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The impact of Trimetazidine on patients' response in patients on EECP therapy

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ABSTRACT— The current study was designed to evaluate the effect of EECP therapy with or without trimetazidine(TMZ) in patients with refractory angina via modulating peripheral monocyte expression of Toll like receptor4 (TLR4) and its downstream signaling. A randomized double blind control trial in which 88 stable refractory angina patients allocated into two groups, Enhanced External Counter Pulsation (EECP) group: include 44 patients with stable refractory angina were treated with EECP-Therapy. TMZ-EECP group: include 44 patients with stable refractory angina which gave TMZ 35 mg twice daily in addition to EECP-Therapy. TLR4 levels were assayed in peripheral monocyte by flow cytometry and 8-iso-prostaglandin F2α (8-iso-PGF2 α), interleukin6 (IL6), high mobility group box-1 protein (HMG- BOX) and monocytes chemoattractant protein-1(MCP-1) were also measured beforethe EECP-therapy and before the TMZ giving to patients, and after 35 hours of EECP treatment (7 consecutive weeks).Inhibition in TLR4 expression in peripheral monocyte in both study groups but significantly lower expression level was observed among the TMZ-EECP group ($P<0.05$). Inflammatory cytokine (HMG Box-1 protein, MCP-1) were remarkably decreased in both study groups but (HMG Box-1 protein, MCP-1 and IL-6) significantly decreasing level was observed among the TMZ-EECP group ($P<0.05$). Also, the oxidative stress biomarker 8-iso-PGF2α were remarkably decreased in both study groups but significantly decreasing level was observed among the TMZ-EECP group ($P<0.05$). Timetizidine and EECP therapy decrease the release of HMGB-1 and also decreases the 8-iso-PGF2α serum level. Thus attenuate the TLR4 expression on peripheral monocytes which affect the downstream signaling of TLR4, resulting in decreasing the TLR4 downstream signaling inflammatory markers such as MCP-1 and IL6 levels which yield improvement in the quality of patient's life by decrease frequency of angina episodes, decreasing the Short-acting nitrate use and change the exercise tolerance and distance.

KEYWORDS: Timetizidine, Enhanced External Counter Pulsation

1. INTRODUCTION

Cardiovascular disease associated with death and disability remains as a serious medical problem. Cardiovascular disease approaching $450 billion a year in 2010 and projected to rise to over $1 trillion a year by 2030 (act as high direct and indirect medical care costs) this make it as a societal and critical medical issue [1]. Angina pectoris risk factors for consist of diabetes mellitus, early coronary artery disease family history, hypercholesterolemia, cigarette smoking, left ventricular hypertrophy, obesity, lipoprotein(a) serum level elevation and homocysteine, plasminogen activator inhibitor, serum triglycerides, or low high-density lipoprotein (HDL) [2].Enhanced external counter pulsation is a non-invasive therapy, for coronary artery disease (CAD) and for patients with refractory angina pectoris who fail to respond to standard revascularization procedures and aggressive pharmacotherapy. Data from the International Patient Registry (IEPR) demonstrate that angina episodes and nitrate usage decreased by the effect of EECP, and exercise tolerance increases in patients with refractory angina [3]. The anti-ischemic benefits occur early and are sustained up to 5 years in patients with a favorable initial response [4]. TLR4 causes intracellular signaling pathways activation, like nuclear factor-κB (NF-κB), which leads to increases in reactive oxygen species/

