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Blocking Leukotrienes Receptors Ameliorates Ischemia/Reperfusion Injury In A Rat Model.

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ABSTRACT

Myocardial ischemic reperfusion injury (I/R) is an obligatory process during cardiac surgery and catheterization of coronaries that induce an inflammatory response. this study was undertaken to determine the protective effect of montelukast (a leukotrienes antagonist) by amelioration of I/R injury in rat model. 28 adult males of Swiss albino rats were randomized in four groups: sham group (n = 7), rats underwent same anesthetic and surgical procedures but without I/R injury. Control group (induced untreated group, n = 7) rat underwent ligation of LAD (left anterior descending artery) for one hour and reperfusion for one hour. Control vehicle group (n = 7), rats received montelukast vehicle (ethanol 2%) 30 minutes before I/R and the same dose repeated before the reperfusion. Montelukast treated group, (n = 7) , the rats received montelukast (7 mg/kg) intraperitoneal injection 30 minutes before the I/R and the same dose repeated just before the reperfusion period. The I/R injury in the rats was done by ligation of LAD under general anesthesia through left thoracotomy for one hour then remove the ligation of prolene to start reperfusion period for one hour then sacrificing the rat, blood sample were collected from heart for measurement of plasma level of cardiac troponin 1 (cTn1) and serum IL-6 and TNF-α. The heart harvested and fixed in 10% formaline for histological examination.comparing with the sham group, the level of serum IL-6, TNF-α and plasma cTn1 were increased significantly ($p<0.05$) in control and vehicle groups. Histologically, the control and vehicle groups showed significant myocardial injury ($p<0.05$). montelukast treated group significantly counteract the increase in serum level of IL-6, TNF-α and plasma cTn1. Histological study of myocardium revealed that the montelukast treated group markedly reduced the severity of myocardial injury in the rats underwent I/R injury. The results of the present study reveal that monteukast may ameliorate I/R injury in the myocardium of rats via interfering with inflammatory reaction induced by I/R injury. These findings suggested that montelukast have a promising protective effect against induced I/R injury.

Keywords: Leukotrienes, Receptors, Ameliorates, Ischemia, Reperfusion.

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INTRODUCTION

Coronary heart disease is the leading cause of death worldwide. The process of restoring blood flow to the ischemic myocardium, can induce injury. This phenomenon termed myocardial reperfusion injury , can paradoxically reduce the beneficial effects of myocardial reperfusion [1] the injury culminates in the death of cardiac myocytes that were viable immediately before myocardial reperfusion, this may explain the rate of death despite optimal myocardial reperfusion [2].

The prolonged ischemia results in a variety of cellular metabolic and structural changes which include alteration in membrane potential, cellular swelling, increase intracellular calcium and sodium, increased hypoxanthine, decreased ATP and cellular acidosis. (164)reperfusion of ischemic tissues results in the formation of toxic reactive oxygen species including superoxide anions(O2 2), hydroxyl radicals, hydrogen peroxide(H2O2) and nitric oxide derived peroxynitrate [3] I/R results in complement activation and the formation of several proinflammatory mediators that alter vascular homeostasis, particularly C3a,C5a, iC3b and C5b-9 [4] I/R results in leukocyte activation, chemotaxis, leukocyte-endothelial cell adhesion and transmigration. The rapid restoration of physiologic pH during myocardial reperfusion which follows the washout of lactic acid and activation of the sodium-hydrogen exchanger contributes to lethal reperfusion injury [5] the mitochondrial PTP (permeability transition pore) is a non selective channel of the inner mitochondrial membrane. Opening the channels collapses the mitochondrial membrane potential and uncouples oxidative phosphorylation resulting in ATP depletion and cell death [6]

