The Effect of Phosphodiesterase-type 5 Inhibitor, Sildenafil and Vitamin E on Isoprenaline-induced Myocardial Infarction in Male Rats

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Abstract:

Background and Aim: Despite great efforts during the last decades, acute myocardial infarction (MI) contributes to significant mortality statistics with growing yearly prevalence. The cardioprotective effect of the phosphodiesterase5-inhibitor, Sildenafil (Sil) against MI and its complications stills a matter of debate and controversy. So, we aimed to investigate the impact of Sil pretreatment on the outcome of isoprenaline (ISO)-induced infarct-like lesions and comparing it to that of vitamin E (Vit.E). Methods: 5 groups of rats were conducted; Control and ISO-injected group (75mg/kg; i.p) to induce infarct-like lesion in addition to 3 groups that were pretreated with either oral Sil (10mg/kg/day) and/or oral Vit.E (100IU/kg/day) for 3 weeks before injection of ISO. Results: the preconditioning with Sil or Vit.E significantly ameliorated ECG alterations, decreased serum levels of the cardiac enzyme CK-MB, and reduced cardiac injury score with no evident necrotic changes on histopathological examination that were observed in the ISO-induced MI. These changes were accompanied by significant increases in the cardiac SOD content and decreases in MDA content in addition to reduced immunohistochemical expression of transforming growth factor β (TGFβ) that plays an outstanding role in post-infarct remodeling. **Conclusion:** Sil and/or Vit.E exerted a prophylactic effect against MI and post-infarct remodeling, at least in part, through preserving the cardiac redox balance and antifibrotic effect by lowering TGFβ.

Key words: Myocardial infarction; Sildenafil; Vitamin E; TGFβ; Oxidative stress.

Abbreviations: CK-MB; Creatine Kinase–MB, CVD; cardiovascular disease, ISO; Isoprenaline, MI; Myocardial infarction, MDA; malondialdehyde, PDE5; Phosphodiesterase-5, Sil; Sildenafil, SOD; superoxide dismutase, TGFβ; Transforming growth factor β,Vit.E; Vitamin E.

Introduction

Myocardial infarction (MI) refers to an irreversible death, necrosis, of the myocardium caused by prolonged imbalance between myocardial demands and coronary blood delivery. It thus results in mechanical, electrical, and structural alterations [1]. MI is becoming a worldwide cause of morbidity and mortality of cardiovascular disease (CVD) in developed as well as in developing countries [2]. In Egypt, the prevalence of Ischemic heart disease is 8.3% [3].

The development of MI is complex and basically involves oxidative stress that stems from an imbalance between free radicals and anti-oxidant enzymes [4]. Another exacerbating factor in MI pathogenesis and complications is the multifunctional cytokine, transforming growth factor β (TGFβ) [5]. In the heart, it is released from cardiomyocytes, endothelial cells, and others. When activated by free oxygen radicals, TGFβ causes myocyte apoptosis and reduces its proliferation [6]. Additionally, it promotes secretion of numerous extracellular matrix proteins including; collagens, fibronectin, and proteoglycans [7]. Subsequently, it plays a key role in post-infarct remodeling in which the necrotic myocardial cells are compensated through fibrosis causing adverse myocardial structures with pump failure [6].

In this context, alternate pharmacological approaches could be introduced to lessen oxidative stress and prevent molecular damage in the heart. With discovery that phosphodiesterase-5 (PDE5) is expressed in the myocardium and upregulated in cardiac hypertrophy and heat failure [8], attention has been moved towards direct myocardial effects of PDE5-inhibitors. The highly specific and commonly available PDE5-inhibitor is Sildenafil (Sil) that sold as Viagra. In addition to erectile dysfunction and pulmonary hypertension [9], latest data have indicated a possible use of Sil in several experimental models of diseases; atherosclerotic mice model [10], ischemia-reperfusion renal injury [11], stenotic kidneys with renovascular hypertension [12], in a rat model of sepsis [13] in addition to obesity and type 2 diabetes [14]. However, the Sil's prophylactic impact against MI stills a matter of debate and controversy in addition to paucity of data concerning Sil's efficacy on cardiac TGFβ expression in the setting of MI. So, we aimed to illustrate this issue.

