1. Introduction

Myocardial infarction has been an alarming health issue as it causes sudden death without warning. The incidence of myocardial infarction and its associated mortality and morbidity have been increasing. Statistics Department of Malaysia reported that ischemic heart disease was the principal cause of death in 2016 and the most common ischemic heart disease is myocardial infarction. The pathogenesis behind myocardial infarction was multifactorial but ischemia is the main cause nonetheless, which ultimately leads to cell death [1].

Isoprenaline (ISO) was used in this study to induce myocardial infarction in rats. ISO is proven to cause a lesion similar to human myocardial infarction in animals [2]. ISO is a non-selective beta-adrenoreceptor agonist, thus it will increase the heart rate and cardiac contractility. Then, it will cause increased myocardial oxygen consumption, leading to myocardial ischemia and injury. The oxidation products may cause further myocardial injury by increasing oxidative stress, leading to myocardial damage. The oxidants and reactive oxygen species may attack cell membrane, causing loss of cell integrity [3], leading to the release of cardiac markers such as ALT, AST, LDH, and CK-MB [4]. The end product of lipid peroxidation is indicated by malondialdehyde (MDA), whereby its level is increased in myocardial infarction [5]. Other macromolecules such as protein and DNA will also be attacked by ROS causing damage and degradation. Advanced oxidation protein products (AOPP) is a new stable marker for protein oxidation that has been used to detect oxidative stress [6]. The activity of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and antioxidant level such oxidized glutathione (GSH) would alter to overcome the oxidative stress [7].

One of the antioxidants in nature is vitamin E that has been proven to reduce the occurrence of coronary heart disease, which at the end, would account for myocardial infarction [8, 9]. It is believed that vitamin E exerts its function as a peroxyl radical scavenger that terminates chain reactions [10] other than inhibit platelet aggregation, endothelial cell nitric oxide production and superoxide production in neutrophils and macrophages [11]. These actions might be able to prevent cardiovascular disease or reduce its effects [12, 13]. The cardioprotective effect of tocotrienol is exerted through its antioxidative, anti-inflammatory and suppression effects on the mevalonate pathway [14]. Tocotrienol is one of the vitamin E isomers that is abundantly found in the palm oil [15]. TRF supplementation has the ability to reduce infarct size of the myocardium, protects the blood vessel wall, prevents the blood pressure increase, reduces plasma lipid peroxidation and increases the antioxidant enzyme activity [16].

Thus, this study aimed to determine the effect of TRF supplementation on oxidative status of heart rats induced by isoprenaline. TRF may have a potential in protecting the cardiac from injury because it was proven to ameliorate the oxidative stress [17].

2. Material and Methods

Chemicals

Isoprenaline (isoproterenol hydrochloride) was purchased from TCI Chemicals. Palm oil tocotrienol rich fraction (Gold Tri. E® 70) was supplied by Sime Darby Malaysia. The TRF content are d-alpha-tocopherol (29%), d-alpha-tocotrienol (25%), d-beta-tocotrienol (3%), d-gamma-tocotrienol (31%) and d-delta-tocotrienol (12%).

Animals

Adult male rats (n=24), weighing 220-250 g were obtained from the Animal Laboratory Unit of The National University of Malaysia. The animals were housed at the animal laboratory room in plastic cages, fed with a standard pellet and water *ad libitum*. The laboratory room was maintained at a temperature of 25˚C to 28˚C and exposed to a 12 h light/dark cycle throughout the experiment. The rats were acclimatized for one week before the experimental period begins. All the experimental work involving animals were performed according to the institutional animal ethics guidelines. The study protocol was reviewed and approved by The National University of Malaysia Animal Ethics Committee (UKMAEC) with certificate approval number, PP/BIOK/2015/ZAKIAH/20-MAY/677-MAY-2015-NOV.-2016

Experimental Design

A total of 24 male Sprague Dawley rats were divided into four groups, each group consisting of five animals, as follows:

Group I	Control-was given vitamin E- stripped oil
Group II	Rats were given vitamin E-stripped oil and induced with isoprenaline (MI group)
Group III	Rats were supplemented with 200 mg/kg of body weight of TRF
Group IV	Rats were supplemented with 200 mg/kg of body weight of TRF and induced with isoprenaline (TRF+ MI)