There are new strategies for preventing lethal reperfusion injury; Ischemic Post conditioning, in 2003, Zhao et al. showed after 45 minute myocardial ischemia, interruption of myocardial reperfusion with three 30 second cycles of I/R can reduce the myocardial infarct size [7]Ischemic Preconditioning refers to phenomenon by which exposure of tissues to brief periods of ischemia protects them from harmful effects of prolonged I/R [8] Targeting the RISK pathway, this pathway refers to a group of protein kinases are activated during myocardial reperfusion [9] also pharmacologic inhibition of mitochondrial PTP opening during myocardial reperfusion reduce infarct size in animals up to 50% [10]. Antileukocyte therapy, experimental therapeutic strategies to limit leukocyte-mediated I/R injury have focused on inhibition of inflammatory mediator release or receptor engagement, leukocyte adhesion molecule synthesis, or eukocyte endothelial adhesion. Leukocyte activation after I/R is facilitated by release of such inflammatory mediators as histamine, platelet activation factor, leukotriene B4, and tumor necrosis factor-α. Inhibition of inflammatory mediators or receptor engagement can attenuates I/R-induced leukocyte activation [11].

Leukotrienes comprise a family of products of 5-lipoxygenase pathway of arachidonic acid metabolism. Synthesis from substrate arachidonic acid is initiated by 5-lipoxygenase-activating protein (FLAP). Leukotriene A4 (LTA4) is converted by LTA4 hydrolase to leukotriene B4 (LTB4), or it can be conjugated with reduced glutathione by leukotiene C4 synthase to yield LTC4. The released LTC4 is converted to leukotriene D4 (LTD4), which undergoes conversion to leukotriene E4 (LTE4) by sequential amino acid hydrolysis [11]. Cysteinyl-leukotrienes (CysLTs) are potent bronchconstrictors, with LTC4 and LTD4 being more potent than E4. In addition, the CysLTs were shown to inuce cell adhesion proteins and promote leukocyte adhesion to vascular endothelial cells. Studies have demonstrated a capacity for CysLTs to induce ROS and NO formation [12] there are two CysLTs receptors termed Cysteinyl leukotriene receptor type 1 and type 2 (CysLT1 and CysLT2).

Montelukast is an orally active, highly selective leukotriene receptor antagonist that inhibits the CysLT1 receptor. It inhibits physiologic actions of LTD4 at the CysLT1 receptor without any agonist activity [13]. Cysteinyl leukotriene receptor 1 exert anti-inflammatory effects through the suppression of TH2 cells that lead to inhibit production of histamines, leukotienes, and pro-inflammatory cytokines such as interleukins 4,5 and 13 [14] high doses of montelukast module the production of IL-6, TNF-α AND MCP-1through the inhibition of NF-κB activation [15].

Tumor Necrosis Factor-α (TNF-α), is a 17-Kd, 157- amino-acid cytokine that is secreted by a wide spectrum of cells. It is a proinflammatory cytokine predominantly secreted by macrophages in response to a variety of pathologic processes.[16] Interleukin-6, is 21-Kd cytokine that is produced by a variety of cells including fibrblasts, mononuclear cells, phagocytes, neutrophils, and T and B lymphocytes, the production increase in response to endotoxin [17]. Cardiac troponin 1 (cTn1) is a basic globular protein containing

approximately 210 amino acids (24 kDa). It is believed that all isoforms of cTn1 are expressed exclusively in cardiomyocytes, thus its detection in the blood is synonymous with myocardial injury [18].

METHOD

A total of 28 adult male Albino rats weighing 150-220 g were purchased from Animal Resource Center, Al-Nahrain University. They were housed in the animal house of Kufa College of Medicine in a temperature-controlled (25 °C) room (humidity was kept at 60-65%) with alternating 12-h light/ 12-h dark cycles. They were randomized into four groups (7 rats in each group) as follow:

- Sham group ; rats underwent the same anesthetic and surgical procedures for an identical period of ischemia time.
- Control group ; rats underwent ischemia by ligation of left anterior descending artery (LAD) for one hour then reperfusion for one hour.
- Control vehicle group ; rats received montelukast vehicle (ethanol 2%) 30 minutes then induction of I/R for one hour and then the same dose was repeated just before reperfusion period (reperfusion time one hour).
- Montelukast treated group ; rats received montelukast (7mg/kg) intraperitoneal injection 30 minutes before induction of ischemia , ischemia for one hour, and the same dose was repeated just before reperfusion period , reperfusion time for one hour. The drug dissolved in one ml (2%) ethanol to form homogenized drug [19,20].

