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Palm Tocotrienol-Rich Fraction Protects Neonatal Rat Cardiomyocytes from H_2O_2-Induced Oxidative Damage

(Fraksi Kaya Tocotrienol Sawit Melindungi Kardiomiosit Tikus Neonatal daripada Induksi Kerosakan Pengoksidaan H_2O_2)

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ABSTRACT

Oxidative stress plays an important role in the pathogenesis of heart disease. Tocotrienol-rich fraction (TRF) is an antioxidant and that has the potential to reduce the risk of heart disease. This study is to determine the protective effects of palm TRF against H_2O_2-induced oxidative damage in neonatal rat cardiomyocytes (NRCM). The NRCM were divided into control, treated with TRF (10 µg/mL), H_2O_2 (0.5 mM) and treated with TRF prior to H_2O_2 induction (pre-treatment). Cell viability was determined by the MTS assay,while the presence of reactive oxygen species (ROS) was determined using fluorescent dihydroethidium (DHE) and 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) dye. Mitochondrial integrity and cell death were determined using JC-1 and Annexin V-FITC staining, respectively. Lactate dehydrogenase (LDH) and superoxide dismutase (SOD) activity were determined by colorimetric assay kit. The concentration of H_2O_2 from 0.5 to 5 mM reduced the cell viability and the H_2O_2 IC_{50} value of 0.5 mM was used in the experiment. H_2O_2 induction increased the intensity of carboxy-H_2DCFDA and DHE-stains; and also the intensity of green fluorescence of J-monomers in JC-1 staining compared to the control group. The activity of LDH increased while the activity of SOD decreased in the H_2O_2 group. Pre-treatment with TRF reduced the intensities of carboxy-H_2DCFDA and DHE-stains, as well as the green fluorescence of J-monomers in JC-1. Meanwhile, the LDH activity was reduced in the pre-treatment group but no changes were recorded in SOD activity compared to the H_2O_2 group. Palm TRF protects cardiomyocytes from oxidative damage by reducing ROS production and maintaining the mitochondrial membrane integrity thus reducing cell death.

Keywords: Cardiomyocytes; H_2O_2; oxidative damage; tocotrienol-rich fraction

ABSTRAK

Tekanan oksidatif memainkan peranan penting dalam patogenesis penyakit jantung. Fraksi kaya tokotrienol (TRF) adalah antioksidan dan berpotensi mengurangkan risiko penyakit jantung. Kajian ini adalah untuk mengetahui kesan pelindung TRF sawit terhadap kerosakan oksidatif aruhan H_2O_2 pada kardiomiosit tikus neonatal (NRCM). NRCM dibahagi kepada kawalan, dirawat dengan TRF (10 µg/mL), H_2O_2 (0.5 mM) dan dirawat dengan TRF sebelum induksi dengan H_2O_2 (pra-rawatan). Kebolehhidupan sel ditentukan dengan ujian MTS. Kehadiran ROS ditentukan menggunakan pewarna dihidroetidium (DHE) dan pewarna 5-(dan-6)-karboksi-2',7'-diklorodihidrofluorescein diasetat (carboxy-H2DCFDA). Integriti mitokondria dan kematian sel ditentukan menggunakan pewarnaan JC-1 dan Annexin V-FITC masing-masing. Aktiviti laktat dehidrogenase (LDH) dan superoksid dismutase (SOD) ditentukan menggunakan kit esei kalorimetrik. Kepekatan H_2O_2 bermula daripada 0.5 hingga 5 mM menurunkan kebolehhidupan sel dan nilai IC_{50} H_2O_2 0.5 mM digunakan di dalam kajian ini. Aruhan H_2O_2 meningkatkan keamatan karboksi-H2DCFDA dan pewarnaan DHE; dan juga keamatan pendarfluor hijau monomer-J dalam pewarnaan JC-1 berbanding kumpulan kawalan. Aktiviti LDH meningkat sementara aktiviti SOD menurun dalam kumpulan H_2O_2. Pra-rawatan dengan TRF menurunkan keamatan karboksi-H2DCFDA dan pewarnaan DHE; dan juga keamatan pendarfluor hijau monomer-J dalam pewarnaan JC-1. Manakala aktiviti LDH menurun dalam kumpulan pra-rawatan tetapi tiada perubahan ditunjukkan dalam aktiviti SOD berbanding kumpulan H_2O_2. TRF sawit melindungi kardiomiosit daripada kerosakan oksidatif melalui pengurangan penghasilan ROS dan mengekalkan integriti membran mitokondria seterusnya mengurangkan kematian sel.

