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EFFECT OF ETANERCEPT AGAINST MYOCARDIAL ISCHEMIA/REPERFUSION INJURY IN MALE MICE

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ABSTRACT: Objective: This study was undertaken to investigate the potential role of etanercept in amelioration of myocardial I/R injury induced by ligation of coronary artery in a mice model. **Materials & methods:** Adult male Swiss Albino mice were randomized into 4 equal groups. Group (1) sham group: mice underwent the same anesthetic and surgical procedure as the control group except ligation of LAD coronary artery , Group(2) control group: mice subjected to regional ischemia for 30 min and reperfusion for 2 hours by ligation of LAD coronary artery ,Group(3) control vehicle group: same control group except mice given IP normal saline before reperfusion 5 minutes, Group(4) Etanercept treated group : mice treated with etanercept 5 mg/kg i.p. 5 minuts before reperfusion of ligation of LAD coronary artery. The heart tissue (below the ligation site) was used for measurement of apoptosis (caspase 3 and Bcl-2)and histopathology study. **Results:** Histologically, all mice in control group showed significant ($p<0.05$) cardiac injury. Furthermore all mice in control group showed significant ($p<0.05$) of caspase 3 while significant decrease ($p<0.05$) of Bcl-2. Etanercept significantly counteract the increase in myocardium level of caspase 3 ($P < 0.05$), etanercept significantly counteract the decrease ($p<0.05$) in myocardium level of Bcl-2. Histological analysis revealed that etanercept markedly reduced ($P < 0.05$) the severity of heart injury in the mice underwent LAD ligation procedure. **Conclusion:** The etanerceptameliorate myocardial I/R injury in mice via interfering with apoptosis which induced by I/R injury.

INTRODUCTION: 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[1].

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When ATP levels decrease, cells lose function of the Na^+/K^+ ATPase and Na^+ levels increase. In an effort to neutralize the decreased intracellular pH, cells activate their sodium ion-hydrogen ion exchanger (NHE). The majority of eukaryotic cells to maintain ionic homeostasis and to protect cardiac myocytes from intracellular acidosis using this exchanger. The NHE is an integral plasma membrane protein that electrochemical exchanges extracellular Na^+ for intracellular H+ with 1:1 stoichiometry. If the intracellular $[Na^+]$ is excessive, osmolar disturbances such as cellular oedema and swelling may occur. The myocyte will correct the osmolality by pumping Na^+ outward and moving Ca^{+2} inward. The osmolar load of the cell with the use of the Na^+/Ca^{+2} exchanger (NCE), which exacerbates the hypercalcemic intracellular state. The Ca^{+2}-ATPase pump will pump excess Ca^{+2} into the sarcoplasmic reticulum (SR). If the

SR overfills with excess Ca^{+2}, a vicious cycle of Ca^{+2} uptake and release will result that uses excessive ATP without achieving calcium ion homeostasis [2].

