The effect of Simvastatin in attenuation of Myocardial Ischemia/ Reperfusion injury

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Abstract:

Background: Myocardial ischemia–reperfusion represents a clinically relevant problem associated with thrombolysis, angioplasty and coronary bypass surgery. Injury of myocardium due to ischemia–reperfusion includes cardiac contractile dysfunction, arrhythmias as well as irreversible myocytes damage.

Objective: This study was undertaken to investigate the potential role of simvastatin in amelioration of myocardial I/R injury induced by ligation of coronary artery in a rat model.

Materials & methods: adult male Swiss Albino rats were randomized into 4 equal groups. Group (I) sham group: rats underwent the same anesthetic and surgical procedure as the control group except ligation of LAD coronary artery , Group(II) control group: rats subjected to regional ischemia for 25 min by ligation of LAD coronary artery and reperfusion for 2 hours ,Group(III) control vehicle group: rats received vehicle of simvastatin (normal saline) via I.P injection and subjected to regional ischemia for 25 min by ligation of LAD coronary artery and reperfusion for 2 hours , Group(IV) simvastatin treated group :rats pretreated with simvastatin 1mg/kg i.p 1 hr before ligation of LAD coronary artery. At the end of experiment (2 hr of reperfusion), the heart was harvested and the part of the heart just below the site of ligation was divided into two parts ,the upper part was homogenized for the measurement of interleukin-1β (IL-1β) and the lower part of tissues fixed in 10% formalin and embedded in paraffin the sections were stained with hematoxylin and eosin (H&E) then used for histopathological study .

Results: Compared with the sham group, levels of myocardial IL-1β, were increased (p<0.001) in the control group. Simvastatin significantly counteract the increase in myocardium level of IL-1B ($P < 0.001$). Histological analysis revealed that simvastatin markedly reduced ($P < 0.001$) the severity of cardiac injury in the rats underwent LAD ligation procedure .

Conclusions:

The results of the present study reveal that simvastatin may ameliorate myocardial I/R injury in rats via interfering with inflammatory responses which induced by I/R injury.

Key words: Ischemia , Reperfusion injury , simvastatin

تأثير السمفاستاتين في تضعيف الإصابة القلبية جراء نقص ألارواء وإعادته لعضلة القلب

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الخلاصة:

يمثل نقص أرواء عضلة القلب واعادة الارواء مشكلة ذات صلة سريريه مرتبطة بالجلطات و القسطرة و جراحة تغيير الشرايين التاجيه. تشمل إصابة عضلة القلب بسبب نقص التروية الدمويه وإعادتها ـ ضعف مقلص القلب، عدم انتظام ضربات القلب وكذلك تلف الخلايا العضلية التي لا رجعة فيه. أجريت هذه الدراسة لمعرفة الدور الوقائي المحتمل للسيمفاستاتين في تحسين حال الإصابة بالنقص القلبي بالإرواء الدموي/ إعادة الإرواء عضلة القلب الناجمة عن ربط الشريان التاجي النموذج الجرذان.

استخدم في هذه الدراسة اربع وعشرون ذكرا بالغا من الجرذان البيض السويسرية. وتم توزيعهم بشكل عشوائي الى اربع مجاميع تشمل مجموعة التظاهر ومجموعة السيطرة (الغير معالجه) ومجموعة المذيب و المجموعة المعالجة بالسمفاستاتين

واظهرت النتائج ان المستوى الى IL-1B قد ازدادت معنويا(p<0.001) في مجموعة السيطرة. أما النتائج النسيجية بعد عملية ربط الشريان التاجي الأمامي النازل الأيسرفقد أظهرت أن جميع قلوب الجرذان قد تضررت معنويا(p<0.001) من خلال نتائج هذه الدراسه يظهر بأن السمفاستاتين يحسن من ضرر القلب الناتج لدى الجرذان التي تعرضت الى عملية ربط الشريان التاجي الأمامي النازل الأيسر المسببة للإصابة بالنقص القلبي بالإرواء الدموي/ اعادة الارواء.

