Etanercept ameliorate cardiac damage and Apoptosis Induced by Myocardial Ischemia/Reperfusion in male mice.

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Abstract:
Ischemia-reperfusion of cardiac tissues may lead to a prominent damage of the cardiomyocyte through either necrosis or apoptosis that seems to be the predominant modes of death during this period. In this study, we investigated the effects of Etanercept in regional ischemia/ reperfusion injury and apoptosis. White albino adult male mice were divided into four groups (six mice per group). Sham group, mice were subjected for all surgical procedure without ligation of the left interior descending coronary artery (LAD). Control group, in which LAD was ligated. Control vehicle and Etanercept treated groups, mice subjected to the surgical procedure with ligation of LAD for 30 minutes followed by 120 minutes reperfusion, mice treated with normal saline (etanercept vehicle) and Etanercept (5 mg/kg, I.P. 5 minute before reperfusion). At the end of reperfusion, cardiac tissue caspase 3 and Bcl-2, as well as plasma cardiac troponin I (cTnI) were measured. It has been found that Etanercept treated group showed significant reduction (P<0.01) in caspase 3 and cTnI but increase (P<0.01) the level of the Bcl-2 as compare with the control groups. Histopathology study revealed that the treatment with Etanercept significantly (P<0.01) improved cardiac injury as compared with control groups and the total severity scores showed showed 16.7 % of the group had no damage and 50% had mild cardiac injury and 33.3% had moderate cardiac injury of Etanercept treated group. It...
is concluded that Etanercept reduces cardiac damage and apoptosis associated with ischemia/reperfusion injury.

**Key word:** Apoptosis, Etanercept, caspase 3, Bcl-2, cTnI, ischemia/reperfusion injury

### Introduction

Paradoxically that leads to enhanced cytotoxicity. Sodium-dependent pH regulatory mechanisms, including the Na⁺-H⁺ exchanger and the Na⁺-HCO₃⁻ transporter is activated and lead to accumulation intracellular sodium. Increase sodium concentrations lead to increases in sarcoplastic reticular Ca²⁺ by the Na⁺ - Ca²⁺ exchanges [2]. 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 In the United States, IHD (ischemic heart disease) accounts for over 500,000 deaths annually. The most frequent complication of IHD is AMI (acute myocardial infarction), 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, reestablishment 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
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].

1. Materials and methods

1.1. Animals
A forty adult males Swiss Albino mice weighing 28-35 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 mice were left for two weeks without interference for acclimatization. They had no manifestation of any illness upon examination.

1.2. 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 100 mg/kg ketamine and 10 mg/kg xylazine [12]. after protein) (2) Down regulation of Bel-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. It 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,
anaesthesia, 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.

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 mouse 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].
1.3. **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 underwent the anaesthetic and surgical procedures but 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 (for 30 min.), then reperfusion for 2 hours 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 (for 30 min.), then reperfusion for 2 hr., 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 (for 30 min.) then reperfusion for 2 hr., mice received normal saline i.p. 5min. before reperfusion [18].

1.4. **Blood Sampling for measurement of plasma cTnI**

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 (22 mg/ ml) as anticoagulant and mixed thoroughly then centrifuged at 3000 RPM for 15 min. Then it was used for the determination of plasma cTnI.

1.5. **Preparation of samples for caspase 3**

Rinse cardiac tissues two times with PBS; remove any remained PBS after the second rinse. Solubilize tissue in Lysis Buffer and allow samples to sit on ice for 15 minutes. Assay stored at ≤ -70° C. Before use, centrifuge at 2000 x g for 5 minutes and transfer the supernate to a clean test tube. Assay was done by diluted the lysates 6-fold with IC Diluent and made further serial dilutions in IC Diluent.

1.6. **Preparation of samples for Bcl-2**

Rinse cardiac tissues two times with PBS; remove any remained PBS after
2. Statistical Analysis
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 histopathological grading of heart changes is a non-normally distributed variable measured on an ordinal level of measurement; therefore non-parametric tests were used to assess the statistical significance involving this variable. 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.