reactive nitrogen species (ROS/RNS) production and pro-inflammatory cytokines release. Therefore, this inflammatory pathway may be associated with a number of diseases such as clinical depression, chronic fatigue syndrome, rheumatoid arthritis and cardiovascular disorders [5]. The endogenous ligands of TLR4 are oxidized low density lipoprotein (oxLDL), Oxidized phospholipids, heat shock protein70 (HSP70) and HMGB1. Peter and Bobik represented the HMGB-1 as a pro-inflammatory cytokine, which act as TLR4 endogenous ligand [6]. In the human with atherosclerosis, lipopro-8-isoprostane plasma and urinary levels increased [7]. The IL-6 can be used as biomarkers for evaluating coronary heart disease severity and inflammatory response [8], MCP-1 serum levels seen to be high in the coronary artery of atherosclerotic patients, myocardial infarction and patients with heart failure [9]. TMZ is a clinically effective antianginal agent that has been used in the prophylaxis and management of angina pectoris. Unlike other classical antianginal drugs, TMZ displays anti-ischemic effects without inducing hemodynamic changes. TMZ is well tolerated and accompanied by minor side effects. Besides, it is freely soluble in water and has a relatively short half-life of 6 h [10]. The cardio protective effect of TMZ has not yet been fully understood; it has been attributed to its direct actions as cytoprotective, which cause a reduction in myocardial cell acidosis and calcium overload reduction, intracellular adenosine triphosphate (ATP) levels preservation, antioxidant capacity increases, and protection against oxygen-free radical induced toxicity. TMZ was shown to favorably alter the level of oxidative stress markers [11]. TMZ exerts a significant, nitric-oxide dependent, cardio protection against ischemia reperfusion injury and preserves the coronary endothelium. Preservation of the production of endothelial nitric oxide synthase and its bioavailability appears also to be a critical factor in the decrease in release of endothelin-1 and the preservation of endothelial function [12]. The pretreatment with TMZ showed decreases in level of nuclear factor kappa-BNF-kB and increase levels of nuclear factor erythroid 2- related factor 2-(Nrf2-) heme oxygenase-1 (HO-1) Nrf2/HO-1, suggesting that the TMZcardioprotective effect against exercise-induced myocardial injury might be achieved by oxidative stress and apoptosis reduction by NF-kB inactivation and promoting the activation of Nrf2/HO-1 signaling pathway [13].

2. Patients and method

2.1 Study design: prospective Randomized double blind Control trial

Eighty-eight patients with chronic stable refractory angina recruited from privet clinic of DR. Prof. Fadhil Ghali Yousif, Al-Najaf Center of Cardiac Surgery, during a clinical screening procedure performed by a cardiologist that is mandatory for all patients referred for EECP. The patients were divided them randomly into two groups after exclusion the patients who suffered from uncontrolled atrial fibrillation, decompensated heart failure, severe aortic insufficiency, severe peripheral arterial disease, severe hypertension, aortic aneurysm, venous disease, severe chronic obstructive pulmonary disease, eplipsy, patients whom already on Monoamine oxidase inhibitors (MAOIs) treatments and allergy of TMZ were excluded. EECP group: include 44 patients with stable refractory angina were treated with EECP-Therapy. TMZ-EECP group: include 44 patients with stable refractory angina which gave Trimetazidine 35 mg twice daily in addition to EECP-Therapy.

2.2 Blood samples collection

For each patient two blood samples were obtained. The first blood sample was taken before the EECP-therapy and before the TMZ giving to patients and the second blood sample was taken after 35 hours of EECP treatment (7 consecutive weeks). Five milliliters of blood drew from a peripheral vein in each case which divided into 2 ml of aspirated blood put in sterile Ethylenediaminetetraacetic acid (EDTA) bottles for flowcytometry analysis TLR4and 3 ml of blood centrifuged at 3000 ×g for 5 min to obtain serum that's kept in -80 C that to be used for the assay of 8-iso-PGF2a IL6, HMGBOX Protein, and MCP-1.

2.3 Patient satisfaction

Subjective evaluation of the Patient satisfactionwas based on the patients' response to a questionnaire administered before EECP and TMZ therapy and after 35 1-hour sessions of EECP therapy and treatment with TMZ. Patient satisfaction is a common used indicator for measuring the improvement in the quality of life, anginal pain, exertional dyspnea, Short-acting nitrate use, five-minute work and Exercise tolerance. Patient satisfaction affects clinical outcomes and determine whether the patients saw improvement, worsening, or no change.

2.4 Flow cytometic analysis

Bricyte E6 flow cytometic used for measuring TLR4 expression on peripheral monocyte cells. Florescent Peridinin chlorophyll protein (PreCP)TLR4 antibody used for blood sample staining at 4 C° for 45 minutes in dark environment. After that the RBC lysis buffer incubated with the mixture, then by using phosphate buffer the mixture washed, then using irrelevant Ecotype-matched control IgG as a control. The washed cells cell-associated fluorescence measured by Bricyte E6 flow cytometic (Mandray, China) and the data were analyzed by MR flow software.