Introduction:

Ischemia/reperfusion injury describes the experimentally and clinically prevalent finding that tissue ischemia with inadequate oxygen followed by successful reperfusion initiates a wide and complex array of inflammatory responses that may aggravate local injury as well as induce impairment of remote organ function[1]. Ischemia-reperfusion injury results from several interdependent mechanisms, namely oxidative stress, intracellular calcium overload and hyper contracture, endothelial cell activation with micro vascular dysfunction and altered myocardial metabolism [2]. Ischemia–reperfusion injury prompts a release of oxygen free radicals, cytokines and other pro inflammatory mediators that activate both the neutrophils and the coronary vascular endothelium. Activation of these cell types promotes the expression of adhesion molecules on both the neutrophils and endothelium, which recruits neutrophils to the surface of the endothelium and initiates a specific cascade of cell–cell interactions, leading first to adherence of neutrophils to the vascular endothelium, followed later by trans endothelial migration and direct interaction with myocytes. This specific series of events is a prerequisite to the phenotypic expression of reperfusion injury, including endothelial dysfunction, micro vascular collapse and blood flow defects, myocardial infarction and apoptosis [3]. I–R injury may occur in a variety of clinical settings, including reperfusion after thrombolytic therapy, coronary angioplasty, organ transplantation, aortic cross-clamping or cardiopulmonary bypass [4]. Adaptive cellular responses activate the innate immune system with its Tall-like receptors and the complement system as well as the adaptive immune system. This results in a profound inflammatory tissue reaction with immune cells infiltrating the tissue. The damage is mediated by various cytokines, chemokines, adhesion molecules, and compounds of the extracellular matrix. The expression of these factors is regulated by specific transcription factor with NF-kB being one of the key modulators of inflammation [5]. Simvastatin , is member

of statins, that competitively inhibit the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the first committed step in cholesterol biosynthesis simvastatin have peliotropic (lipid lowering –independent) encompass anti inflammation, correction of endothelial dysfunction, increase in nitric oxide bioavailability, anti-oxidation, and stabilization of atherosclerotic plaques(6)

Naidu *et al.* (**2003**) demonstrated that after treatment with simvastatin, the expression of NADPH oxidase is inhibited, which would result in a decrease in mitochondrial the transcription factors nuclear factor NF-κB and activator protein (AP)-1 (7). In addition, there are Several mechanisms have been proposed for statin-elicited beneficial effects, including the prevention of mevalonate formation and subsequently the synthesis of isoprenoid farnesyl pyrophosphate and geranylgeranyl pyrophosphate (GGPP), which leads to inhibition of the isoprenylation of small guanosine triphosphate-binding proteins, such as Rho or Ras proteins involved in cell differentiation, apoptosis, and inflammatory response (8).

Materials and Methods:
2.1. Animals
A forty-six adult males Swiss Albino rat weighing 180-220 g were purchased from Animal Resource Center, the National Center for Drug Control and Researches. The animals were apparently healthy and they were housed in the animal house of College of Medicine/University of Kufa in a temperature-controlled (24 ± 2 °C) room with ambient humidity and alternating 12-h light/12-h dark cycles and were allowed free access to water and standard chow diet until the start of experiments. The rat were left for two weeks without interferance for acclimatization. They had no manifestation of any illness upon examination.
2.2.In vivo myocardial I/R model

The *in vivo* myocardial I/R model was modified from a previous study (9). Briefly, rats were anesthetized with with 100mg/kg ketamine and 5mg/kg xylazine (10). The rats were intubated and Mechanical ventilation is then achieved by connecting the endotracheal tube to Scientific ventilator (Harvard Model) at a respiratory rate of 138 breath/minute with a tidal volume of 20 mL/kg body weight(11). A left thoracotomy was carried out to expose the heart. The LAD is then transiently ligated (or can be tied with a slipknot) using a 6-0 polyprolene suture for a 25-minute ischemic period (12). After a 25-min ischemia, by Microsurgical scissors are used to cut the knot in the ligature (or by releasing the slipknot) the heart was reperfused for 2 h. Immediately after finishing the reperfusion time the rat was sacrificed ,starting by injection of high dose from ketamine and xylazine , after giving good time for the animal to go into deep anesthesia , the rat is positioned and the chest is opened in flap like manner revealing the heart then a needle of the syringe is introduced into right ventricle to aspirate around 2 ml of blood for later plasma analysis. After that hearts were rapidly removed for quantification of myocardial injury by biochemical studies
2.3.Experimental groups and protocols
After the two week of acclimatization the rats were randomized into 4 groups, 6 rates in each group as follow
I.   **Sham group**: rats underwent anesthetic and surgical procedures same as control but without ligation of (LAD) coronary artery.
II.   **control group**(**Untreated group**): rats underwent Myocardial ischemia for 25 minutes by ligation of (LAD) coronary artery .& reperfusion fore 2 hr.
III.   **control vehicle group** : rats pretreated with normal saline(vehicle for simvastatin) via IP rout & underwent Myocardial ischemia for 25 minutes by ligation of LAD coronary artery. & reperfusion fore 2 hr.