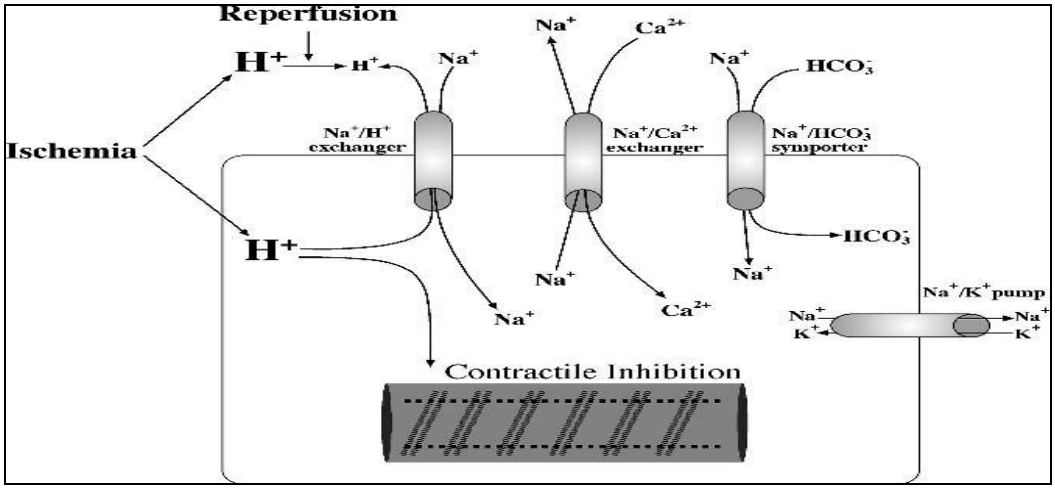


FIG.1: ELECTROLYTES CHANGE DURING ISCHEMIA& REPERFUSION (HA AND KIM 2010)

Cardiomyocyte apoptosis is one of the major mechanisms underlying I/R injury. The progressive loss of cardio -myocytes due to apoptosis plays a critical role in cardiac dysfunction after acute myocardial infarction[3]. B cell lymphoma-2 (BCL-2): The protective role of anti-apoptotic Bcl-2 in the heart is demonstrated by the fact that cardiac-specific over expression of Bcl-2 significantly reduces infarct size after I/R [4]. The decreased bcl-2 content facilitates MPT opening in the presence of oxidative stress. The current results indicate that the ischemia induced decrease in bcl-2 content in combination with increased ROS generation from the damaged electron transport chain will trigger MPT opening and increase cardiac injury during early reperfusion [5]. In the ischemia/reperfusion injury tissue, the intracellular calcium and ROS level will increased, these factors all contribute to induce apoptosis induce factor (AIF) to release and translocate to the nucleus, in the end, the caspase-independent apoptosis will happen, if we prevent this cell death, the prognosis of ischemia/reperfusion will be favourable [6].

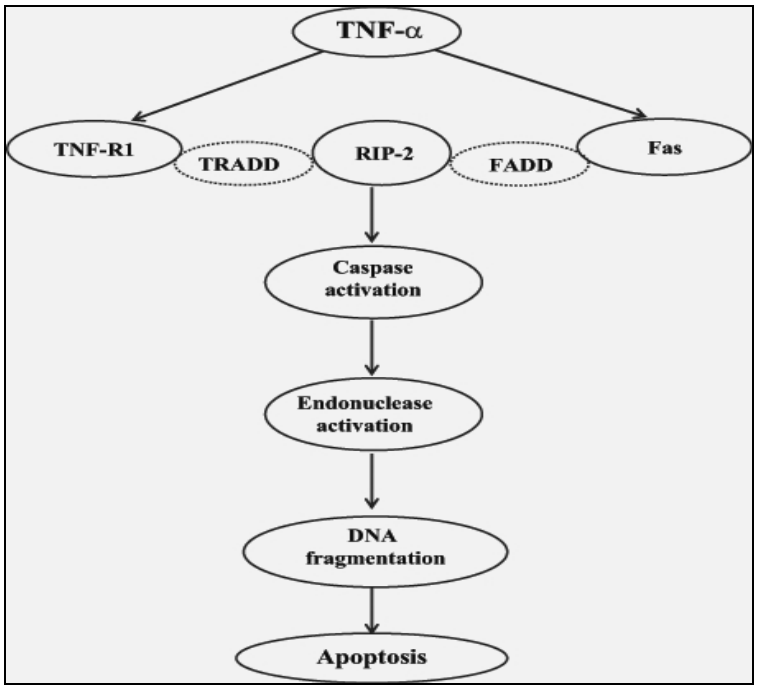


FIG.2: TNF-α INDUCE APOPTOSIS

Etanercept is a fully humanized, dimeric, recombinant protein generated by the fusion of 2 soluble (p75) TNF receptors with the constant fragment (Fc) of human immunoglobulin G1 (IgG1), which includes the CH_2 and CH_3 domains of the immunoglobulin. This is the fraction that provides stability to the molecule [7].

