin also showed a significant reduction in MCP-1 gene expression compared with obese IRI.

Effects on myocardial infarct size:
As shown in **Fig. 3**, pre-treatment either with rivaroxaban or r- hirudin caused significantly reduce the infarct size ($p<0.05$) by 33.25 % and 45.22 % respectively, when compared with obese rats subjected to IRI at 65.65 %. However there was a non significant difference between rivaroxaban and r- hirudin pretreated rats.

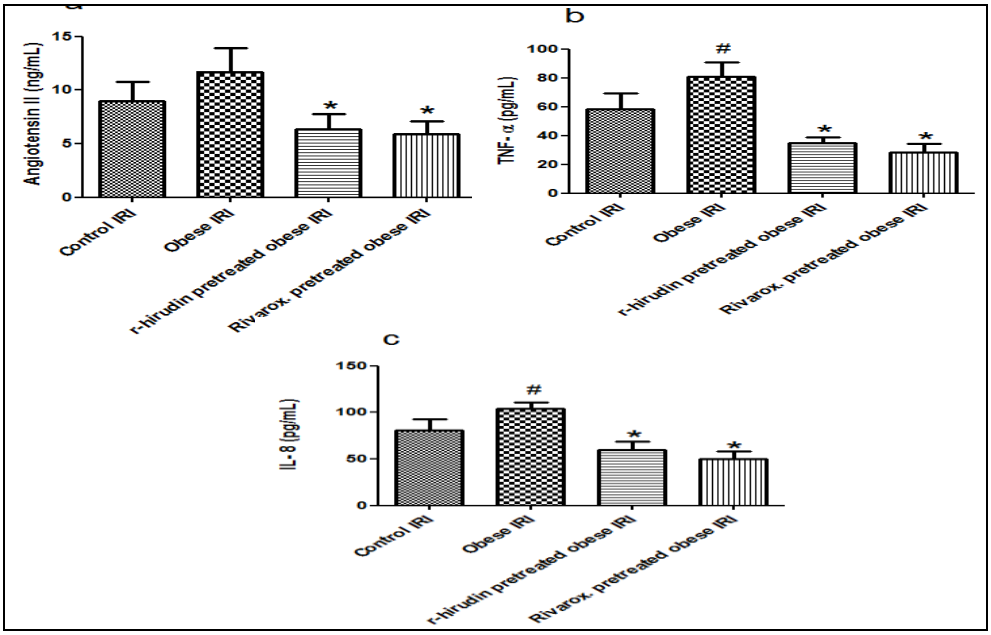

FIG.1: EFFECT OF RIVAROXABAN AND r- HIRUDIN PRETREATMENT ON CARDIAC ANGIOTENSIN II, TNF- α AND IL-8 CONTENTS IN RATS SUBJECTED TO IRI.

Data are represented as means ± SD, n= 6 per group. # Significant at p< 0.05 versus control IRI, * P< 0.05 versus obese IRI.
a: Angiotensin II, b; TNF-α, c: IL-8

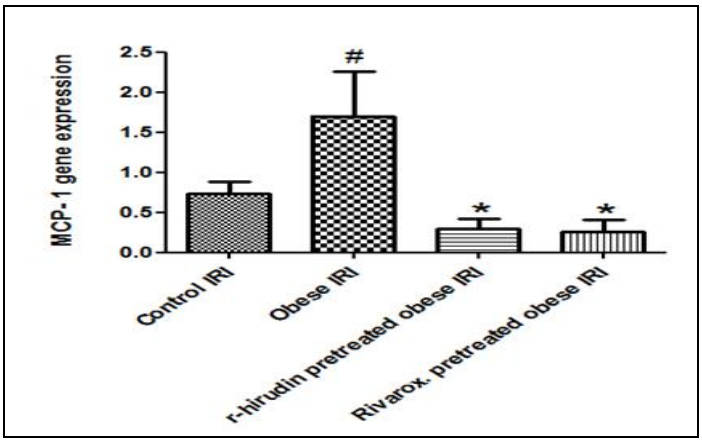

FIG.2: EFFECT OF RIVAROXABAN AND r- HIRUDIN PRETREATMENT ON CARDIAC MCP-1 GENE EXPRESSION IN RATS SUBJECTED TO IRI.

Data are represented as means ± SD, n= 6 per group. # Significant at p< 0.05 versus control IRI, * P< 0.05 versus obese IRI.