IV.   **Simvastatin treated group** : rats pretreated with simvastatin 1mg/kg (13) at 1 hr before ligation of LAD coronary artery via intraperitoneal injection (14). Dissolved simvastatin in normal saline (14) & given in a dose (1mg\kg) via IP route at 1 hr before occlusion of LAD ,simvastatin prepared immediately before injection.

2.4.Tissue Preparation for measurement of (IL-1B)

Cardiac tissues collected 120 minutes after reperfusion were homogenized in a solution containing 1:10(w/v) phosphate buffered saline that contain 1% triton X-100 and protease inhibitor cocktail   (15). by using high intensity liquid processor , After homogenization, samples were centrifuged at 14,000 rpm for 20 min at 4°C (16)   the supernatant was collected and used in IL-1B measurement using commercially available ELISA kits (signosis) according to the manufacturer's instructions.

2.5.Histopathological Analysis and Damage Score

Tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were stained with hematoxylin and eosin for histological evaluation of tissue damage. In order to have a quantitative estimation of cardiac damage, sections (n=6 for each animal) were scored by 2 independent observers blinded to the experimental protocol. The following morphological criteria were considered: score 0, no damage; score 1 (mild), interstitial edema and focal necrosis; score 2 (moderate), diffuse myocardial cell swelling and necrosis; score 3 (severe), necrosis with the presence of contraction bands and neutrophil infiltrate; and score 4 (highly severe), widespread necrosis with the presence of contraction bands, neutrophil infiltrate, and hemorrhage.

2.6. Statistical Analysis

Statistical analyses were performed using SPSS 20.0 for windows.lnc. Data were expressed as mean ± SEM. Analysis of Variance (ANOVA) was used for the multiple comparisons among all groups followed by post-hoc tests using LSD method.

Results:
3.1.simvastatin reduced myocardial IL-1B following MI/R injury

Myocardium levels of inflammatory cytokines following MI/R were analyzed by ELISA. **Figure(1)**shows that MI/R injury increased significantly (p<0.001) the levels of Myocardium IL-1B compared with the Sham group ($P < 0.001$). In the simvastatin treatment group, Myocardium levels of IL-1B, were reduced significantly compared with the Control group ($P < 0.001$).
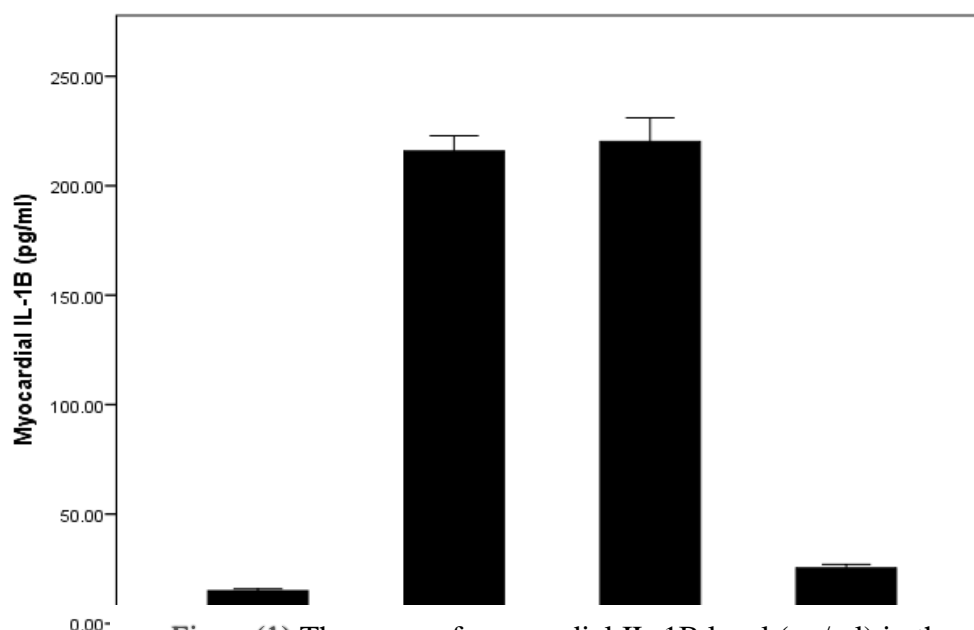
Figure(1) The mean of myocardial IL-1B level (pg/ml) in the six experimental groups   at the end of the experiment

3.2. Histopathological findings

Treatment of rats with simvastatin improved cardiac injury significantly ($P < 0.001$) as compared with control vehicle group and the total severity scores mean of this group showed 16.7% of the group had no damage, 66.7% had mild cardiac injury and 16.7% had moderate cardiac injury. A cross section of sham rat's heart showed a normal cardiac structure. All rats in this group showed normal hearts 100% as shown in table(1). There was statistically insignificant difference between control vehicle (1) group (III) and control group (II) ($P>0.001$) and the total severity scores of the control group showed 16.7% of the group had moderate cardiac.