Vitamin E (Vit.E) is the most abundant and important lipophilic radical-scavenging

antioxidant. It is incorporated into cell membranes and therefore, protecting them from oxidative damage [15]. It is vital for good health and reported to be effective for the primary and secondary prevention of CVD [16]. Subsequently, this study was designed to answer the question concerning the prophylactic impact of the PDE5-inhibitor, Sil against experimentally-induced infarct-like lesions in male rats, and also to compare it to the well-known antioxidant Vit.E if found. ECG monitoring, serum levels of a cardio-specific enzyme, histopathological examination, cardiac redox status, and TGFβ immunohistochemical expressions were assessed to explore such effect.

Materials and method

Animals:

This prospective study was conducted on 30 adult male albino rats, 6-8 weeks old, weighing between 200±20 g. They were obtained from the Experimental Animal Unit of Faculty of Agriculture, Benha University, Egypt. The rats had free access to water and diet. They were hosted in metallic cages (3 per cage), at the prevailed room temperature, 25 ±1°C with a 12:12-h light/dark cycle all through the experiment. The animals were acclimatized to the laboratory conditions for 10 days prior to the initiation of the experiment. This experiment was performed at Physiology department, Faculty of Medicine, Benha University. The protocol was revised and approved by the Ethical Committee of Animal Experiments, Faculty of Medicine, Benha University, Egypt.

Drugs

Isoprenaline hydrochloride (ISO) purchased from (Sigma–Aldrich, USA Lot NO: 5984-95-2). The PDE5-inhibitor, Sildenafil citrate (Viagra)® tablets purchased from (Pfizer, Egypt, Lot NO:0069-4220-30). Vit.E soft gelatinous capsule purchased from (Pharco Company for Pharmaceutical, Egypt, Lot NO: 00904-0274-60). Urethane purchased from (Sigma-Aldrich, USA, Lot NO: 51-79-6)

Experimental groups and procedure:

The rats were classified into 5 equal groups (n=6) as follow;

Control group; formed of normal rats that received oral gavage distilled water for 3 weeks, MI group; formed of rats that received ISO (75 mg /kg; i.p; once) to induce infarct-like lesion [17], Sil +MI group; formed of rats pretreated with oral gavage Sil dissolved in distilled water (10 mg/kg/day) for 3 weeks then injected with ISO [18], Vit.E +MI group; formed of rats pretreated with oral gavage Vit.E dissolved in olive oil (100 IU/kg/day) for

3 weeks then injected with ISO [19], and Sil+ Vit.E+ MI group; formed of rats received oral gavage Sil and Vit.E as described previously for 3 weeks then then injected with ISO.

On the 22nd day and 2 hours after ISO injection, the rats were anaesthetized by urethane (1.25g/kg; i.p) for ECG monitoring [20]. Thereafter, blood samples were collected via cardiac puncture and allowed to clot at room temperature then centrifuged at 3000 rpm for serum preparation. The sera were then separated and stored at -20ºC for biochemical analysis of the cardio-specific enzyme creatin kinase-MB. The rats were then sacrificed by decapitation and their hearts were rapidly isolated and divided into two halves; one for cardiac super oxide dismutase and malondialdehyde assay, that was immediately washed with normal saline, kept in liquid nitrogen, and stored at -20° C while, the other half was kept in formaldehyde for histopathological and immunohistochemical analysis.

ECG Monitoring

The anesthetized rats were placed in the supine position on a board and ECG was traced continuously by means of needle electrodes. These electrodes were inserted subcutaneously into the paw pads of the rat and connected to Software Lab Chart 8 power lab recorder and analyzer (AD Instruments, CA, USA). For each rat, Lead II was recorded being the most informative one (right forelimb to left hind-limb) [21]. ECG tracing was analyzed for Q, R, T waves and heart rates.