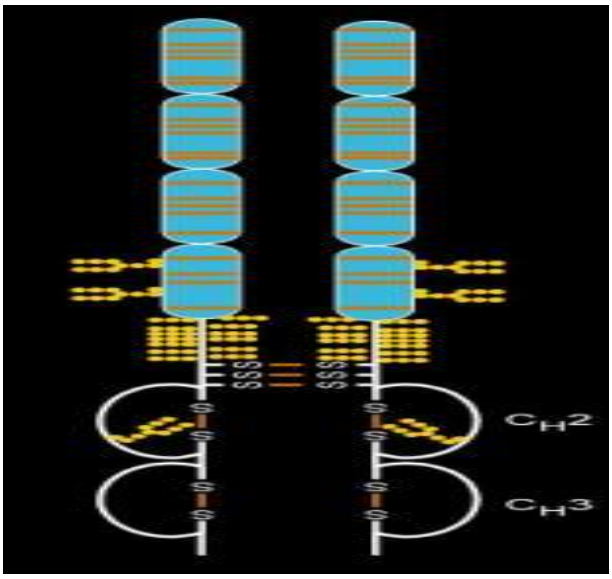


FIG.3: STRUCTURE OF ETANERCEPT

MATERIALS AND METHODS:

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**.**

In vivo myocardial I/R model:

In vivo myocardial I/R model was modified from a previous study [8]. Briefly, Animals were intraperitoneally anesthetized with 100 mg/kg ketamine and 10 mg/kgxylazine [9]. after anaesthesia, shave the neck area and the left side of the rib cage and disinfected by 80% ethanol [10]. 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 weightb [11]. 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 polyprolene suture for a 30 -minute ischemic period [12]. 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 highdose 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 [13].

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 [14].

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 [15].

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 [15].

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 #6 and allow samples to sit on ice for 15 minutes. Assay stored at ≤ -70° C. Before use, centrifuge at 2000x 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 #8 and made further serial dilutions in IC Diluent #3.

Preparation of samples for Bcl-2:
Rinse cardiac tissues two times with PBS, remove any remained PBS after the second rinse. Solubilize tissue in Lysis Buffer #12 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 #4.

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 [16] 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 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. 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, $P < 0.05$ was considered to be statistically significant.

RESULTS

Etanerceptreduced cardiac caspase 3:
At the end of the experiment, the level of myocardial Caspase 3 was significant ($p < 0.001$) 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 ($p < 0.001$) lower than that in the control group. Etanercept treated group showed a significant ($p < 0.001$) increase in the level of caspase 3 as compared with the sham group, **Fig. 4** and **Table 1**.

Etanercept increase cardiac Bcl-2:
At the end of the experiment, the level of myocardial Bcl-2 was significantly ($p < 0.001$) 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 ($p < 0.001$) higher than that in the control group.

Etanercept treated group showed significant (p <0.001) increase in the level of Bcl-2 as compared with the sham group, **Fig.5** and **Table 2**.

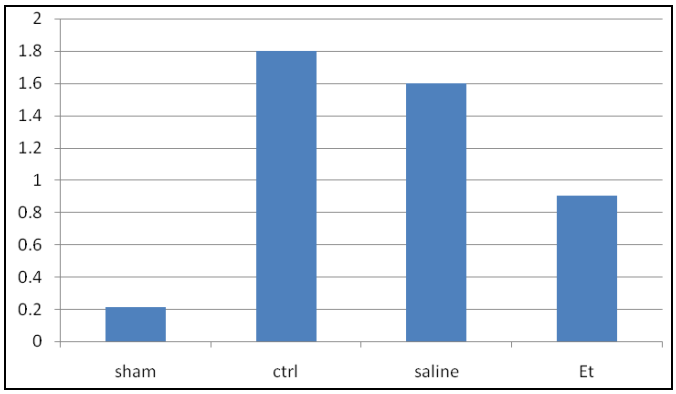


FIG. 4: THE MEAN OF CASPASE 3 (pg/ mg) IN THE FOUR EXPERIMENTAL GROUPS AT THE END OF THE EXPERIMENT.