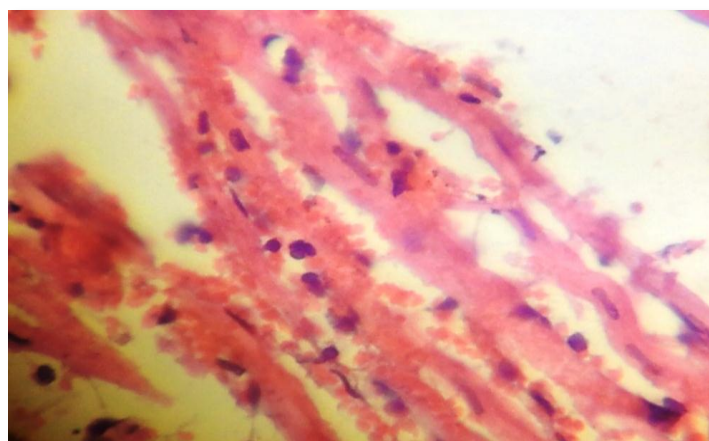|	Sham group		Control group		Control Vehicle group		Simvastatin treated group	
Normal	6	100%	0	0	0	0	1	16.7%
Mild	0	0	0	0	0	0	4	66.7%
Moderate	0	0	0	0	1	16.7%	1	16.7%
Sever	0	0	4	66.7%	4	66.7%	0	0
Very Sever	0	0	2	33.3%	1	16.7%	0	0
Total cardiac injury	6	100%	6	100%	6	100%	6	100%

Table (1): The differences in histopathological scoring of abnormal heart changes among the four experimental groups
injury, 66.7% had severe cardiac injury and 16.7% had highly severe.
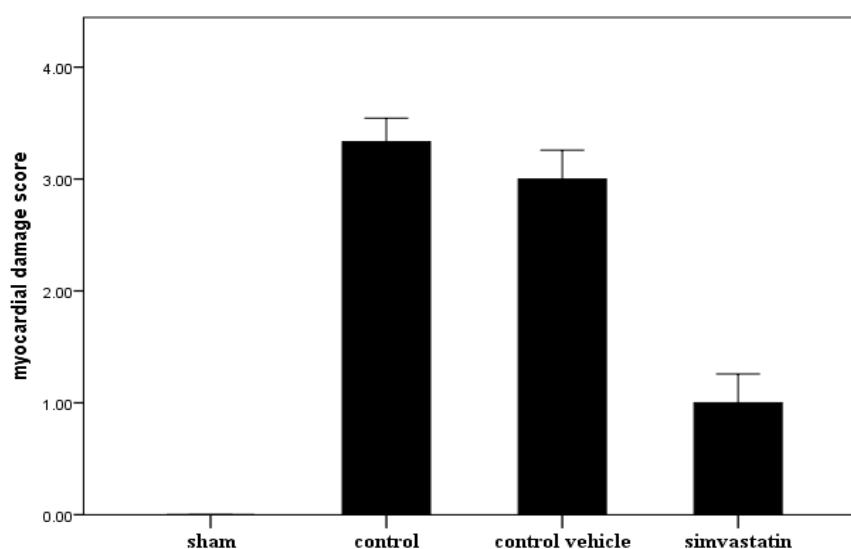

Figure (2): Error bar chart shows the difference in mean± SEM values of total severity scores in the four experimental groups.
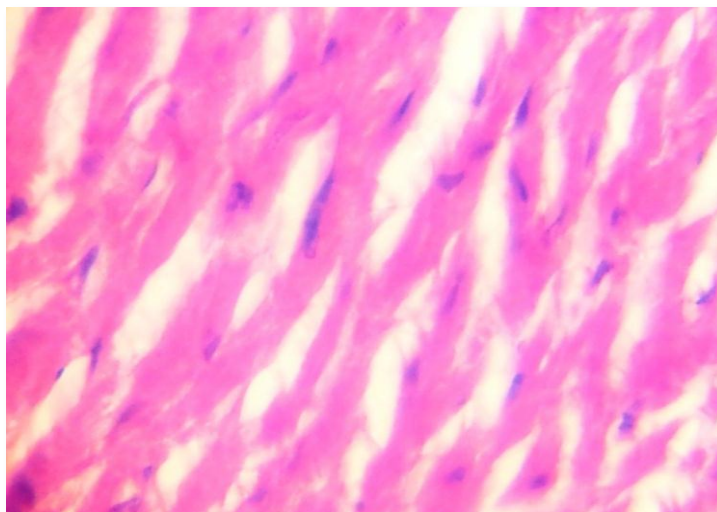
Figure (3): Photomicrograph of heart section of normal rats shows the normal architecture. The section stained with Haematoxylin and Eosin (X 10).