Rats in TRF+MI groups were supplemented with TRF for 84 consecutive days before the administration of ISO for two consecutive days after the TRF supplementation period ended. The dose of the ISO chosen in this study was according to the previous study, which showed that 85 mg/kg would induce biochemical and histopathological alterations without causing lethality to rats [3]. In contrast to isoprenaline, group I and III were injected with normal saline. Meanwhile, TRF dose was based on the study of Budin et al. [19] which showed that 200 mg/kg of TRF could reduce oxidative damage in rats. Following 84 days of treatment, the body weight was taken and the rats were

were anaesthetized with ketamine and dissection was performed. The blood sample was collected into an EDTA tube and centrifuged at 3500 rpm for 15 minutes at 4˚C to separate the serum. The rats were sacrificed and the heart was dissected immediately and heart weight was taken. The heart tissue was used for histological analysis. The serum samples were stored at -40˚C for cardiac marker enzyme assay (AST, ALT, LDH, CKMB). The excised heart tissue was homogenized in chilled Tris -HCl buffer (0.1 M) pH 7.4. The homogenate was then centrifuged at 8000 rpm for 2 min at 4˚C using the Eppendorf high-speed cooling centrifuge and stored at -80°C until further analysis. The clear supernatant obtained was used for the assay of malondialdehyde (MDA) level, protein oxidation (advanced oxidation protein product, AOPP) and antioxidant status (superoxide dismutase, SOD; catalase CAT; and reduced glutathione, GSH).

Cardiac Marker Enzyme Assays

Activity of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine kinase MB (CKMB) enzyme activity was assessed using commercially available diagnostic kits (Biosystems, Spain) and analyzed using semi-automated laboratory bioanalyzer (BTS-350, Spain). Serum lactate dehydrogenase (LDH) activity was estimated based on the method of Wróblewski and Ladue [20]. Plasma total protein was determined according to the Bradford method using bovine serum albumin as standard [21].

Histopathological Analysis by Hematoxylin and Eosin Staining

The heart tissues were cut into 10 mm^3 and were preserved in 10% formalin for two days. Dehydration process was done by using alcohol from low to high concentration. Cleaning process was done by using toluene before embedded in paraffin wax. The block was cut about 3-5 µm thick and stained with Hematoxylin and Eosin (H & E) staining. The evaluation of myocardial injury was done under a light microscope.

Determination of Inflammation Marker of CD68

The inflammation marker, CD68 was determined immunohistochemically using commercial kit of CD68 (Abcam) to detect the presence of macrophage at the area of myocardial injury.

Oxidative Stress Markers Level

Malondialdehyde (MDA), the end product of lipid peroxidation, was determined using thiobarbituric acid reactant substance (TBARS) assay by Hunter and Jamaludin [22]. The chromogenic pink colour formed during the assay, indicates the reaction between TBARS and MDA through the heating process. The absorbance was read using spectrophotometer at the wavelength of 532 nm. Advanced oxidation protein product (AOPP) was used to determine the protein oxidation level using Witko-Sarsat, Friedlander [23] method. The absorption was determined at 340 nm. Protein oxidation of heart tissues was also determined by immunohistochemistry using nitrotyrosine antibody purchased from Santa Cruz, Biotechnology as previously described [24].

Antioxidant Enzymes Activity

The antioxidant enzyme, superoxide dismutase (SOD) was assayed according to method of Beyer and Fridovich [25] based on the inhibition or reduction of nitro blue tetrazolium (NBT) measured spectrophotometrically at 560 mm. Catalase was estimated using the method by Aebi [26] and the absorbance read at 240 nm. One unit of catalase was defined as the amount of enzyme which liberates half the peroxide oxygen from a H_2O_2 solution in 30 s. Reduced glutathione (GSH) was determined by the method of Ellman [27] to measure the level of reduced glutathione conjugates with 5,5-dithiobis-2-nitrobenzoic acid (DTNB) indicated by the yellow conjugate GSH-DTNB at 412 nm.

Statistical Analysis

Statistical analyses were performed using SPSS 22.0 software. Data are expressed as mean ± standard error of mean (SEM). Normal distribution of the data was analysed using Shapiro Wilk test. Parametric data were analyzed using one-way ANOVA followed by Tukey's post hoc multiple comparisons using GraphPad Prism (version 6.00, GraphPad Software, California USA). The results with the value of $p < 0.05$ were considered to be statistically significant.

3. Results

Body Weight, Heart and Left Ventricle Weight

There was no significant difference in body weight between the groups (Table 1). Heart and left ventricle weight of ISO-treated myocardial injury (MI) rats showed a significant increased as compared to control. Supplementation of TRF showed no significant decreased in heart and left ventricle weight in MI.

