Etanercept ameliorate inflammatory responses and apoptosis induces by myocardial ischemia/reperfusion in male mice

Najah R. Hadi ¹, Fadhil G. Al-Amran ², Maitham G Yousif ³, Saif M. Hassan ¹

Abstract

Ischemia/reperfusion of cardiac tissues may lead to a prominent damage of the cardiomyocyte through either necrosis or apoptosis. In this study, we investigated the effects of Etanercept in myocardial ischemia/reperfusion injury. Acute myocardial injury was induced by ligation of the left anterior descending coronary artery (LAD) for 30 minutes followed by 120 minutes reperfusion in white albino adult male. At the end of reperfusion, cardiac tissue TNF-α, IL-1β, IL-6, caspase-3 and Bcl-2, as well as plasma cardiac troponin I (cTn-I) were measured. It has been found that Etanercept treated group showed significant reduction in proinflammatory cytokines, caspase-3, Bcl-2 and cTn-I with respect to the control groups (P<0.01). Histopathology study revealed that the treatment with Etanercept significantly improved cardiac injury as compared with control groups (P<0.05). Further, the total severity scores showed 16.7% of the group had no damage, while 50% had mild cardiac injury and only 33.3% with moderate cardiac injury in Etanercept treated group. We demonstrated that Etanercept reduces inflammatory reaction, cardiomyocyte apoptosis and myocardial damage induced by ischemia/reperfusion injury.

Keywords: Etanercept; Caspase-3; Ischemia/reperfusion; LAD; Myocardial injury

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Introduction

In the United States, ischemic heart disease (IHD) accounts for over 500,000 deaths annually. The most frequent complication of IHD is acute myocardial infarction (AMI), known as a heart attack. AMI usually results from plaque rupture with thrombus formation in a coronary vessel, resulting in an acute reduction in blood supply to the downstream myocardium. Paradoxically, re-establishment of the blood supply can exacerbate vascular injury [1]. The myocardial ischemia is energetic stress, while reperfusion is associated with abrupt ionic shifts and considerable oxidative stress. Cells die by necrotic and apoptotic pathways after the acute injury, the healing myocardium is undergone to biomechanical stress and inflammation, which can cause by a smaller but more sustained wave of cell death, these changes in the metabolic and functional characteristics of surviving cells [2].

Ischemia causes accumulation of intracellular sodium (Na⁺), hydrogen (H⁺), and calcium (Ca²⁺) ions culminating in tissue acidosis. While during reperfusion rapid alterations in ion flux, and renormalization of pH paradoxically that leads to enhanced cytotoxicity. Sodium-dependent pH regulatory mechanisms, including the Na⁺/H⁺ exchanger and the Na⁺/HCO⁻³ transporter is activated and lead to accumulation intracellular sodium. Increase sodium concentrations lead to increases in sarcoplasmic reticular Ca²⁺ by the Na⁺/Ca²⁺ exchanges [3]. Cardiac ischemia-reperfusion (IR) injury causes a decrease of ATP, irreversible proteins oxidation, lipids, and DNA inside the cardiomyocyte, and can trigger apoptosis by excessive generation of reactive oxygen species (ROS), overload of intracellular Ca²⁺, H⁺ leakage in the mitochondrial, inflammation, and metabolic products lead to the opening of the mitochondrial permeability transition pore (PTP) [4].

Reperfusion induced vascular injury and this injury may result in the recruitment and activation of neutrophils, release of inflammatory molecules and further injury to the tissues or blood vessels [5]. Apoptosis has led to the suggestion that ischemia–reperfusion mediates apoptosis by or in combination with: 1) Up regulation of Bax (proapoptotic protein). 2) Down regulation of Bcl-2 (anti-apoptotic protein). 3) Activation of Fas or TNF-α receptors. 4) Activation of p53 and c-Jun kinase pathways. 5) Neutrophil and/or macrophage activation and infiltration [6]. Tumor necrosis factor
alpha (TNF-α), a proinflammatory cytokine, is produced by activated macrophages, monocytes, and mast cells and is considered to have an important role in the regulation of host defense response. The adult heart also synthesizes TNF-α under *in vitro* and *in vivo* conditions [7]. TNF-α which causes myocardial dysfunction by reducing intracellular Ca+2 level, triggering apoptosis and increasing inducible nitric oxide synthetase (iNOS) mediated nitric oxide (NO), peroxynitrite (ONOO) levels [8].