Figure (4): Photomicrograph of cardiac section for the control vehicle group showed interstitial edema ,haemorrhage and PMN infilteration. The section stained with Haematoxylin and Eosin (X 40).
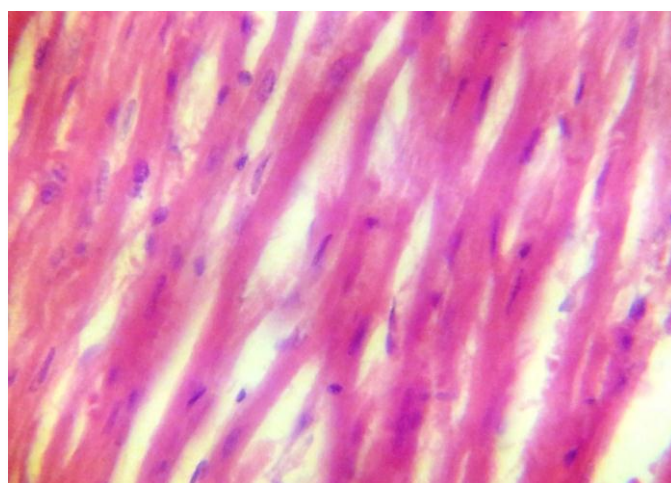
Figure (5): Photomicrograph of cardiac section in simvastatin treated group. The section show almost normal cardiac tissue, the section stained with Haematoxylin and Eosin (X 40)

Discussion:

The major findings of the present study are as follows. Firstly, that the inflammatory cytokine (IL-1B) play important role in the pathology of myocardial I/R Secondly, simvastatin pretreatment played a protective role against myocardial I/R injury., the protective effects of Simvastatin during myocardial I/R injury were correlated with the attenuation of inflammation and Myocardial damage.

Coronary arterial occlusion after atherosclerotic plaque rupture is the major cause of myocardial infarction. this acute events represent the leading cause of death worldwide. Early reperfusion is the best method to salvage the ischemic organ; however, it leads to additional damage known as reperfusion injury (17).The early reperfusion phase is characterised by enhanced release of ROS from endothelial cells and cardiomyocytes, as well as enhanced expression of cytokines and adhesion molecules, The enhanced expression of chemokines during the first hours of reperfusion triggers further recruitment of neutrophils and monocytes into the infracted myocardium which lead to increase the cardiac damage by further releasing ROS, inflammatory mediators and proteases (18).

Zhang et al. (2005 a) showed that Simvastatin markedly attenuated the production of TNF-alpha, IL-1beta, IL-6 and increased IL-10 levels in the no infarcted and infarcted regions, reduced collagen deposition in the no infarcted myocardium and improved left ventricular function (19).

Dantas *et al.* (**2010**) found that simvastatin pretreatment attenuated Cyclophosphamide -induced urotelium inflammation in an experimental rat model, through significantly reduction in plasma level of pro- inflammatory cytokines (TNF-α, IL-6 and IL-1β) (20).

Naiyra. abd elbaky *et al.* (**2010**)studied the possible protective effect of simvastatin (SIM), against doxorubicin induced cardiotoxicity, He found that Rats received simvastatin alone showed apparently normal myocardial features similar to that of normal control while Rats administered doxorubicin showed typical myocardial toxicity in a form of myocardial muscle coagulative necrosis with focal areas of fibrosis, vascular dilatation and congestion, valves edema, and massive mononuclear cellular infiltration. Meanwhile, rats received doxorubicin and pretreated with simvastatin showed few inflammatory cells infiltration, little edema and improvement of myocardial cell necrosis (21). Statin mediated inhibition of Rho kinase leading to activation of phosphatidylinositol-3 kinase (PI3K)/protein kinase Akt pathway that promotes survival of the myocardial tissue (22).

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