Cardiac Marker Enzymes

The activity of AST was increased significantly ($p<0.05$) in MI and in MI supplemented with TRF as compared to control (Table 2). MI significantly increased CKMB activity as compared to control and the enzyme activity was decreased with TRF supplementation. However, TRF supplementation caused no significant changes for all the serum marker enzymes as compared to control.

Histological Study

Hematoxylin and eosin staining revealed a clear integrity of the heart cell membrane with no inflammatory cell infiltration observed for control and TRF supplemented groups (Figure 1). Heart tissue for MI showed a heavy infiltration of inflammatory cells with a focal necrosis area, infiltration of inflammatory cells, separation of cardiac muscle fibres as well as the absence of nuclei. There was a presence of inflammatory cells infiltration, separation of cardiac muscle fibres, necrotic cells and debris in MI with TRF supplementation.

Abbreviation: N: nucleus, C: cardiomyocytes, Black arrow: brown immunostaining, White arrow: inflammatory cells

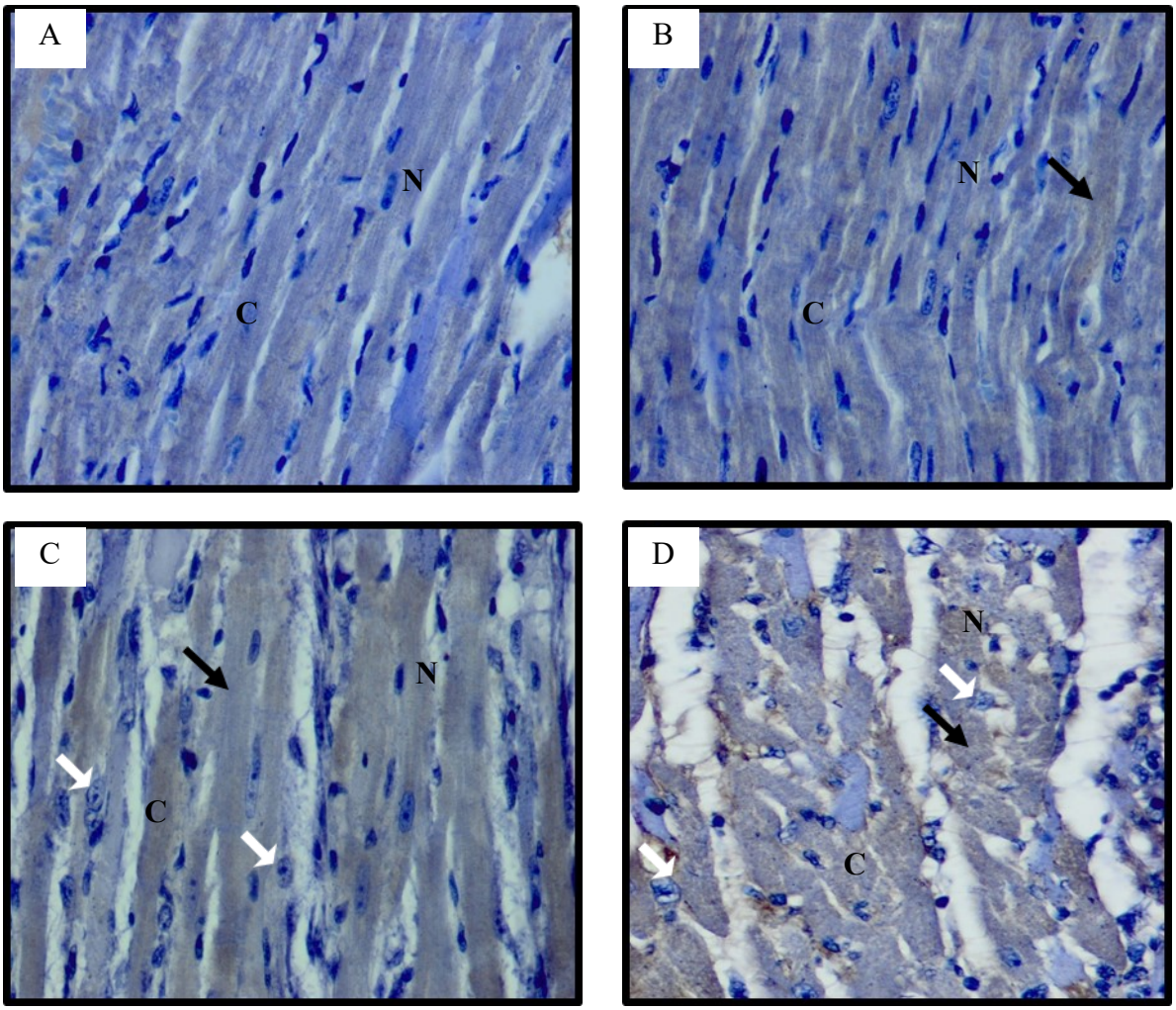

Figure 3: Immunohistochemistry localization of nitrotyrosine in endomyocardial tissue of A) control group, B) TRF group, C) ISO-induced myocardial injury in rats and D) ISO-induced myocardial injury and with TRF supplementation at 40x magnification.