The surgical procedure and anesthesia; rat were anasthesized by intraperitoneal injection with a ketamine in a dose of 100 mg/kg and xylazine in a dose 10 mg/kg. when the rat anesthesized, the trachea explored by longitudinal incision in the neck, then the trachea intubated with a canulae sized 20 FG by help of forceps to hold the trachea. This canula connected to the ventilator which ranged between 110 – 140 breath/minute according to weight of rat with 100% oxygen. Left thoracotomy done, incision from left anterior axillary fold to the anterior midline, cutting the overlying muscles, intercostals muscle separated in the third or fourth interspace, lung pushed by swap, opening the pericardium, the left anterior descending coronary artery (LAD) is faint, pinkish with intramural position in contrast to the vein which is superficial, dark red. The LAD is pulsatile and run from below the left auricle to the apex. The LAD is ligated with 8/0 prolene , ischemic time for one hour then release of suture to begin the reperfusion time for one hour. Thoracotomy incision closed, the rate of ventilation decreased and watching for spontaneous breathing and careful extubation done. Finally, the were sacrificed by taking blood sample directly from heart then harvesting the hear for histopathology. About 3 ml of blood was collected from the heart of each rat, the blood then centrifuged at 3000 rpm for 15 minute, serum separated and analyzed for determination of TNF-α and IL-6 level and part of sample separate plasma for cTn1 level by ELISA test.

All specimens of heart were immediately fixed in 10% buffered formaline. After fixation they were processed in usual manner. The sections were examined by microscope under magnification power of (×10 and ×40) then the histological changes were determined. After fixation, evaluation of scores were performed by investigator blindly to the experimental groups. Zingarelli et al were used to assess the histopathological damage score: score 0; no damage, score 1; (mild) interstitial edema and focal necrosis , score 2; (moderate) diffuse myocardial cell swelling and necrosis , score 3; (sever) necrosis with the presence of contraction bands, neutrophil infiltration and compressed capillaries, score 4; (highly sever) wide spread necrosis with presence of contraction bands, neutrohpil infiltration, compressed capillaries and hemorrhage.

Statistical analysis data of studied group were entered and analyzed using SPSS (statistical package for social sciences) software for windows. Descriptive statistics were presented as (mean ± standard error of mean (SEM), ANOVA test was used for comparison among the four groups of study and to find the significance of the differences. In all statistical procedures and tests level of significance was set at P≤0.05 to be considered as significant difference. Finally all data and results were presented in tables.

RESULTS

Effect of myocardial I/R injury on inflammatory mediators. The effect on serum TNFα level and serum IL-6 level, the mean value of serum TNFα and IL-6 levels was significantly(p≤0.05) increased in control and

control vehicle with sham group. There is insignificant difference between both control group and control vehicle group. The TNFα and I-6 levels of montelukast treated group was significantly (p≤0.05) lower than that of control and control vehicle group. Changes in serum TNFα are summarized in table 1 and in serum IL-6 in table 2

Table 1: Serum TNFα level (pg/ml) of four experimental groups at the end of experiment(n=7). * control vehicle vs. sham group. ** treated vs. control vehicle. $ treated vs. control. $$ treated group vs. sham group.

Group	Mean ± SE	P.value
Sham	3.36 ± 0.39	<0.05*
Control vehicle	24.43 ± 1.06	< 0.05**
Control	25.8 ± 1.14	<0.05$
Treated	11.9 ± 0.52	<0.05$$

Table 2: Serum IL-6 level (pg/ml) of four experimental groups at the end of experiment(n=7). * control vehicle vs. sham group. ** treated vs. control vehicle. $ treated vs. control. $$ treated group vs. sham group.