Serum creatin kinase-MB (CK-MB) measurement:

The cardiac specific enzyme CK-MB kits (Spinreact Company, Spain, Cat. No: 9001-15-4), were used to assess the serum levels of CK-MB according to manufacturer instructions.

Cardiac superoxide dismutase (SOD) and malondialdehyde (MDA) assay:

Portions of the myocardium were homogenized in a saline solution (0.9%) and centrifuged at 3000 rpm for 15 min; the supernatant was kept at –20 o C. The antioxidant SOD kits (Kamiya Biomedical Company, U.S.A, Cat. No. KT-745) and the MDA kits (Cambridge, UK, Cat. No.ab118970) were used to assess the cardiac content according to the enzymatic colorimetric assay method [22, 23] respectively.

Histopathological examination of the cardiac tissue:

Cardiac tissue sections were dehydrated in a series of alcohols, 70% to 95% to 100%, then cleared in xylene for 15 min followed by

embedding in paraffin wax for 1 h. Sections were cut at 4 μm thickness using a HistoRange microtome (model: LKB 2218, LKB-Produkter AB, Bromma, Sweden) and stained with hematoxylin and eosin [24]. Heart tissue sections were examined under light microscope (Olympus BX50, Tokyo, Japan) by a blinded pathologist. The cardiac histopathological injury assessed according to the following score (0): nil, (1): mild, small multifocal degeneration, (2): moderate myofibrillar degeneration, (3): severe necrosis [21].

Immunohistochemical analysis of cardiac TGFβ:

The TGFβ primary antibodies (Abcam biochemical, UK) and Goat anti-mouse peroxidase-conjugated secondary antibody (Glostrup, Denmark), were used to assess the TGFβ expression in accordance with the manufacturer's instructions. Sections were assessed using light microscopy (Olympus Corporation, Tokyo, Japan). The expression score of TGFβ was assessed according to the degree of cellular brown staining (1): weak, (2): moderate, (3): strong [25].

Statistical analysis:

The collected data were summarized in terms of mean ± Standard Deviation (SD). Comparisons between the different study groups were carried out using the one-way analysis of variance (ANOVA) followed by post hoc tests using the LSD method using the Statistical Package for Social Science (SPSS) program, version 19 (Chicago IL USA, 2000). P value < 0.05 was considered statistically significant.

Results

Effect of ISO injection, Sil and Vit.E pretreatment on ECG tracing (Fig. 1&2)

ISO injection to induce infarct-like lesion showed ST segment elevation, significant Q wave amplitude increase and R wave amplitude decrease, T wave inversion with increased amplitude in addition to significant increases in heart rate when compared to the control group ($P<0.05$).

On the contrary, rats pretreated with Sil for 3 weeks then injected with ISO showed reduction in the ST segment elevation and significant decrease in Q wave amplitude, an increase in R wave amplitude, and a positive T wave with significant decrease in the heart rate as compared to the MI group ($P<0.05$). These findings were non-significantly changed when compared to their corresponding in the Vit.E-pretreated group ($P<0.05$). Moreover, the combined Sil and Vit.E-pretreatment preserved

the normal pattern of ECG wave amplitudes and heart rate following ISO injection.

- Effect of ISO injection, Sil and Vit.E pretreatment on the serum CK-MB levels (Fig. 3A)

The level of cardiac specific enzyme CK-MB was significantly increased in the serum of ISO-injected group when compared to the control group ($P<0.05$). Conversely, Sil-pretreatment resulted in a significant reduction in CK-MB serum levels when compared to the MI group. Moreover, this reduction was non-significantly changed when compared to its corresponding in the Vit.E pretreated group ($P<0.05$). Additionally, Both Sil and Vit.E pretreatment produced further significant decrease in the serum CK-MB levels when compared to the MI group ($P<0.05$) and were neat to normal values.