TABLE 1: MYOCARDIAL CASPASE 3(pg/mg) OF THE FOUR EXPERIMENTAL GROUPS AT THE END OF THE EXPERIMENT (N = 6 IN EACH GROUP)

GROUP	Caspase 3 (pg/ mg)	P value
Sham	0.200 ± 0.24	
Control	2.05 ± 0.19	P<0.01*
Control vehicle (saline)	1.82 ± 0.66	
Etanercept	0.29 ± 0.20	P<0.01**

* Vs. sham group, **vs. Control group. The data expressed as mean ±SEM.

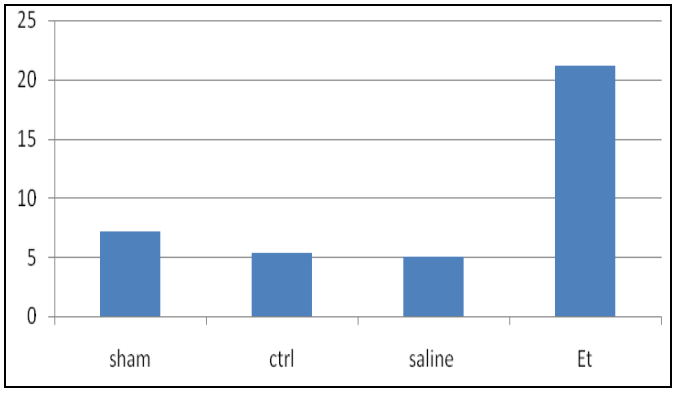


FIG.5: THE MEAN OF Bcl-2 (pg/ mg) IN THE FOUR EXPERIMENTAL GROUPS AT THE END OF THE EXPERIMENT.

TABLE 2: MYOCARDIALBCL-2(pg/mg) OF THE FOUR EXPERIMENTAL GROUPS AT THE END OF THE EXPERIMENT (N = 6 IN EACH GROUP)

GROUP	Bcl-2 (pg/mg)	P value
Sham	9.15 ± 0.51	
Control	6.17 ± 0.53	P<0.01*
Control vehicle (saline)	5.83 ± 0.54	
Etanercept	11.08 ± 0.44	P<0.01**

* Vs. sham group, **vs. Control group. The data expressed as mean ±SEM.

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 3**.

There was statistically insignificant difference between control vehicle 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 injury, 66.7% had severe cardiac injury and 16.7% had a high severe cardiac injury.

TABLE 3: THE DIFFERENCES IN HISTOPATHOLOGICAL SCORING OF ABNORMAL HEART CHANGES AMONG THE FOUR EXPERIMENTAL GROUPS

Histopathological Scoring	Study groups							
	Sham		Control		Control vehicle		Etanercept	
	N	%	N	%	N	%	N	%
Score 0 (no damage)	6	100	0	0	0	0	1	16.7
Score 1 (mild)	0	0	0	0	0	0	3	50
Score 2 (moderate)	0	0	1	16.7	1	16.7	1	16.7
Score 3 (severe)	0	0	4	66.7	4	66.7	1	16.7
Score 4 (high severity)	0	0	1	16.7	1	16.7	0	0
Total	6	100	6	100	6	100	6	100