Kata kunci: Fraksi kaya tokotrienol; H_2O_2; kardiomiosit; kerosakan oksidatif

Introduction

Cardiovascular disease is one of the most prevalent ailments associated with high morbidity and mortality in both developing and developed countries (WHO 2016). Studies have reported that oxidative stress plays a central role in the pathophysiology of heart disease and causes cell death (Taverne et al. 2013). Accumulation of ROS increases the oxidative stress and causes oxidative damage that leading to detrimental modifications in cellular macromolecules. ROS such as superoxide anions (O^{2-}), hydroxyl radicals (OH^{-}), and hydrogen peroxide ions (H_2O_2) are produced as part of physiological processes. Lipid peroxidation, DNA damage, mitochondrial dysfunction and loss of enzymatic activity caused by the instigation of ROS lead to necrosis and/or apoptosis (Biswas 2016). In pathological condition, ROS such as $ONOO^{-}$ from the reaction of superoxide and free radical nitric oxide (NO) causes endothelial dysfunction, which is a predictor of various cardiovascular diseases (CVDs). NO is a potent vasodilator produced by the endothelium. In atherosclerosis, high level of ROS is produced by phagocytosis as part of inflammatory responses by adhesion molecules (Panth et al. 2016).

Cardiomyocytes are a cardiac muscle cells responsible for generating contractile force, and some are responsible for the rhythmic beating of the heart. It requires a constant supply of oxygen for its function. In situation with less oxygen supply or hypoxic stress, such as in acute myocardial infarction, ROS increases rapidly and plays a major role in tissue necrosis and apoptosis, leading to cardiomyocytes cell death (Zhou et al. 2015). In hypoxic stress, ROS induces both the extrinsic- and intrinsic apoptosis pathways. Pro-apoptotic proteins that control the permeability of mitochondrial membranes (Webster 2012) are activated to change the integrity and permeability of the mitochondrial membrane, initiating the apoptotic mitochondrial pathway and promotes cardiomyocyte cell death that may increase infarct size (Condorelli et al. 2001). Since the heart comprises of cells with low regeneration capacity, regardless of the scale of cardiomyocyte loss, the contractile efficiency would be affected (Tham et al. 2015).

The levels of ROS are controlled by antioxidant enzymes such as catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD). They catalyse the conversion of these ROS to less-toxic products, apart from protecting cells against free radical-induced damage (Lobo et al. 2010). SOD is a main intracellular antioxidant defence mechanism which catalyses the dismutation of superoxide radicals into H_2O_2 and oxygen. Overexpression of SOD3 was reported to decrease infarct size and increased cardiac regeneration after myocardial ischaemia (Obal et al. 2012). Therefore, interventions that involve antioxidants or natural compounds with free radical-scavenging activities may provide beneficial effects either by increasing the activity of an antioxidant enzyme or acting directly against oxidative stress and reducing the oxidative damage.

Studies in humans and animal models have showed that vitamin E exerts antioxidant, anticancer, anti-inflammatory, antimicrobial activities, and other biological activities, apart from protecting the cardiovascular system (Vasanthi et al. 2012). Vitamin E has been suggested as a valuable compound with many medical applications. It is a fat-soluble vitamin, which is composed of naturally-occurring α-, β-, γ-, and δ-tocopherols as well as -tocotrienols (Fu et al. 2014). The difference between tocopherols and tocotrienol is that tocotrienol has an unsaturated isoprenoid side chain with three carbon-carbon double bonds at positions 3', 7', and 11' of hydrocarbons, whereas tocopherol side chains are saturated. Although both have an antioxidant function, each form has different biological activities and physical properties. Alpha-tocopherol is the most readily absorbed and retained by the body (Kamal-Eldin & Appelqvist 1995). Hence, it is the most nutritionally beneficial form of vitamin E. Tocotrienol has been shown to act as serum cholesterol-lowering agents by inhibiting the activity of HMG-CoA reductase and LDL-cholesterol level (Khor et al. 1995). *In vivo* studies showed that the cardioprotective effect of tocotrienol is associated with the inhibition of HMG-CoA reductase activity. Therefore, it decreases cholesterol synthesis and scavenges peroxynitrite through the activation of the NO-cGMP pathway. As a consequence, tocotrienol has been shown to reduce myocardial reperfusion injury (Berbee et al. 2011; Esterhuyse et al. 2005), oxidative stress and inflammation (Kuhad & Chopra 2009), and restored endothelium-dependent relaxation in arteries and rats with streptozotocin-induced diabetes (Muharis et al. 2010). Bester et al. (2010) reported that supplementation with red palm oil reduces infarction size in the rat. Although the effects of tocotrienols on myocardial injury are well documented, knowledge on the effect of cardiac mitochondrial function is still lacking.

Mitochondria is the main power house for cardiac functions and produces about 95% of the required levels of ATP through the process of oxidative phosphorylation, ROS generation occurred as a normal by-product of the energy production process (Sabbah et al. 2016). The previous study by Krager et al. (2015) reported that rice bran tocotrienol-rich fraction (TRF) protects H9c2 cells from oxidative injury (through H_2O_2 or ischemia injury) by preserving the mitochondrial function of the

cells Mitochondrial protection was observed through the restoration of mitochondrial respiration by increased oxygen consumption rate and reversed mitochondrial uncoupling following ischemia-reperfusion exposure. Furthermore, the study by Ali and Woodman (2015) showed that tocotrienol rich tocomin was more effective than α-tocopherol and the isomers of tocotrienols alone in reducing oxidative stress and restoring endothelium-dependent relaxation in rat aorta. A combination of tocotrienol and α-tocopherol isomers were suggested to give better protective effects. Therefore, the objective of this study was to determine the effects of palm TRF on the oxidative status of neonatal rat cardiomyocytes (NRCM) induced with H_2O_2.