2.5 ELISA technique

Sandwich enzyme immune assay was performed for measuring serum level concentrations of 8-iso- PGF2a, IL6, HMGBOX Protein, and MCP-1 using Elabscience Elisa kit. At room temperature, 100µl serum was added to each well and incubated for 1.5 hours. After that, the prepared biotinylated detection antibody as 100µl added to each well and then incubated at room temperature for 1 hour, aspirate and wash three times. HRP conjugated solution as 100 µl was added and then incubated for 30 minutes at room temperature aspirate and wash five times. Substrate reagent as 90 µl was added and incubated for fifteen minute in 37 degrees. Finally, added 50 µl from stop solution. Color intensity was measured at 450nm.

2.6 Statistical analyses

Statistical analyses were performed using statistical package for social science (SPSS) version 20. Categorical variables were presented as number and percentage. Chi-square test was used to express the association between categorical variables. Continuous variables were expressed as Mean ± stander error of the mean. Pairedt-test was used for comparison of means at a various time point in the same group. The independent sample t-test used for comparison between two groups. P-value
<0.05 was regarded as statistically significant.

3. Result

All the baseline parameter of the EECP-group and TMZ-EECP group are not significant statistically regarding gender, age, smoking, history of diabetes mellitus, hypertension, drugs intake, total cholesterol, renal function tests this details in table (1).

Table1 Demographic characteristics of participated patients

patients characteristics	TMZ-EECP	EECP	p
Male	40 (90.9%)	39 (88.6%)	N.S
Female	4 (9.1%)	5 (11.4%)	N.S
Age(years)	57.27±6.4	57.84±7.12	N.S
BMI Kg/m2	26.35±4.43	27.21±3.40	N.S
Smoker	1 (2.3%)	2 (4.6%)	N.S
X-smoking	12 (27.3%)	12 (27.3%)	N.S
Insulin	2 (4.6%)	3 (6.9%)	N.S
Oral hypoglycemic drugs	20 (45.5%)	14 (31.8%)	N.S
Aspirin	10 (22.7%)	9 (20.5%)	N.S
Clopidogril	8 (18.2%)	14 (31.8%)	N.S
B-blocker	3(6.9%)	6 (13.6%)	N.S
Angiotensin-converting enzyme inhibitors ACEI	7 (15.9%)	8 (18.2%)	N.S
Calcium channel blocker	4 (9.1%)	7 (15.9%)	N.S
Nitrate	10 (22.7%)	12 (27.3%)	N.S
Antihyperlipidemic drugs	6 (13.6%)	7 (15.9%)	N.S
Diuretics	5 (11.3%)	12 (27.3%)	N.S
Hypertension	24 (44.4%)	30 (55.6%)	N.S
Diabetes	36 (50%)	36 (50%)	N.S
B. urea mg/dl	44.75±13.961	45.82±7.146	N.S
B. sugar mg/dl	183.0357±17.708	175.2581±30.7	N.S
S. creatinine	0.968±0.0174	0.9621±0.111	N.S
Cholesterol	185.9286±40.295	166.9167±34.486	N.S
TG	167.55±25.129	180.5556±74.061	N.S
EF	51.42±6.619	51.9±9.939	N.S

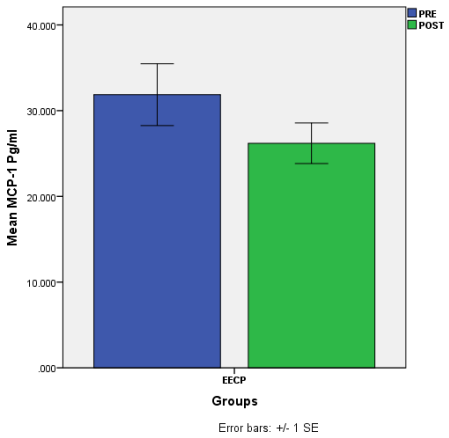

Figure (1): Effect of EECP-therapy on MCP-1 serum level, comparison between Pre-EECP therapy and Post-EECP therapy

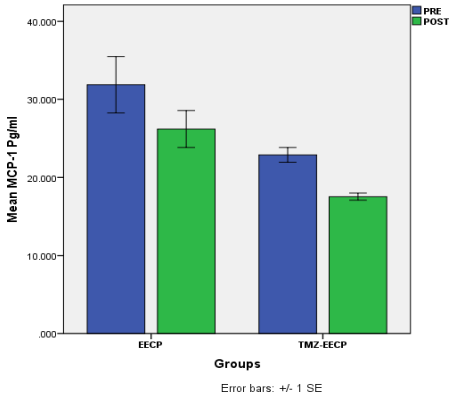

Figure (2): Effect of TMZ and EECP therapy on MCP-1 serum level, comparison between Post TMZ-EECP therapy and Post-EECP therapy