Immunohistochemistry for CD68
Immunohistochemistry for CD68 detected no macrophage accumulation in any area for MI, as well as for the other groups (Figure 2).

Oxidative Stress Markers Level
The oxidative status of heart tissue for MI and TRF supplementation were shown in Table 3. No significant difference was observed for MDA and AOPP levels between the groups. The activity of SOD and CAT also did not show significant changes between the groups ($p>0.05$). However, supplementation of TRF significantly increased GSH level compared to the control ($p<0.05$).

Protein Oxidation by Immunohistochemistry
Immunohistochemistry of the localization of nitrotyrosine (NT) in endomyocardial tissue showed brown staining indicating the presence of protein oxidation in Figure 3. Brown staining was showed in MI (Fig 3C) and MI with TRF supplementation group (Fig 3D). Absence of brown immunostaining was observed in control (Fig3A) and TRF group (Fig 3B) with magnification 40x**.**

4. Discussion

Isoprenaline (ISO) is a synthetic sympathomimetic catecholamine that may produce `infarct-like' myocardial necrosis resembling human myocardial infarction [28]. Administration of ISO at a moderate dose (85 mg/kg) was reported to show changes in biochemical parameters, and caused hypertrophy cardiomyopathy [29], moderate necrosis in the heart and left ventricle dilatation due to high oxygen demand [30]. These changes are responses to cellular damage of the heart [5]. It explained the reason for the increased of rat heart and left ventricle weight in this study but the body weight was not affected. Necrosis of cardiomyocyte may cause aspartate aminotransaminase (AST) level and CKMB activity increment in the serum of ISO-induced myocardial injury. It was confirmed by the histological observation, showing myocyte membrane damage, cardiac cell coagulated necrosis [31] and inflammatory cells infiltration [7] as proven and consistent with the increase of heart and left ventricle weight. The cardiomyocyte fibre was seen separated from each other [32] while necrosis is marked with the absence of the nucleus in some cells suggesting karyolysis, the prominent marker of necrosis, has occurred . Microscopic observation of immunohistochemistry stained heart tissue with anti-CD68 showed no labelling of CD68 (a marker of macrophage) in myocardial infarction. This is probably because macrophage usually appears three days to two weeks after myocardial infarction [33, 34], specifically after the inflammation phase is finished [35].

Myocardial infarction is a result of cardiac tissues necrosis due to oxygen and nutrient deprivation because of the interruption of coronary blood. It is detected by measuring the cardiac marker enzyme, such as lactate dehydrogenase, CKMB, ALT, AST and protein level, such as troponin T. Unfortunately, both alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) showed no significant difference changes in this study. This could be due to the ALT arrives at its peaks after the second day of MI induction [36] while LDH peaks about 2-3 days after the myocardial infarction induction [37]. However, a more specific enzyme such as creatinine kinase myocardial band (CK-MB) was increased. The release of CK-MB to the circulation is quick onset; ranging from four to six hours after the MI induction and reverts back to the normal level around 48 to 72 hours after that [38].

Tocotrienol has shown antioxidative, anti-inflammatory and cardioprotective effects in numerous study [39]. Cardioprotective properties of tocotrienol were reported to be able to reduce myocardial injury due to oxidative stress [40], reduce cholesterol level [41], down-regulates vascular adhesion molecule (VCAM-1) and intercellular adhesion molecule (ICAM-1) expression in endothelium cells [42]. In contrast, in this study that the supplementation of TRF could not prevent the myocardial injury induced by ISO.