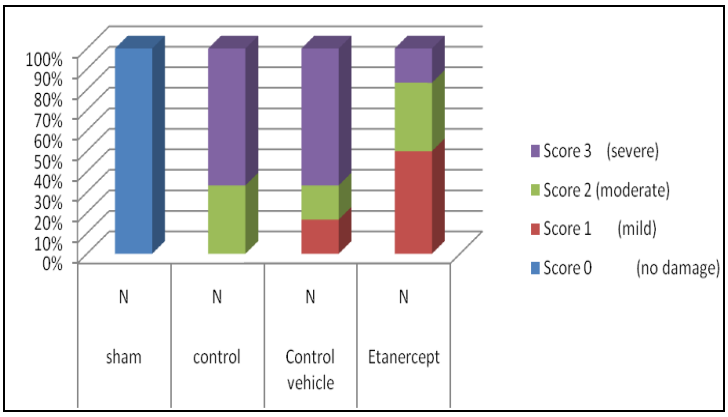
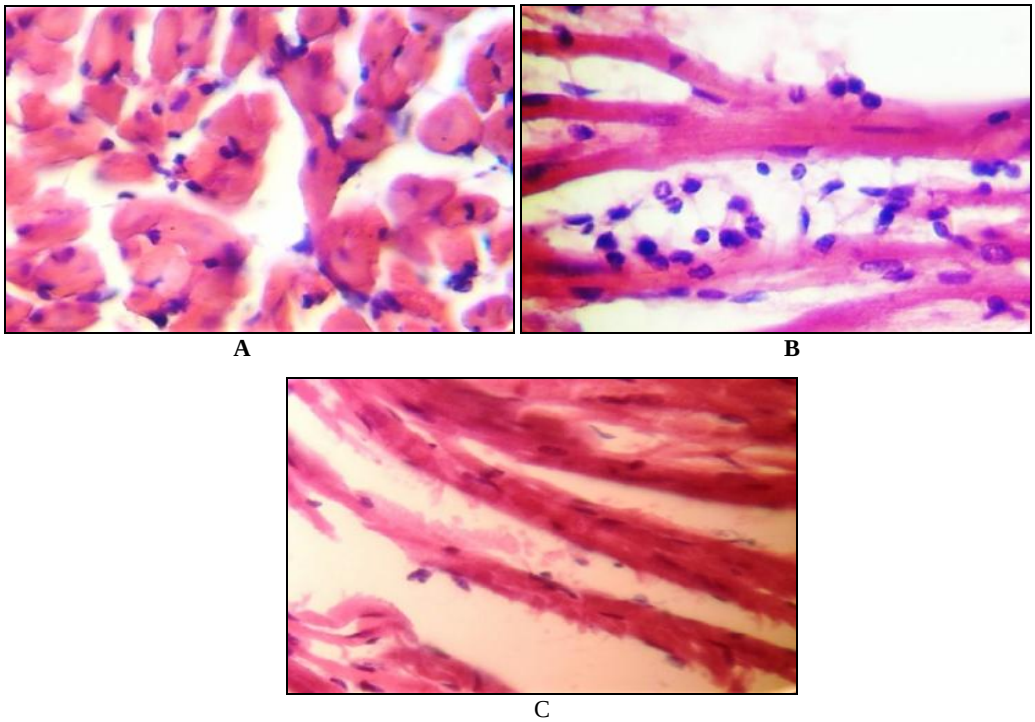


FIG.6: COMPONENT BAR CHART SHOWS THE RELATIVE FREQUENCY OF DIFFERENT HISTOPATHOLOGICAL GRADING OF ABNORMAL HEART CHANGES AMONG THE FOUR EXPERIMENTAL GROUPS



A **B**

C

FIG.7: 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 changes.

DISCUSSION*:* The major findings of the present study are caspase 3 and Bcl-2 play important role in the pathology of myocardial I/R. Etanercept treatment played a protective role against myocardial I/R injury. The protective effects of etanercept during myocardial I/R injury was attenuation apoptosis.

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 [17].

In ischemia-reperfusion (IR) injury, tumor necrosis factor (TNF) -α mediates apoptosis. A soluble TNF-αreceptor (Etanercept) has shown anti-inflammatory and anti-apoptotic effects in several animal models [18].

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 ischmia / reperfusion improve outcome of myocardial infarction patients [19]. 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 [15]. Also, (Genovese, Mazzon et al. (2006) found that etanercept significantly increases the level of spinal cord Bcl-2 expression [20]. 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 [21].

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 [15]. Also Genovese, Mazzon et al. 2006) **s**howed that when mice are treated with etanercept significantly reduced the degree of spinal cord inflammation and tissue injury histological score and neutrophil infiltration [20]. 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 [22]. 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 [21].

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