3. Results

3.1. Etanercept reduced cardiac troponin I in MI/R injury
At the end of the experiment, the level of plasma (cTnI) was significantly (p < 0.01) increased in induced untreated (control) group as compared with the sham group. There was an insignificant difference between control vehicle (saline) and the second rinse. Solubilize tissue in Lysis Buffer and allow samples to sit on ice for 15 minutes. Assay stored at ≤ -70° C. Before use, centrifuged samples at 2000 x g for 5 minutes and transfer the supernate to a clean test tube. Sample protein concentration may be quantified using a total protein assay; dilution is made by IC Diluent.

1.7. 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 [19] 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.
Etanercept treated group developed a significantly ($p < 0.01$) increased the plasma cTnI levels as compared with the sham group. For etanercept treated group was a significantly ($p < 0.01$) lower than that of control vehicle (saline) and control group.

**Figure (1):** The mean of plasma cTnI level (ng/ml) in the four experimental groups at the end of the experiment. *Vs. sham group, **vs. Control group.

### 3.2. Etanercept reduced cardiac caspase 3

At the end of the experiment, the level of myocardial Caspase 3 was significant ($p < 0.01$) increased in induced untreated (control) group as compared with the sham group. There was an insignificant difference between control vehicle (saline) and control group. The myocardial Caspase 3 of etanercept treated group was significantly ($p < 0.01$) lower than that in the control group. Etanercept treated group showed a significant ($p < 0.01$) increase in the level of caspase 3 as compared with the sham group, figure (2).
Figure (2): The mean of Caspase 3 (pg/ mg) in the four experimental groups at the end of the experiment. * Vs. sham group, **vs. Control group.

3.3. **Etanercept increase cardiac Bcl-2**

At the end of the experiment, the level of myocardial Bcl-2 was significantly (p < 0.01) reduced in induced untreated (control) group as compared with the sham group. There was an insignificant difference between control vehicle (saline) and control group. The myocardial Bcl-2 in etanercept treated group was significantly (p < 0.01) higher than that in the control group. Etanercept treated group showed significant (p <0.01) increase in the level of Bcl-2 as compared with the sham group, figure (3)

![Graph showing Bcl-2 levels](image)

Figure (3): The mean of Bcl-2 (pg/ mg) in the four experimental groups at the end of the experiment. * Vs. sham group, **vs. Control group.

4. **Histological finding**

Treatment of mice with etanercept improved cardiac injury significantly (P < 0.01) as compared with the control vehicle group and the total severity score mean of
this group showed 16.7% of the group had no damage and 50% had mild cardiac injury and 33.3% had moderate cardiac injury.

A cross section of sham mice’s heart showed a normal cardiac structure. All mice in this group showed normal hearts 100% as shown in table (1).

There was statistically insignificant difference between control vehicle group (III) and control group (II) (P>0.01) and the total severity scores of the control group showed 16.7% of the group had moderate cardiac injury, 66.7% had severe cardiac injury and 16.7% had high severe cardiac injury.

<table>
<thead>
<tr>
<th>Histopathological Scoring</th>
<th>Study groups</th>
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<tbody>
<tr>
<td></td>
<td>Sham</td>
</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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<td>Score 4 (high severity)</td>
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</tr>
<tr>
<td>Total</td>
<td>6</td>
</tr>
</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.

![Component bar chart](image1)

A

B

C

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.

5. Discussion

The major findings of the present study are as follows (1) caspase 3 and Bcl-2 play important role in the pathology of myocardial I/R. (2) 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 apoptosis. (4) Etanercept ameliorate myocardial I/R injury as evidenced by reduce the release of cardiac specific enzyme troponin I and Myocardial damage.

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 [20]. Many factors such as antigen-independent inflammatory condition has been characterized the ischemia/reperfusion injury lead to increase proinflammatory cytokines synthesis and release [21]. 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 [22]. In ischemia-reperfusion (IR) injury, tumor necrosis factor (TNF) -α mediates inflammation and apoptosis. A soluble TNF-receptor (Etanercept) has shown anti-inflammatory and anti-apoptotic effects in several animal models [23].

Gu, Yang et al. (2006) found that etanercept reduced NF kappa B activation, ICAM-1 upregulation and myocardial injury.following isch mia-reperfusion in dogs ischemia reperfusion model. 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.

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 [24]. 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 [25]. 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 [26].

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 splanchic [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 [25]. 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 [27]. 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 [26].

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].

6. References