- Effect of ISO injection, Sil and Vit.E pretreatment on cardiac histopathological findings (Fig. 4)

Histopathological examination of the cardiac tissue in the ISO-injected group revealed existence of necrotic areas, sever hydropic degeneration, inflammatory cellular infiltrate, and interstitial edema with an evident thrombus in coronary blood vessel concomitantly with significant increase in the cardiac injury score ($P<0.05$) when compared to the control group (Fig. 4B, F).

Conversely, rats pretreated with Sil for 3 weeks then subjected to infarct-like lesion by ISO showed improvement of histopathological findings in the form of mild hydropic degeneration without necrotic changes or cellular infiltration concomitant with significant decrease in the cardiac injury score when compared to the MI group ($P<0.05$) (Fig. 4 C, D, F). In additional, similar histopathological findings were noticed in the Vit.E-pretreated group with non-significant change in the cardiac injury score when versus Sil-pretreated group ($P<0.05$). Moreover, when rats pretreated with both Sil and Vit.E, normal myocardial striation with no pathological changes observed (Fig. 4 E, F).

- Effect of ISO injection, Sil and Vit.E pretreatment on the cardiac redox status (Fig. 3B) and TGFβ expression (Fig. 5)

The cardiac content of the MDA was significantly increased concomitantly with significant decreases in SOD levels in ISO-injected group with respect to the control group ($P<0.05$). Conversely, rats pretreated with either Sil and/or Vit.E then subjected to infarct-like lesion showed significant drop in the cardiac MDA levels while significant rise in the cardiac SOD contents in comparison to the

MI group (P<0.05). Noteworthy, the cardiac contents of both MDA and SOD were non-significantly changed in the Vit.E pretreated group when compared to the Sil pretreated group and in the combined pretreated group versus the control group (P<0.05) (Fig. 3B).

- Effect of ISO injection, Sil and Vit.E pretreatment on the cardiac TGFβ expression (Fig. 5)

As regard immunohistochemical expression of the cardiac TGFβ, ISO-injected group exhibited strong positive TGFβ-expressing cells in the cardiac tissue with significant rise in the TGFβ-expression score when compared to the control group (P<0.05) (Fig. 5 B, F). On the other hand, pretreatment by either Sil or Vit.E led to weak positive TGFβ-expressing cells with significant decline in the TGFβ-expression score in comparison to the MI group (P<0.05). Moreover, there was a non-significant change in the Sil-pretreated group when compared to the Vit.E-pretreated group (P<0.05) (Fig. 5 C, D, F). Additionally, rats received both Sil and Vit.E showed negative immunohistochemical staining of TGFβ and significant decreases in TGFβ-expression score to near normal values when compared to the MI group (P<0.05)(Fig. 5E,F).