In addition, administration of ISO was reported to induce oxidative stress as a result of autooxidation mechanism of ISO. This may cause infarct-like necrosis of the heart muscle [43], which closely resembles histological damage seen in human myocardial infarction [44]. In oxidative stress, the excessive production of free radicals may attack lipid membrane, protein and DNA [45], causing lipid peroxidation, protein oxidation [46] and DNA damage [19]. MDA, the end product of lipid peroxidation, may further increase free radical generation and reduce the activity of the antioxidant enzyme [47, 48]. In contrast to previous studies, this study showed no significant changes in MDA levels of the heart tissue after ISO administration. The same finding was seen in AOPP level. Moreover, ISO administration causes no marked changes in antioxidant enzyme activity (SOD and CAT) as well as non-enzymatic antioxidant, glutathione level in cardiac tissue. Nonetheless, a previous study in ISO rat model showed the opposite finding. Depletion of antioxidant and increased oxidative stress as shown by a rise in myocardial MDA level could be due to the generation of free radicals from autooxidation of ISO. This exceeds the capacity of the antioxidant system in scavenging the toxic radicals, thereby causing oxidative damage to the myocardium membrane [49,50].

No significant changes of MDA after ISO administration is probably due to the thiobarbituric acid (TBA) test reaction. The substances in the biological sample or in the reactants, such as metal ions and iron cations, may initiate and fasten the decomposition process, leading to a diminished level of MDA-adduct [51]. Artefact formed in TBA assay may interfere with the measurement of MDA [52,53]. Its non-specificity is also contributed by the formation of several MDA-unrelated products causing an underestimated amount of MDA present in the sample [54]. As for TRF-supplemented group, the lack of any significant changes to the MDA is probably because of TRF providing sufficient antioxidant effect in reducing or improving the oxidative stress. The absence of marked changes in antioxidant enzyme activity (SOD and CAT) as well as the non-enzymatic antioxidant, glutathione is probably because of the low level of oxidative stress as reflected from the MDA level itself. Hence, level

of antioxidant activity too, is maintained as the minimal oxidative stress, controlled by the cellular antioxidant defence [55].

Positive immunostaining localization of nitrotyrosine, which is a marker of oxidative stress, was observed in ISO administration group reflects the presence of oxidative damage [56]. This suggests that ISO administration induces oxidative stress in myocardial tissue via immunohistochemistry method as reported by previous studies [56, 57].

TRF supplementation could not reduce the oxidative stress and modulate antioxidant activity to ISO-induced myocardial injury. At 200 mg/kg body weight, TRF might not be enough to protect the myocardial from injury or it might be required a longer time to see the TRF effect on the injury. Even though many studies have used the same dose and shown very positive findings, they were used in different conditions [58]. Basically, TRF could exert its antioxidant activity by donating the hydrogen at its hydroxyl group in its chemical structure. The hydrogen donation is made to the free radicals [59]. By donating hydrogen, lesser cytotoxic free radicals are available to damage the cells. Other possible reason might be due to the administration of ISO did not cause significant changes on the oxidative stress of the cardiac tissue. As a result, TRF supplementation is unable to exert its antioxidant activity. This is in contrast with previous studies that reported supplementation of TRF ameliorated the oxidative damage in a variety of rat models [60, 62] due to its potent antioxidant activity. It can donate the phenolic hydrogen to the toxic radicals, thereby scavenge and neutralize these free radicals generated, thus producing less reactive metabolites products [63]. TRF supplementation on normal rats significantly increased glutathione level as reported in other studies [62, 64]. This may suggest that TRF is capable to enhance GSH content through its potent and effective antioxidant activity of TRF [65] contributed by tocotrienol rather than tocopherol [18] due to the high content of tocotrienol in palm oil [66]. While, Budin et al [61] reported that lower level of oxidative stress in normal rats causes the GSH level to be maintained in a normal state to counteract the endogenous action of free radicals that continuously produced in low level in an aerobic organism, to limit the generation of free radicals in excessive amount.

However, TRF pretreated ISO-induced rats did not experience attenuated myocardial oxidative stress as presented with nitrotyrosine brown immunostaining of myocardial tissue similar to those observed in the ISO group, suggesting that the antioxidant activity of TRF was not shown in this model. However, another study reported that raxofelast, a new synthetic hydrophilic vitamin E-like antioxidant agent, may yield protective effects against acute inflammation induction through reducing nitrotyrosine immunostaining, an indicator of peroxynitrate formation in inflammation. In many previous studies, TRF supplementation has been recognized to exert potent antioxidant activity through its amelioration effect against oxidative damage in many animal models including diabetes and chemical-induced rat [67, 68].

As the conclusion, TRF supplementation was able to reduce myocardial injury seen in MI model as evident in the improved histological structure compared to MI model alone. Nevertheless, TRF did not seem to alter the oxidative stress and antioxidant status. The exact mechanism of action of TRF on heart remained unknown. Hence, further studies are definitely needed to elucidate the mechanism of actions.

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6. Conflict of Interest

The authors declare that they have no competing interests.