Etanercept is a fusion protein produced by recombinant DNA and fuses the TNF receptor to the constant end of the IgG1 antibody. First, the developers isolated the DNA sequence that codes the human gene for soluble TNF receptor 2, which is a receptor that binds to tumor necrosis factor-alpha [9]. Etanercept acts by reversible, competitive binding of both circulating and membrane/bound TNF-α and TNF-β. This prevents binding to specific cell-surface receptors on the target cells and thus inhibits cell activation and, as a result, the proinflammatory effect of TNF. Etanercept may also modulate biological responses controlled by additional downstream molecules (e.g., cytokines, adhesion molecules, or proteinases) that are released or regulated by TNF [10].

**Method**

**Animals**

Forty adult males Swiss Albino mice weighing 28-35g 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 (24±2°C) room temperature with ambient humidity and alternating 12h light/12h dark cycles and were allowed free access to water and standard chow diet until the start of experiments. The mice were left for two weeks without interference for local acclimatization and they had no manifestation of any illness upon examination.

**In vivo myocardial I/R model**

*In vivo* myocardial I/R model was modified from a previous study [11]. Briefly, animals were intraperitoneally anesthetized with 100mg/kg ketamine and 10mg/kg xylazine [12]. After anesthesia, shave the neck area and the left side of the rib cage and disinfected by 80% ethanol [13]. Place the mouse on its back checked the reflexes by pinching the tail and hind feet to be sure that the mouse has sufficient anesthesia. Under microscopic view, perform a midline cervical incision separating the skin, muscle, and
tissue covering the trachea. When the trachea was exposed, the trachea was intubated through oral route with a cannula sized either 22 or 20 G according to the weight of the animal. As the small catheter was reserved for the smaller animal, the tube was visible through the trachea which was already exposed. The mice 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 [14]. A left thoracotomy was carried out to expose the heart. Zoom in the microscope on the heart, the LAD is then transiently ligated (or can be tied with a slipknot) using a 6-0 polypropylene suture for a 30 -minute ischemic period [15]. The chest wall closed by the enclosing the ribs with figure eight of 5-0 silk suture. The pectoral muscles should be returned back into the original position (first the minor, then the major partly overlying it), then the skin was closed with 5-0 silk suture. The rate of ventilator was gradually decreased until reach 80 breaths /min. and watched for the spontaneous breathing, and when it was sufficient, and the decision was made for gentle and careful extubation after free the mouse from tapes. Finally, the mouse should be transferred into a clean cage oxygenated with 100% oxygen and placed near the fair heating lamp. Immediately after finishing the reperfusion time the moue 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 mouse 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 0.5 ml of blood for later plasma analysis. After that hearts were rapidly removed for quantification of myocardial injury and apoptosis and biochemical studies [16].

**Experimental groups and protocols**

After the 1st week of acclimatization, the mice were randomized into four groups as follows:

1. Sham group: this group consisted of six mice. Mice are underwent the anaesthetic and surgical procedures without left anterior descending (LAD) coronary artery occlusion.

2. Control group (induced untreated group): this group consisted of six mice. Mice underwent LAD coronary artery occlusion (30 min), then reperfusion for 2 h and left until the end of the experiment [17].
3. Drug treated group: this group consisted of six mice. Mice underwent LAD coronary artery occlusion (30 min) then reperfusion for 2h, mice received Etanercept 5 mg/kg i.p. 5min before reperfusion [18].

4. Vehicle treated group: this group consisted of six mice. Mice underwent LAD coronary artery occlusion (30 min) then reperfusion for 2h, mice received normal saline i.p. 5min before reperfusion [18].

Blood sampling for measurement of plasma cTn-I

At the end of reperfusion, the blood from the apex of the heart was collected; about 0.5 ml of blood was collected from the heart. The blood sample was placed in a tube contain disodium EDTA (22mg/ml) as anticoagulant and mixed thoroughly then centrifuged at 3000 RPM for 15 min.