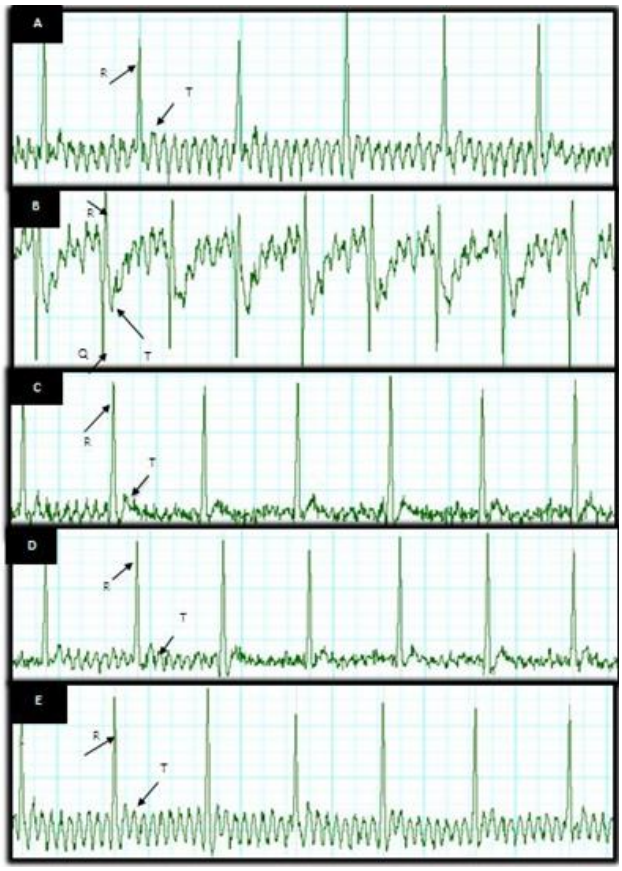

Fig.1 ECG tracing in the experimental groups. Lead II trace showing ECG changes in different experimental groups; (A) Control group showing normal pattern ECG (B) MI group showing deep Q, short R and inverted T waves with ST segment deviation (C) Sil+ MI group showing nearly normal Q, R and T waves (D) Vit.E+ MI group showing nearly normal Q, R, and T waves. (E) Sil+ Vit.E+ MI group showing normal pattern Q, R, T waves. MI; myocardial infarction, Sil; sildenafil, Vit.E; vitamin E. Width (Px): 421, Height (Px): 584Color Depth

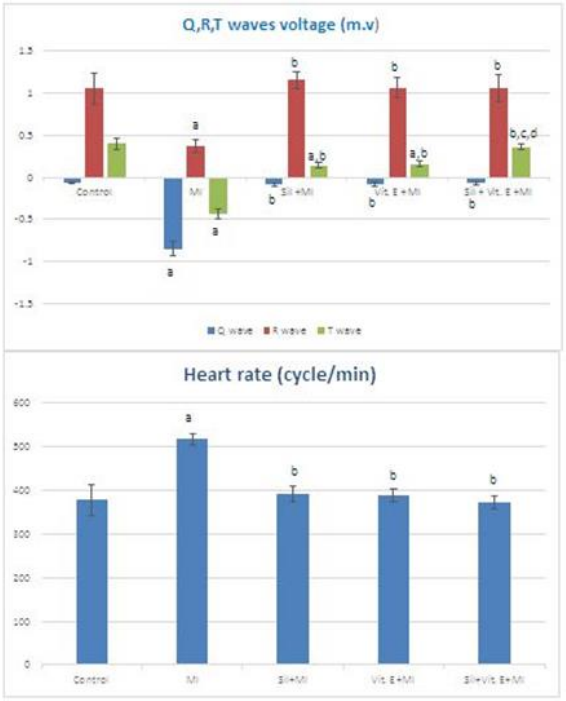

Fig.2 Q,R,T waves' voltage (m.v) and Heart rate (cycle/min) in the experimental groups. Data are expressed as mean ± standard deviation (SD); n = 6; P value = probability of chance, P < 0.05 is significant tested by one-way analysis of variance (ANOVA) and post hoc multiple comparison LSD method. a P < 0.05 vs. control group, b P < 0.05 vs. MI group, c P < 0.05 vs. Sil +MI group, d P < 0.05 vs. Vit.E +MI group. MI; myocardial infarction, Sil; sildenafil, Vit.E; vitamin E Width (Px): 408, Height (Px): 500 Color Depth