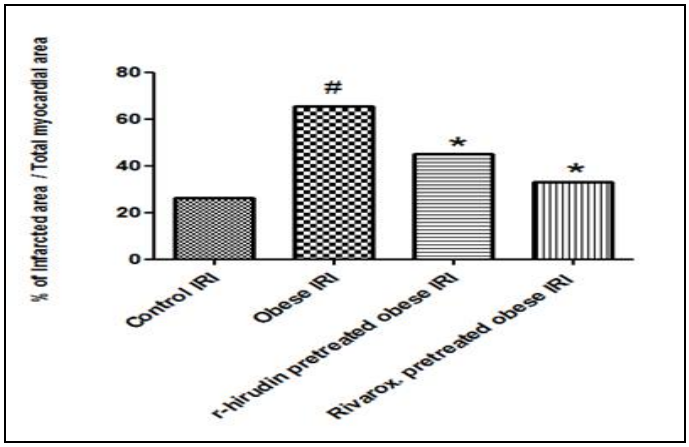

FIG.3: EFFECT OF RIVAROXABAN AND r- HIRUDIN PRETREATMENT ON PERCENTAGE OF MYOCARDIAL INFARCT SIZE IN RATS SUBJECTED TO IRI.

Data are represented as means ± SD, n= 6 per group. # Significant at p< 0.05 versus control IRI, * P< 0.05 versus obese IRI.

Histopathological examination:

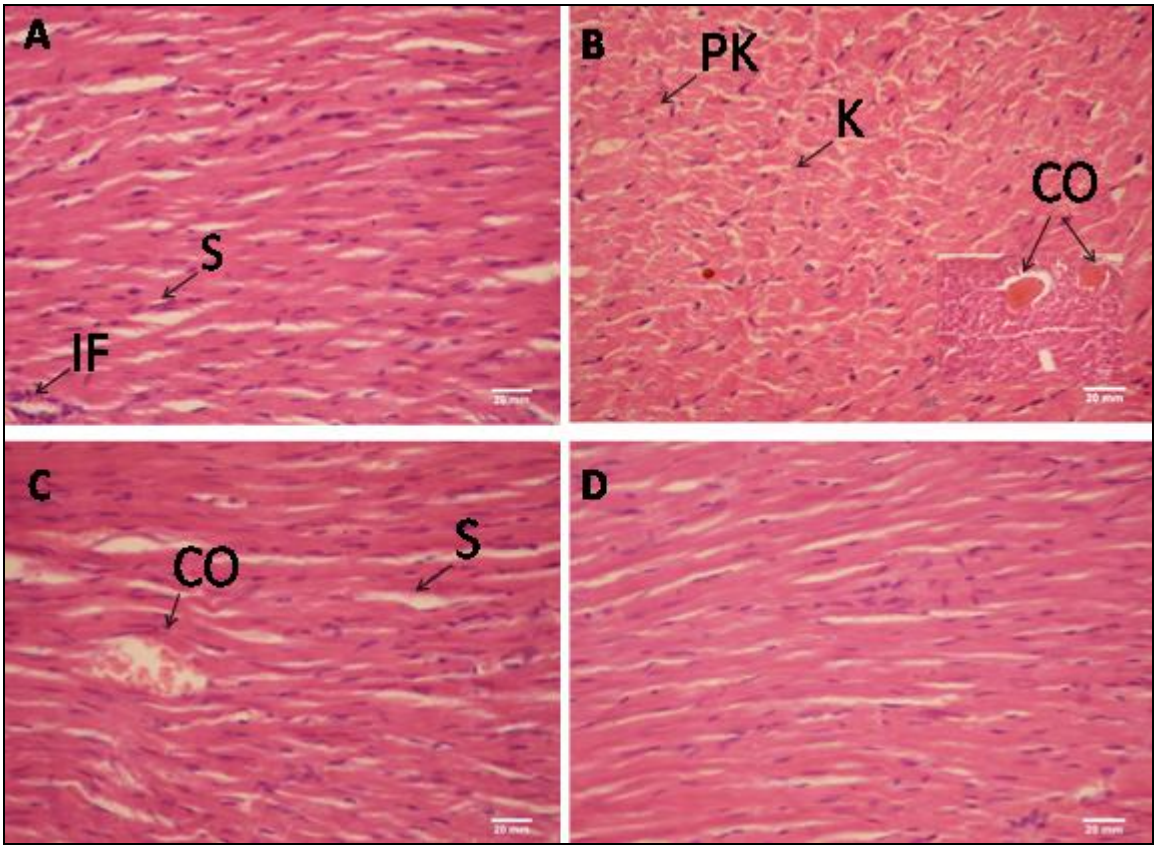

FIG.4: HAEMATOXYLIN AND EOSIN STAINED SECTIONS OF THE EXPERIMENTAL RATS. OBESE IRI RATS SHOWED A MARKED KERATOLYSIS, NEUTROPHIL INFILTRATION AND CONGESTION, IN ADDITION TO THE LOSS OF THE NORMAL CARDIAC STRIATIONS OF THE MYOCYTES. AN INCREASE IN THE NUMBER OF THE APOPTOTIC CELLS, WITH SMALL PKYNOTIC NUCLEI WAS NOTICED. PRE-TREATMENT WITH r-HIRUDIN PRESERVED THE NORMAL CARDIAC STRIATIONS WITH A REDUCTION IN THE NUMBER OF PKYNOTIC CELLS BUT SOME SEPARATION AND CONGESTION IN THE CARDIAC MUSCLE FIBERS WERE OBSERVED. PRE-TREATMENT WITH RIVAROXABAN SHOWED A SIGNIFICANT REDUCTION IN THE NUMBER OF PKYNOTIC CELLS, CONGESTION, NEUTROPHILS INFILTRATION AND CARDIAC MUSCLE SEPARATION COMPARED WITH OBESE IRI RATS.
A: control IRI, **B**: obese IRI, **C**: r-hirudin pretreated obese IRI, **D**: rivaroxaban pretreated obese IRI, **S**: separation, **IF**: neutrophils infiltration, **K**: keratolysis, **CO**: congestion, **PK**: pkynotic nuclei. Scale bar 20 mm

DISCUSSION: Reperfusion of the ischemic myocardium is accompanied by an inflammatory response that results from the actions of certain cytokines (e.g. TNF- α, IL-8) and chemokines (e.g.MCP-1) in addition to the action of Angiotensin II and the increased expression of adhesion molecules on cardiomyocytes.[19]

Previous study stated that cytokines enhance thrombin formation[20], thrombin in turn stimulates PAR-1 receptors which are found on endothelial cells, fibroblasts, T-lymphocytes and smooth muscle cells to express MCP-1 and adhesion molecules, such as intercellular adhesion molecule–1 (ICAM–1) and P-selectin to the site of injury in addition to the stimulation of IL-6 and IL-8 production from monocytes, which constitute more and more inflammatory response.[21, 22] During myocardial IRI, increase thrombin formation significantly contributes to the adverse functional consequences of ischemic myocardium.[8, 23] Previous studies stated that obesity increased myocardial susceptibility to develop IRI than normal heart [5, 6, 7, 24, 25] which manifested here by increased serum CK-MB activity, myocardial Angiotensin II, TNF- α and IL-8 contents, in addition to increase myocardial MCP-1gene expression and the percentage of infarct size in obese IRI rats compared with control IRI rats.

In the present study, cardiac IRI caused an elevation in tissue Angiotensin II by stimulating cardiac mast cells which are an important source of the aspartyl protease rennin to release rennin that

initiates the activation of a local renin-angiotensin system.[26, 27] Additionally acute myocardial ischemia reperfusion results in a prolonged autocrine upregulation of TNF- α formation and release from the heart [28], which in turn increased the upregulation of MCP-1.[29] Myocardial IL-8 level was also markedly increased in the reperfused myocardium.[30] All that resulted in increasing thrombin formation that developed myocardial necrosis and tissue damage. This can be indicated here by increased CK-MB activity and infract size. These findings were in line with previous studies which stated the release of cytokines as TNF-α during myocardial infarction, mediated progressive impairment in cardiac structure and increased the permeability of the injured myocytes membrane. That was accompanied with an elevation in CK-MB activity.[30, 31, 32]

Many studies suggested a significant role of thrombin in myocardial IRI through a proinflammatory mechanism independent of coagulation and thrombus formation.[8, 33] Thrombin had significant pro-inflammatory effects on endothelial cells which result in the expression of several adhesion molecules and inflammatory cytokines and chemokines. Thrombin also stimulates the generation of reactive oxygen species by smooth muscle cells.[34] Additionally many of the thrombin pro inflammatory effects had implications on the promotion of atherosclerosis.[35] Inhibition of thrombin or decreasing its synthesis resulted in a useful strategy to ameliorate the consequences of IRI.[8, 23] That was proven in this study as pretreatment with rivaroxaban, factor Xa inhibitor, showed a significant improvement in myocardial IRI. That was manifested by a significant decrease in the myocardial Angiotensin II, TNF α, IL-8 levels as well as mRNA expression of MCP-1 and percentage of infarct size.

Additionally a significant decrease in the serum CK-MB activity was observed in obese IRI rats pretreated with rivaroxaban compared to obese IRI. The histological examination confirmed our findings, as shown in (**Fig.5**). Obese rats subjected to IRI showed a marked increase in keratolysis of the cardiac muscles cells that might be a result of ischaemia in addition to neutrophils infiltration and congestion. The normal cardiac striations were lost accompanied by reduction in the pumping capacity of the heart. An increase in the number of the apoptotic cells, with small pkynotic nuclei was also noticed. Pre-treatment with r- hirudin drug, preserved to some extent the normal cardiac striations with a reduction in the number of pkynotic cells. However, some separation in the cardiac muscle fibers and congestion were observed in r- hirudin pretreated. The pre-treatment with rivaroxaban showed a great reduction in the number of pkynotic cells, congestion and neutrophils infiltration, in addition to restoring the normal cardiac muscle striations.

CONCLUSION: Our results indicated a beneficial role for rivaroxaban as a protective agent against consequences of myocardial IRI through inhibiting thrombin synthesis and reducing its pro-inflammatory effects.

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