Measurement of proinflammatory, caspase-3 and Bcl-2

Myocardial tissues, collected 120 min 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 [19] by used a high intensity liquid processor. After homogenization, samples were centrifuged at 14,000 RPM for 20 min at 4°C and used according to the manufacture instructions [20].

Histopathological analysis and damage score

Cardiac tissue was fixed in 10% formalin, processed by routine histological methods, and embedded in paraffin block (Bancroft and Stevens, 1982), 5μm-thick horizontal sections were cut and stained with hematoxylin- eosin (H&E) for subsequent histological examination. After fixation, an investigator who was blind to the experimental treatment groups performed evaluations of scores. The following morphological criteria [21] were used to assess the histopathological damage: 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 neutrophil infiltration; and score 4 (high sever), hemorrhage.

Statistical analyses

Statistical analyses were performed using SPSS 20.0 for windows. Inc. 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. The statistical significance of difference in total score between more than 2 groups was assessed by Kruskal-Wallis test, while
Mann-Whitney U test was used for the difference between 2-groups. In all tests; $P<0.01$ was considered to be statistically significant.

**Results**

Figure 1.
The mean of myocardial proinflammatory cytokines level **A**) TNF-α, **B**) IL-1β, **C**) IL-6 (pg/mg) in the four experimental groups at the end of the experiment. $^*P<0.05$ vs. sham group; $^*^P<0.05$ vs. Ctrl vehicle group.

Figure 2.
The mean of plasma cTn-1 level (ng/ml) in the four experimental groups at the end of the experiment. $P<0.05$ vs. sham group, $^*P<0.05$ vs. Ctrl vehicle group.
Figure 3.

The myocardial mean of: A) Caspase-3 and B) Bcl-2 (pg/mg) in the four experimental groups at the end of the experiment. *P<0.05 vs. sham group, **P<0.05 vs. Ctrl vehicle group.

<table>
<thead>
<tr>
<th>Histopathological Scoring</th>
<th>Study groups</th>
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<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>N</td>
</tr>
<tr>
<td>Score 0 (no damage)</td>
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</tr>
<tr>
<td>Score 1 (mild)</td>
<td>0</td>
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<tr>
<td>Score 2 (moderate)</td>
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<tr>
<td>Score 3 (severe)</td>
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<td>Score 4 (high severity)</td>
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<tr>
<td>Total</td>
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</tbody>
</table>

Table 1.

The differences in histopathological scoring of abnormal heart changes among the four experimental groups.
Figure 4.
Component bar chart shows the relative frequency of different histopathological grading of abnormal heart changes among the four experimental groups.

Figure 5.
Representative photomicrograph of a section of the heart tissue section stained with Haematoxylin and Eosin (X 40). A) The sham group shows normal architecture (score 0); no interstitial edema, no diffuse myocardial cell swelling and necrosis, no neutrophils infiltration, no hemorrhage, and no evidence of apoptosis. B) Photomicrograph of cardiac section for the control group showed hemorrhage, necrosis and neutrophil infiltration. C) Photomicrograph of cardiac section in etanercept treated group show almost normal cardiac structure; most tissues reveal a mild histological change.
Discussion

Decrease of blood flow and oxygen to the cardiac muscle by partial or complete blockage of an artery carrying blood to the myocardium leads to death of an affected cardiac muscle. This condition called myocardial ischemia. While restorations of blood flow to an ischemic heart refer to myocardial reperfusion. Early reperfusion minimizes the extent of myocardial damage whereas reperfusion after a prolonged period of ischemia produces marked damage in myocardial [22]. Many factors such as antigen-independent inflammatory condition has been characterized the ischemia/reperfusion injury lead to increase proinflammatory cytokines synthesis and release [23]. TNF-α and IL -1β are proinflammatory cytokines known to depress myocardial contractility [24].