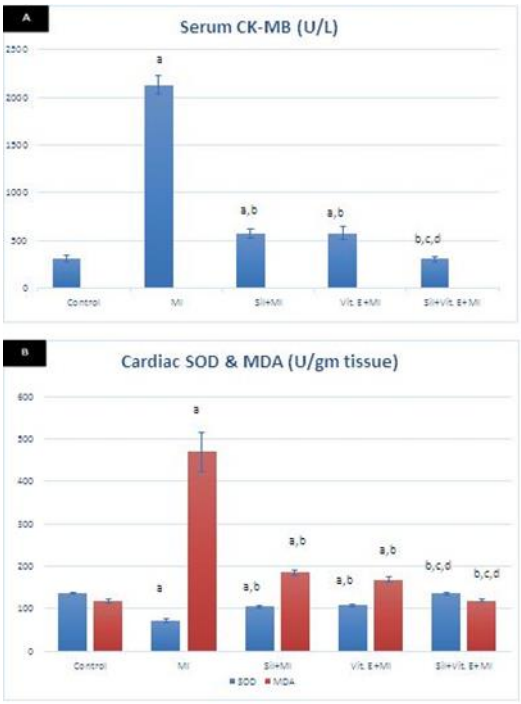

Fig.3 Serum CK-MB (U/L) and Cardiac SOD and MDA (U/gm tissue) contents in the experimental groups. Data are expressed as mean ± standard deviation (SD); n = 6; P value = probability of chance, P < 0.05 is significant tested by one-way analysis of variance (ANOVA) and post hoc multiple comparison LSD method. a P < 0.05 vs. control group, b P < 0.05 vs. MI group, c P < 0.05 vs. Sil +MI group, d P < 0.05 vs. Vit.E +MI group. MI; myocardial infarction, Sil; sildenafil, Vit.E; vitamin E Width (Px): 396, Height (Px): 535 Color Depth

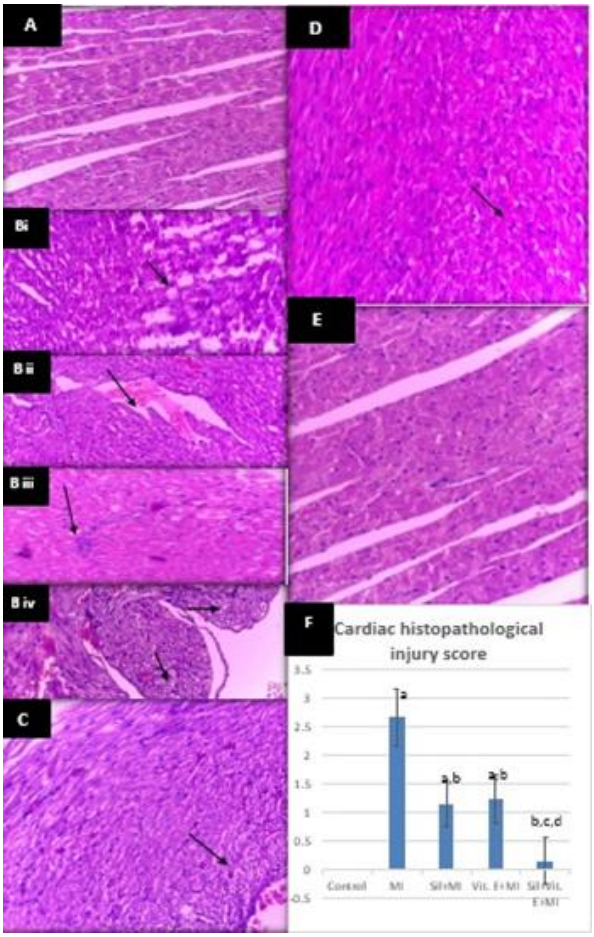

Fig. 4: H&E-stained sections and score of cardiac injury in experimental groups.(A); Control group:normal myocardium, (B); MI group:i) Necrosis, ii)Coronary dilatation with thrombus,iii)inflammatory cells,iv)Marked hydropic degeneration and edema (C); Sil+MI group showing mild hydropic degeneration, (D); Vit.E+MI group:mild hydropic degeneration, (E); Sil+Vit.E+MI group:quite normal myocardium(X 200). (F);histopathological cardiac injury score, data are expressed as mean ± standard deviation. a P<0.05 vs. Control, b P<0.05 vs. MI group, c P<0.05 vs. Sil+MI group, d P<0.05 vs. Vit.E+MI group. Width (Px): 331, Height (Px): 520 Color Depth