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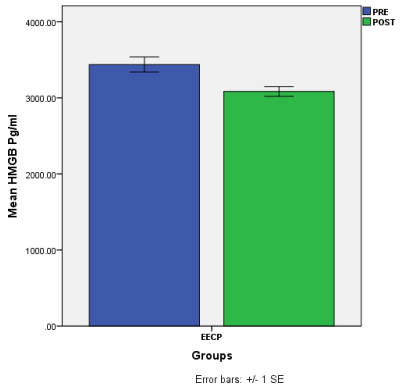

Figure (3): Effect of EECP-therapy on HMGBox-1protein serum level, comparison between Pre-EECP therapy and Post-EECP therapy

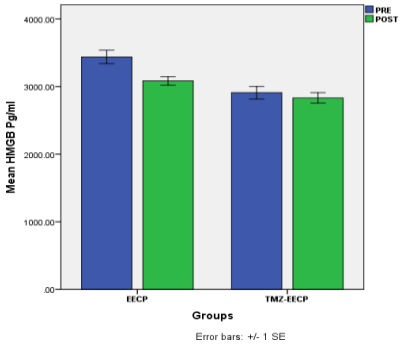

Figure (4): Effect of TMZ and EECP therapy on HMGBox-1protein serum level, comparison between Post TMZ-EECP therapy and Post-EECP therapy

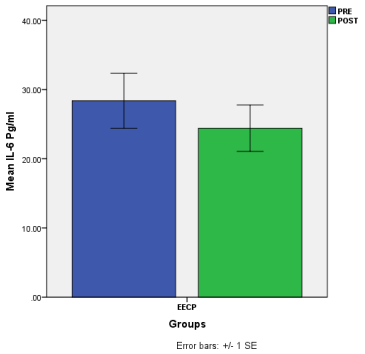

Figure (5): Effect of EECP-therapy on IL-6 serum level, comparison between Pre-EECP therapy and Post-EECP therapy

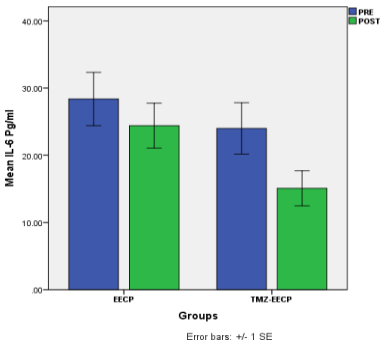

Figure (6): Effect of TMZ and EECP therapy on IL-6 serum level, comparison between Post TMZ-EECP therapy and Post-EECP therapy

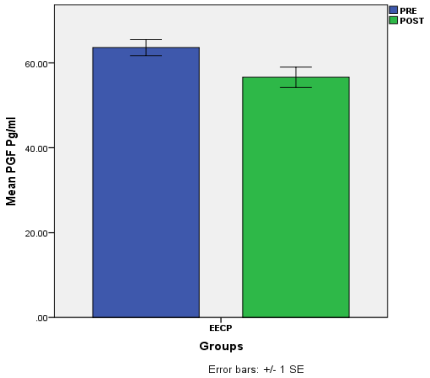

Figure (7): Effect of EECP-therapy on 8-iso-PGF2α serum level, comparison between Pre-EECP therapy and Post- EECP therapy

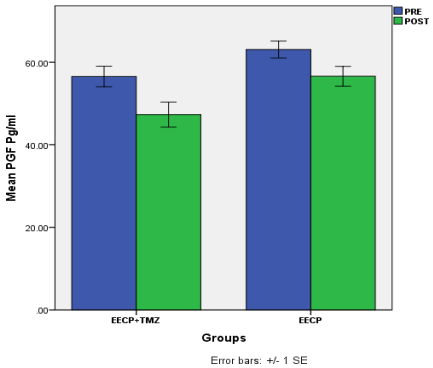

Figure (8): Effect of TMZ and EECP therapy on 8-iso-PGF2α serum level, comparison between Post TMZ-EECP therapy and Post-EECP therapy