Group	Mean ± SE	P.value
Sham	3.36 ± 0.39	<0.05*
Control vehicle	24.43 ± 1.06	< 0.05**
Control	25.8 ± 1.14	<0.05$
Treated	11.9 ± 0.52	<0.05$$

Effect on plasma cTn1 level , the level of plasma cTn1 is significantly (p<0.05) increase in control and control vehicle group as compared with the sham group. there is no difference between both control and control vehicle group. The level of pasma cTn1 for montelukast treated group was lower than that of control and control vehicle group. The changes in plasma cTn1 level are summarized in table 3.

Table 3: Plasma cTn1 level (ng/ml) of four experimental groups at the end of experiment (n= 7) * control vehicle vs. sham group. ** treated vs. control vehicle. $ treated vs. control. $$ treated group vs. sham group.

Group	Mean ± SE	P.value
Sham	1.12 ±0.08	<0.05*
Control vehicle	7.17 ± 0.33	< 0.05**
Control	7.32 ±.29	<0.05$
Treatment	3.79 ± 0.29	<0.05$$

Histopathological findings, a cross section of sham myocardium showed a normal appearance (85.7%) and mid injury (14.3%). In control group showed sever myocardial injury (57.1) and highly sever injury (42.9). the score in control vehicle group showed sever myocardial injury (57.1), highly sever injury (28.6) and moderate myocardial injury (14.3). montelukast treated group showed mild myocardial injury (28.6%), moderate myocardial injury (14.4%) and (57.1) normal myocardium. All are shown in table 4.

Table 4: the differences in histopatholoical grading of abnormal myocardium changes among the four experimental groups

Histopathological	Sham		Control vehicle		Control		Treatment	
	N	%	N	%	N	%	N	%
Normal	6	85.7%	0	0.0%	0	0.0%	4	57.1%
mild	1	14.3%	0	0.0%	0	0.0%	2	28.6%
moderate	0	0.0%	1	14.3%	0	0.0%	1	14.3%
severe	0	0.0%	4	57.1%	4	57.1%	0	0.0%
highly severe	0	0.0%	2	28.6%	3	42.9%	0	0.0%
Total	7	100%	7	100%	7	100%	7	100%

The myocardial damage score shows significant (p<0.05) increase in control and control vehicle group compared to the sham group. The level of myocardial damage score for montelukast treated group was lower

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than that of control and control vehicle group. The changes in myocardial damage score level are summarized in table 5

Table 3: Myocardial damage score of the four experimental groups (n=7) * control vehicle vs. sham group. ** treated vs. control vehicle. $ treated vs. control. $$ treated group vs. sham group.

Group	Mean ± SE	P.value
Sham	0.16 ± 0.03	<0.05*
Control vehicle	4.56 ± 0.23	< 0.05**
Control	5.05 ± 0.22	<0.05$
Treatment	1.14± 0.09	<0.05$$

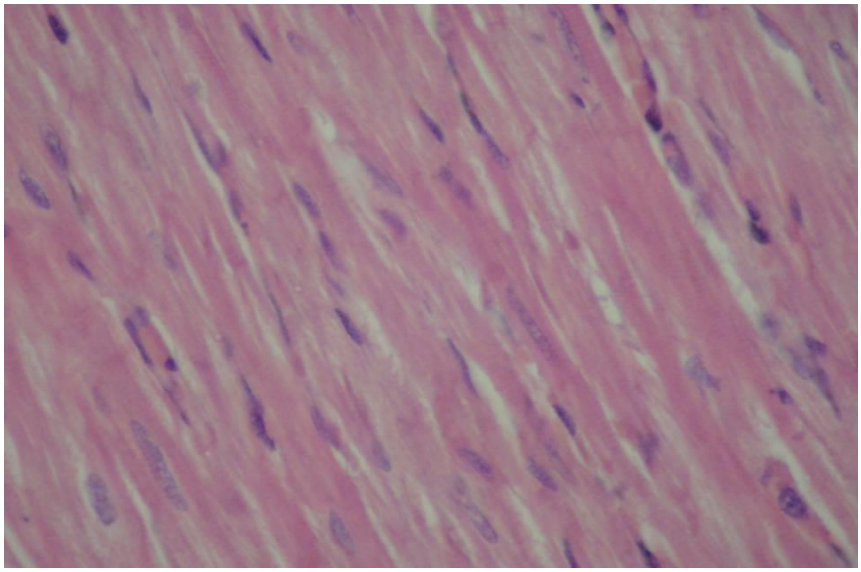

Figure 1: Photomicrograph of section of normal rats myocardium shows the normal architecture . The section stained with H & E (X 40).