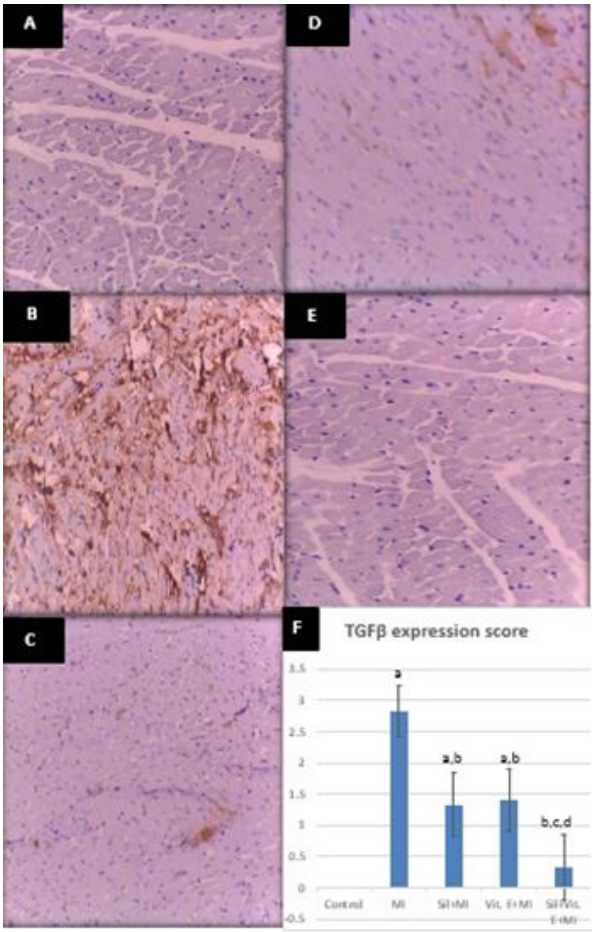

Fig.5: Immunohistochemical staining of cardiac tissue for TGFβ expression and its scoring in experimental groups. (A)Control group:negative TGFβ expression(B);MI group: strong positive TGFβ expression(C); Sil+MI group: weak positive TGFβ expression(D); Vit.E+ MI group: weak positive TGFβ expression (E); Sil+Vit.E+MI group: negative TGFβ expression(X 200).(F); TGFβ expression score, data are expressed as mean ± standard deviation. a P<0.05 vs. control group, b P < 0.05 vs. MI group, c P<0.05 vs. Sil +MI group, d P<0.05 vs. Vit.E +MI group. MI; myocardial infarction, Sil; sildenafil, Vit.E; vitamin E

Discussion

Despite great efforts during the last decades, the annual incidence of MI is nearly 6 million people worldwide and the fatal outcome happens in 25% of cases or even more according to the World Health Organization's estimates [26] thus, searching for prophylactic agents has gained great interest

ISO is a widely used medication to induce infarct-like lesions in animals. It is a very potent β adrenergic receptor agonist. It rapidly stimulates cardiac β1 and β2 receptors, inducing positive inotropic and chronotropic effects (β1 activity) and hypotension (β2 activity) resulting in an increase of oxygen demand of cardiac muscle concomitantly with reduced delivery causing myocardial injury [27].

In the current study ISO injection was seen to cause significant alterations in the ECG pattern presented by elevation of ST-segment and significant deep Q, short R, inverted T waves in addition to tachycardia. These ISO-induced ECG alterations were in agreement with that reported by previous study [21] and used for diagnosis of infarct-like lesions.

An increase in the myocardial-specific enzyme CK-MB in the serum is considered a hallmark for acute myocardial infarction [27]. When myocardial cells are metabolically damaged, due to a deficiency in the oxygen supply or glucose, the cardiac membrane becomes permeable or may entirely ruptures leading to the leakage of enzymes into the extracellular fluid [28]. In our study, marked elevation in the CK-MB levels in serum of ISO-injected group was found.

In agreement with those ECG alterations and biochemical rise in CK-MB serum levels, histopathological examination of the cardiac tissue revealed obvious necrotic areas, sever hydropic degeneration, inflammatory cellular infiltrate with interstitial edema and significant increase in the cardiac injury score.

Based on the aforementioned data, our findings revealed the efficacy of ISO injection to induce infarct-like lesion at all electrical, biochemical, and histopathological levels and are in agreement with those of other investigators [29, 30].