TNF-α which causes myocardial dysfunction by reducing intracellular Ca²⁺ level, triggering apoptosis and increasing inducible nitric oxide synthetase (iNOS) mediated nitric oxide (NO), peroxynitrite (ONOO) levels [8]. The inflammatory cytokines (TNF-α, IL-1β and IL-6) have depressor effects on myocardial function and have been suggested to mediate I/R injury [25]. In ischemia-reperfusion (IR) injury, tumor necrosis factor (TNF)α mediates inflammation and apoptosis. A soluble TNF-α receptor (Etanercept) has shown anti-inflammatory and anti-apoptotic effects in several animal models [26]. Gu, Yang et al. (2006) found that etanercept reduced NF kappa B activation, ICAM-1 upregulation and myocardial injury following ischemia-reperfusion in dogs ischemia reperfusion model [27]. Furthermore Chen, Xia et al. (2011) showed that etanercept significantly lowered IL-1 and IL-6 compared with those of I/R group when rats subjected to ligation of left anterior descending coronary for 30 min and then reperfusion for 2 h [28]. Choi, Jeong et al (2009) found that renal mRNA levels of TNF-α in etanercept-treated IR rats were significantly lower than those in control IR rats.

In addition Genovese, Mazzon et al (2006) found that treatment of the mice with etanercept significantly reduced the degree of cytokines expression (TNF-α, interleukin-1β) in spinal cord ischemia reperfusion [29]. Furthermore Paola, Mazzon et al (2007) showed that when mice treated with etanercept (5 mg/kg administered i.p. 5 min prior to reperfusion) significantly reduced the level of periodontitis cytokines (eg.
TNF-α) [30]. Also White, Santora et al (2012) showed that lung NF-κB expression was decreased in etanercept-treated mice compared with vehicle-treated mice during kidney IRI, when animal subjected to 60 min ischemia followed by 60 min reperfusion [31]. Gao, Liu et al (2011), when adult male mice were subjected to 30 min MI followed by 3h or 24h reperfusion, etanercept decreased apoptosis (caspase-3 activity 21% vs 35% reduction) and concluded that upregulated adiponectin was involved in cardioprotective effect of etanercept and suggested that single administration of etanercept during ischemia-reperfusion improve outcome of myocardial infarction patients [32].

Furthermore Esposito, Mazzon et al (2007) showed that Bcl-2 expression increases a significantly in whole extracts obtained from ischemia/reperfusion-injured in splanchnic mice [18]. Also, (Genovese, Mazzon et al (2006) found that etanercept significantly increases the level of spinal cord Bcl-2 expression [29]. In addition Paola, Mazzon et al. (2007) showed that when mice are treated with etanercept significantly increases the level of Bcl-2 level in periodontitis [30]. Esposito, Mazzon et al (2007) who showed when mice are treated with etanercept reduced the histological score and reduced the neutrophil infiltration when mice subjected to ischemia/reperfusion-injured in splanchnic [18]. Also Genovese, Mazzon et al 2006) showed that when mice are treated with etanercept significantly reduced the degree of spinal cord inflammation and tissue injury histological score and neutrophil infiltration [29].

In addition Chiang (2006) found that when male rats are treated with etanercept, reduce edema and leukocyte infiltration are reduce particularly when they were subjected to acute lung injury [33]. Furthermore Paola, Mazzon et al (2007) showed that treatment of the rats with etanercept significantly reduced the degree of periodontitis inflammation and tissue injury (histological score) and infiltration of neutrophils [30]. Chen, Xia et al (2011) who found that etanercept has significantly lower troponin compared with those of I/R group when rats were subjected to ligation of left anterior descending coronary was performed for 30 min and then the perfusion [28].

In conclusion, the major findings of the present study are as follows: 1) the inflammatory cytokine. 2) caspase-3 and Bcl-2 play important role in the pathology of myocardial I/R. 3) Etanercept treatment played a protective role against myocardial I/R injury. The protective effects of etanercept during myocardial I/R injury were correlated with the attenuation of inflammation and apoptosis. 4) Etanercept ameliorate
myocardial I/R injury as evidenced by reduce the release of cardiac specific enzyme troponin I and myocardial damage.

Competing interests

The authors declare that there is no conflict of interest.

Author Contributions

All authors wrote, read and approved the final manuscript.

References