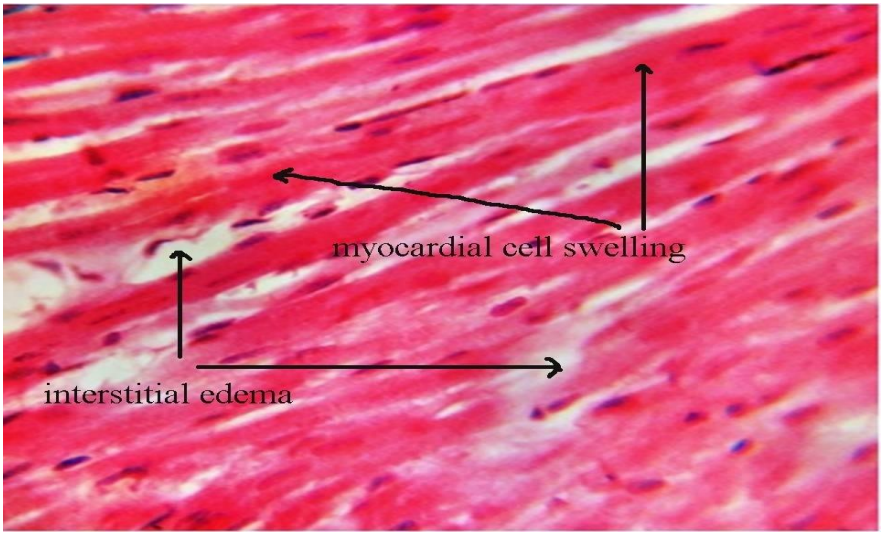

Figure 2: Photomicrograph of rats myocardium shows section after treatment with montelukast showing mild to moderate injury. The section stained with H & E (X 40).

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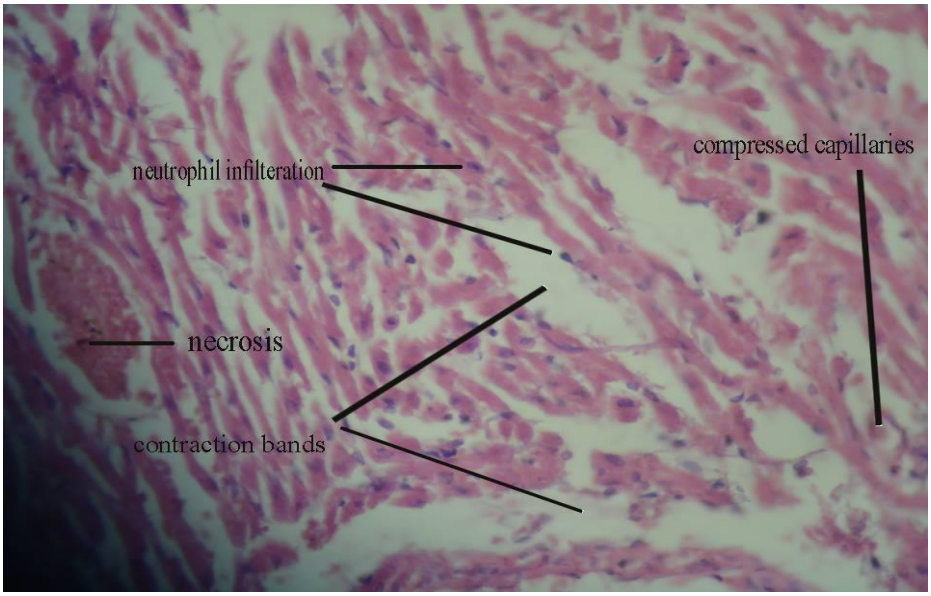

Figure 3: Photomicrograph section of rats myocardium shows the sever and highly sever injury in control untreated group. The section stained with H & E (X 40).