Oxidative stress plays a key role in mediating myocardial tissue damage following an ischemic event [4]. In the current study, ISO produced an increase in the cardiac MDA content concomitantly with reduced the cardiac SOD content. In addition to its potent β-receptors agonistic activity, ISO upon injection

undergoes auto-oxidation resulting in generation of free radicals that cause lipid peroxidation and destruction of the myocardial cell membrane with depletion of antioxidants [31]. Our results were in agreement with that reported before [4].

Free oxygen radicals, as they are found in myocardial infarction, can induce TGFβ activation [6]. It thus, explains the augmented TGFβ expression score of the infarcted myocardium in the ISO-injected group. These results were consistent with others that reported acute MI is associated with TGFβ over expression [32, 33].

To explore the impact of the PDE5-inhibitors in the setting of MI, administration of Sil for 3 weeks before induction of infarct-like lesion was performed. A preventable effect on ISO-induced cardiac injury was found as evidenced by improved ECG parameters. Additionally, pre-treatment with Sil lowered serum levels of the cardiac enzyme CK-MB indicating that Sil helps in maintaining the membrane integrity, thereby restricting the CK-MB leakage. Moreover, this goes hands with the histopathological findings that revealed lowered cardiac injury score with no necrotic changes.

Rising evidences from animal models support our findings regarding the cardioprotective action of PDE5-inhibitors [34, 35] in addition to double-blind placebo-controlled trials that reported PDE5-inhibitors have proven safety profiles with low incidence of adverse cardiovascular events [36].

To understand the possible mechanism underlying the prophylactic effect of Sil against MI and the progression to myocardial fibrosis, the cardiac contents of SOD and MDA in addition to TGFβ expression were assessed. The redox balance of the cardiac muscle was maintained in the Sil-pretreated rats even after injection of ISO. It was documented by cardiac SOD rise coupled with MDA drop.These findings are in line with a recent study by previous authors [27]. Sil mediates activation of redox-sensitive transcription factors leading to enhancement of SOD activity while down regulating NADPH oxidase reducing free radicals generation [12, 37]

Concerning TGFβ, pre-treatment with Sil was found to be associated with a significant decline in TGFβ expression score. Such finding raises the possibility that Sil could prevent both complications of post-infarct remodeling and future cardiovascular events. The reduction of TGFβ expression as evidenced in our study has previously been reported by previous authors [38]. They found that Sil prevented cardiac fibrosis through inhibition of TGFβ.

Administration of the well-known antioxidant, Vit.E for 3 weeks before injection of ISO exerted a cardio-protective effect at all electrical, biochemical and histopathological levels. Our findings are in line with those reported by previous authors [39]. Vit.E pretreatment augmented cardiac SOD and reduced MDA contents compared to the MI group. It thus, improved cardiac redox status and helped the heart to antagonize the cardiotoxic effect of the injected ISO. The hydroxyl group from the aromatic ring of tocopherols donates hydrogen to neutralize any given free radicals [40]. Also, it improves SOD activity [41] and attenuates gene expression of NADPH oxidase [42]. As a consequence, Vit.E significantly reduced TGFβ expression [41].

By comparing the assessed parameters in Sil pre-treated rats to their corresponding in Vit.E pre-treated one, a non-significant change was observed denoting potent antioxidant properties of Sil. Preconditioning with both Sil and Vit.E exerted the most cardioprotective effect presented by maintaining ECG parameters, serum CK-MB, cardiac SOD and MDA, histopathological findings, and TGFβ expression, near normal values. This finding might be supposed to be due to an additive or synergistic effect. Further studies would be conducted to explore other mechanisms underlying the cardioprotective effects of Sil or to investigate a possible curative impact in a post-MI model.

Conclusion

From our study we can conclude that Sil administration prior to MI induction exerts a cardioprotective effect, at least in part, by preserving cardiac redox balance and TGFβ suppression. Thus, it would be expected that people indicated for Sil medication or at high-risk for new MI will show damped incidence of MI and post-infarct complications. An effect would be better when Sil-combined with Vit.E-rich diet.

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