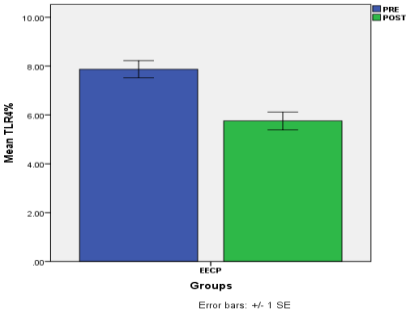

Figure (9): Effect of EECP-therapy on peripheral blood monocyte expression of TLR4, comparison between Pre-EECP therapy and Post-EECP therapy

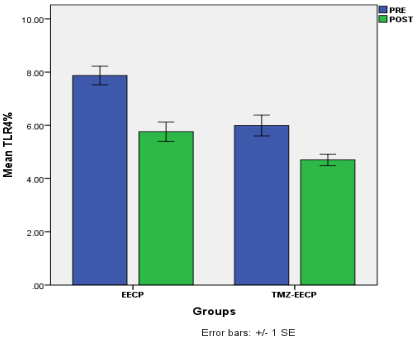

Figure (10): Effect of TMZ and EECP therapy on peripheral blood monocyte expression of TLR4, comparison between Post TMZ-EECP therapy and Post-EECP therapy

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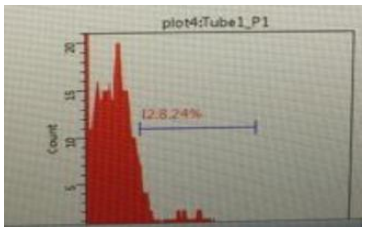

Figure (11): Toll like receptor 4 expression in peripheral monocytes in EECP-group,Pre-EECP therapy

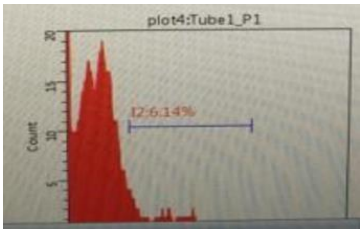

Figure (12): Toll like receptor 4 expressions in peripheral monocytes in EECP-group, Post-EECP therapy

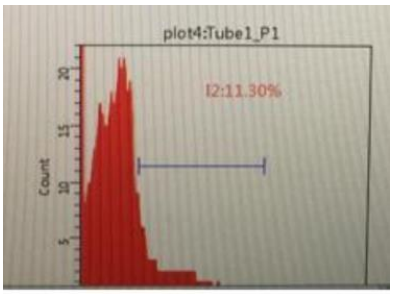

Figure (13): Toll like receptor 4 expressions in peripheral monocytes in TMZ-EECP group, Pre TMZ-EECP therapy

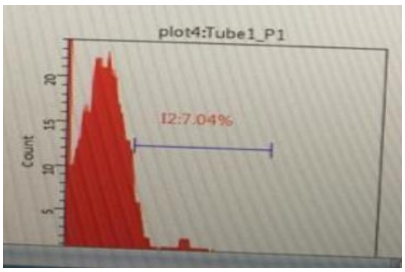

Figure (14): Toll like receptor 4 expressions in peripheral monocytes in TMZ-EECP group, Post TMZ-EECP therapy

4. Patient satisfaction

In our study, in EECP-group we found there was a 70.5% of patients satisfy to the (quality of life, anginal pain, exertional dyspnea, short acting nitrite use, five-minute work and exercise tolerance) after 35 1-hour sessions of EECP-therapy, and 88.6% of patients satisfy in TMZ-EECP group after TMZ 35mg twice a day with 35 1-hour sessions of EECP therapy. There was a significant difference between EECP-group and TMZ-EECP group (p-value = 0.034) in the patient's satisfaction.

4.1 Effect of EECP and TMZ therapy on MCP-1 serum level

In EECP-group, there was a significant decrease ($P<0.05$) in the serum level of MCP-1 after 35-1 hour sessions of EECP therapy (Post EECP therapy) in comparison with Pre-EECP therapy and these results

shown in Figure1. In TMZ-EECP group, there was a highly significant decrease (P<0.05) in the serum level of MCP-1 in post TMZ-EECP combination therapy in comparison with EECP-group (post-EECP therapy) and these results shown in figure (2).