DISCUSSION

Effect of myocardial I/R injury on inflammatory markers

Effect of serum I/R on TNF-α , in our study a significant increase in inflammatory mediator (TNF-α) level in serum ($p<0.05$) was found in control group as compared with sham group. Gurevitch et al 1996[22]were the first demonstrate a significant release of TNF-α in the rat coronary effluent at 1 minute after reperfusion. Meldrum et al [23] demonstrate that TNF-α protein is elevated in the myocardium itself after crystalloid-perfused global ischemia-reperfusion. In 2011 , they confirmed by immunohistochemical analysis of myocardial sections taken from I/R rats showed increased levels of TNF-α and IL-1B [24].

Effect of myocardial I/R on plasma cTn1, in present study, a significant increase in plasma level of cTn1($p<0.05$) was found in I/R rats as compared with sham group. In 2009 a group was measured serum levels of cTn1 after 30 min of ischemia and 6 hr reperfusion and found a significant increase in serum cTn1 level as compared with sham group [25]. The results in the present study are in agreement with that reported by H.Oksuz et al 2009 and YOU Yun-tai 2010, their results showed that serum levels of cTn1 increased markedly after I/R injury [26,27].

Effect of myocardial I/R on serum IL-6 level, In the present study, a significant increase in serum level of IL-6 ($p <0.05$) was found in the I/R rats as compared with sham group. Rajesh Aneja et al. (2004)[28] found that IL-6 levels were significantly elevated after myocardial ischemia and reperfusion in vehicle treated rats (1312.83 + 224.6 pg/mL) when compared with sham control animals (132 + 21 pg/mL; $P < 0.05$). Yamauchi TK et al (1995) [29] Hypoxic cardiomyocytes have been shown to produce IL-6 which could contribute to ventricular dysfunction as observed after myocardial ischemia and reperfusion.

Effect of myocardial I/R injury on heart parenchyma

In our study there was statistically significant difference between induced untreated group and normal sham group ($p <0.05$). Zingarelli et al. (2002)[30] showed that a marked disruption of the myocardial structure in myocardial I/R injury was characterized by appearance of extensive necrosis and contraction bands. Zhu J. et al. (2008)[31] studied the histological changes in transient I/R, (after 30 min ischemia and 3,12,24 hr of reperfusion) and found that after 3 hr myocardial destruction with focal infiltration of PMNs was evident in the left ventricular wall. According to Jiang WL et al [32,33] , (after 30 min of ischemia and 24 hr of reperfusion) pathological features of the infarct area became apparent with widespread tissue necrosis.

Effect on treatment study parameters

Effect of montelukast on inflammatory markers, In this study montelukast significantly reduced the elevation of proinflammatory markers (IL-6 & TNF-α) level ($P < 0.05$) in the I/R(treated) rats. Maeba *et al.* (2005) showed that high doses of montelukast modulate the production of IL-6 and TNF-α through the inhibition of NF-κB activation [34].

Effect of montelukast on heart parenchyma of rats and cTn1

Treatment of rats with montelukast ameliorates I/R injury significantly ($P < 0.05$) as compared with induced untreated group. Chen S et al [35] Stated that decreased serum level of LDH, CK, MDA and attenuated myocardial necrosis area were found in rats pretreated with montelukast sodium 10 and 30 mg.kg-1. They conclude that Montelukast sodium is cardioprotective during myocardial injury in rats by halting the leukotrienes-induced inflammatory response and upregulating the eNOS expression as well as downregulating the iNOS expression. This may represent an approach to the treatment of myocardial ischemia with leukotriene antagonists. In present study we found that, there is a significant strong correlation between level of cTn1 and cardiac injury score ($p<0.05$) and treatment of rats with montelukast ameliorate myocardial injury so it might result in lowering plasma level of cTn1.

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