4.2 Effect of EECP and TMZ therapy on HMGBox-1protein serum level

In EECP-group, there was a significant decrease (P<0.05) in the serum level of HMGBox-1protein after 35-1 hour sessions of EECP therapy (Post EECP therapy) in comparison with Pre-EECP therapy and these results shown in figure (3). In TMZ-EECP group, there was a highly significant decrease (P<0.05) in the serum level of HMGBox-1protein in post TMZ-EECP combination therapy in comparison with EECP-group (post-EECP therapy) and these results shown in figure (4).

4.3 Effect of EECP and TMZ therapy on IL-6 serum level

In EECP-group, there was a non-significant decrease (P<0.05) in the serum level of IL-6 after 35-1 hour sessions of EECP therapy (Post EECP therapy) in comparison with Pre-EECP therapy and these results shown in figure (5). In TMZ-EECP group, there was a significant decrease (P<0.05) in the serum level of IL-6 in post TMZ-EECP combination therapy in comparison with EECP-group (post-EECP therapy) and these results shown in figure (6).

4.4 Effect of EECP and TMZ therapy on 8-iso-PGF2α serum level

In EECP-group, there was a significant decrease (P<0.05) in the serum level of 8-iso-PGF2α after 35-1 hour sessions of EECP therapy (Post EECP therapy) in comparison with Pre-EECP therapy and these results shown in figure (7). In TMZ-EECP group, there was a highly significant decrease (P<0.05) in the serum level of 8-iso-PGF2α in post TMZ-EECP combination therapy in comparison with EECP-group (post-EECP therapy) and these results shown in figure (8).

4.5 Effect of EECP and TMZ therapy on peripheral blood monocyte expression of TLR4

In EECP-group, there was a significant decrease (P<0.05) in the peripheral blood monocyte expression of TLR4 after 35-1 hour sessions of EECP therapy (Post EECP therapy) in comparison with Pre-EECP therapy and these results shown in figures (9,11,12). In TMZ-EECP group, there was a significant decrease (P<0.05) in the peripheral blood monocyte expression of TLR4 in post TMZ-EECP combination therapy in comparison with EECP-group (post-EECP therapy) and these results shown in figures (10,13,14).

5. Discussion

Enhanced external counter pulsation therapy increases venous return, raises cardiac preload, increases cardiac output, and decreases systemic vascular resistance. EECP therapy affect as anti- inflammatory [14-15]. Trimetazidine by antioxidant effects its protect tissue from free radicals and limit the reactive oxygen species inducing membrane damage. It has been suggested that the proinflammatory mediators such as C-reactive protein (CRP), tumor necrosis factor alpha (TNF-a), interleukin 1 (IL-1), and IL-8 release from macrophages in inflammation and ischemia enhanced by reactive oxygen species–mediated and nitric oxide (NO)-mediated damage [16,17]. In this prospective randomize study, in EECP-group we found that, there was a significant decrease (P<0.05) in the serum level of MCP-1 in the post-EECP therapy in comparison with a pre-EECP therapy, thus agreed with (Braith et al. 2010), who observed that after 35 sessions of EECP treatment, there was a reduction in plasma levels of TNF-α, hsCRP and MCP-1 [18-19]. Serum levels of MCP-1 seen to be high in the coronary artery of atherosclerotic patients, myocardial infarction and patients with heart failure [9]. MCP-1 expression from macrophages, vascular smooth muscles cell, and endothelial cells induced by oxidized-LDL, this expression is level and time-dependent manner [20]. The EECP anti-inflammatory action mechanism is likely associated with the intermittent bouts of shear stress created with

each inflation/deflation cycle of the cuffs. The endothelial-derived nitric oxide (NO) synthesis and release stimulated by shear stress [21]. In addition to NO vasodilatation effect, it also had an anti-inflammatory and ant atherosclerotic role by reducing the expression of VCAM-1 and inhibiting the MCP-1 expression [22]. In this study, we also found there is a highly significant decrease ($P<0.05$) in the serum level of MCP-1 in the post TMZ-EECP group in comparison with a post EECP-group, thus due to trimetazidinecardioprotective effect such as oxidative stress reduction, which reduces lipid oxidation, then inhibits monocyte/macrophage stimulation for chemokine and inflammatory cytokines production [23]. Also, TMZ reduces the inactivation rate of NO by enhancing the endothelial function, NO serve an anti-inflammatory and anti-atherosclerotic role by inhibiting the MCP-1 expression and reducing the expression of VCAM-1[24]. Finally, TMZ decreases the vascular cell adhesion molecules-1 and MCP-1 by its inhibitory effect on NF-kB [25]. In the EECP-group of the present study we found there was a non-significant decrease ($P<0.05$) in the serum level of IL-6 in the post-EECP therapy in comparison with a pre-EECP therapy but there was a significant decrease in the serum level of IL-6 in post TMZ-EECP group in comparison with post EECP-group and this agreed with Kuralay et al. which supposed that the trimetazidine inhibits the inflammatory markers like nitric oxide products (nitrite and nitrates), IL-1, IL-6 and TNF alpha [16]. Direct pro and anti atherogenic effects of IL-6 may be associated with atherosclerosis progression and development [26]. Zou et al improved the anti-inflammation, anti-oxidation and anti-apoptosis effect of trimetazidine, and explained the cardio protective effect of TMZ by its role on myocardial metabolism and energy production which control the myocardial fibrosis and cardiomyocyte apoptosis [27]. In the current study there was a significant decrease ($P<0.05$) in the serum level of HMGB1in the post-EECP therapy in comparison with a pre-EECP therapy and a highly significant reduction in serum level of HMGB1 in post TMZ-EECP group in comparison with post-EECP treated group. The correlation between HMGB-1 and coronary heart disease improved by Yao et al. which found that, there was a positive correlation between HMGB-1 expression and inflammatory cytokines like hs-CRP, TNF-an and IL-6 in coronary artery disease patients [28]. The anti-inflammatory effects of EECP may be clinically relevant with regard to decreasing the risk for future cardiovascular events in this patient population [29]. Wang et al. improved that, the Nrf2/HO-1 pathwayplays an important role in the HMGB1 secretion, which explained the HMGB-1 release inhibition due to increase expression of HO-1. Nrf2/HO-1/HMGB-1 axis represented as cardio protective [30]. Zhang et al. explained the cardio protective effect of TMZ, which suggested that, the TMZ increased levels of Nrf2/HO-1 and decreased nuclear NF-kB level [25]. In this study 8-iso-PGF2 serum level significantly decrease ($P<0.05$) in post-EECP therapy in comparison with a pre-EECP therapy, these results were in agreement with Braith et al.2010, which observed that after 35 sessions of EECP treatment, the 8-iso-PGF2a serum level decreased.

Also there was a highly significant reduction in serum level of 8-iso-PGF2a in post TMZ-EECP group in comparison with post-EECP group. 8-iso-PGF2 represented as the most valid plasma marker to assess the systemic oxidative stress which is a potent vasoconstrictor and it associated with CAD [31]. Belardinelli et al reported that in patients with ischemic cardiomyopathy, the endothelium dependent relaxation improved by TMZ, thus related to antioxidant properties [32]. In our randomized study the peripheral blood monocyte expression for TLR4 significantly decreased ($P<0.05$) in the post-EECP therapy in comparison with a pre-EECP therapy and also there was a significant reduction in peripheral blood monocyte expression of TLR4 in post TMZ-EECP group in comparison with post-EECP group. In atherosclerosis observed that the TLR4 expression increase after oxLDL stimulation [33]. TLR4 causes intracellular signaling pathways activation, like nuclear factor-κB (NF-κB), which lead to increases in ROS/RNS production and pro-inflammatory cytokines release. Therefore, this inflammatory pathway may be associated with a number of diseases such as clinical depression, chronic fatigue syndrome, rheumatoid arthritis and cardiovascular disorders [34]. The endogenous ligands of TLR4 and TLR2 are oxLDL, Oxidized phospholipids, HSP70 and HMGB1 [6].

Braith et al. explained the EECP effects on lipid peroxidation, which observed that the 8-iso-PGF2a plasma levels decreased after 35 sessions of EECP treatment [18]. Trimetazidine suppress inflammatory markers elevation such as CRP, TNF-a and also significantly lowering the levels of IL-6 [16,15]. Trimetazidine inhibits the production of deleterious lipid metabolites and it can also reduce mitochondrial damage, inhibiting mitochondrial permeability transition-pore opening [23]. TMZ and EECP both of them had anti-inflammatory and antioxidant effects through hemodynamic effect and increase endothelial nitric oxide and through the regulation of Nrf2/HO-1 pathway which affects the HMGB-1 level, thus, attenuate the TLR4 expression on CD14 monocytes in the present study patients.

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