Materials and Methods

Isolation of neonatal rat cardiomyocytes (NRCM)

Using a modified protocol described by Salameh and Dhein (2005), NRCM were isolated from 1- to 2-day old Sprague-Dawley rats. The experimental protocol was approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (FP/BIOK/2012/ZAKIAH/18-JULY/450-APRIL-2013-APRIL-2016-AR-CAT2).

Neonatal rat ventricles were cut into small pieces using a pair of scissors of about 1 mm length in cold ADS buffer. Then, the minced tissues were transferred into a 50 mL bottle, and 10 mL enzyme solution containing collagenase type II (Worthington) and pancreatin (Sigma) was added. Then, the samples were enzymatically digested in a shaker incubator at 200 rpm for 20 min at 37 °C (cycle 1). The supernatant (containing isolated cells) was collected and placed into a centrifuge tube containing 4 mL fetal bovine serum (FBS) to terminate the digestion. Then, 8 mL of enzyme solution was added to digest the tissue at 180 rpm for 25 min (cycle 2). The same step was repeated until all tissue was digested. All supernatant from each cycle was then pooled and centrifuged at 800 rpm for 5 min to collect the cells. The serum was removed, and the cell pellet was resuspended in media containing DMEM, M199, 10% horse serum, 5% FBS, 100 U/L of streptomycin, and 100 U/L of penicillin. Pre-plating was performed by incubating the cells for 45 min in a cell culture flask at 37 °C in a humidified atmosphere containing 5% CO_2. This was done to reduce contamination by fibroblasts and to obtain pure cardiomyocytes. Subsequently, the supernatant was collected and centrifuged at 800 rpm for 5 min. The resultant cell pellet was resuspended with the mentioned media and cultured overnight before transferred to a new media containing 5% FBS. The NRCM were seeded at a density of 2×10^4 cells/well in the 96-well plate and 4×10^5 cells/well in the 6-well plate according to experimental conditions. The culture medium was changed every other day, and the cells were cultivated for three to four days until the synchronised beating of NRCM were obtained before they were randomly grouped for further experiment.

Experimental groups

The treatment of the cells was performed according to the assigned groups; control group (incubation of NRCM in media), H_2O_2 group (NRCM were subjected to 0.5 mM H_2O_2 for 30 min), TRF group (NRCM were supplemented with 10 μg/mL palm TRF for 24 h), and pre-treatment group (NRCM were supplemented with 10 μg/mL palm TRF for 24 h and were subjected to 0.5 mM H_2O_2 for 30 min after TRF withdrawal).

Determination of cell viability by MTS assay

Various concentrations of H_2O_2 (0-5 mM) and palm TRF (0-40 μg/mL) were used to treat the cells for 30 min and 24 h, respectively. The degree of cytotoxicity was measured using the CellTiter 96® Aqueous Nonradioactive Cell Proliferation Assay (MTS; Promega, USA) according to the manufacturer's protocol. Briefly, 20 μL of MTS solution was mixed with 100 μL of media before added to each well and incubated for 2 h. Next, the absorbance of MTS formazan produced was measured at 490 nm using a microtiter plate reader (VersaMax Molecular Devices, USA). Only one concentration of H_2O_2 and TRF was used for subsequent experiments.

Determination of TRF concentration on NRCM prior to addition of H_2O_2

TRF concentration on NRCM for subsequent experiments was chosen by treating the cells with 10, 15, and 25 μg/mL before the addition of H_2O_2 IC_{50} (0.5 mM) and incubated for 24 h.

Determination of lactate dehydrogenase activity

The cells were cultured in 6-well plates at a density of 4×10^5 cells/well. After treatment, the supernatant was collected and measured for lactate dehydrogenase (LDH) activity using a detection kit according to the manufacturer's instruction (Sigma, USA). LDH activity was expressed as international units per litre (IU/L).

Determination of ROS

The generation of ROS in NRCM was assessed using 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) and dihydroethidium (DHE) (Molecular Probes, USA) dye. Carboxy-H_2DCFDA

is oxidised by various ROS, including H_2O_2, hydroxyl radicals, and peroxynitrite. Meanwhile, DHE detected the production of superoxide. In terms of the procedure, NRCM were washed with PBS at the end of the treatment period and incubated with 40 µM of carboxy-H_2DCFDA and 20 µM of DHE for 45 min. Then, the cells were washed with PBS, and the intensity of fluorescence was measured using a microplate reader (Infinite® 200, Tecan, USA) at the excitation/emission wavelength (EX/EM) of 488/521 nm and 518/600 nm, respectively.

DETERMINATION OF SUPEROXIDE DISMUTASE ACTIVITY (SOD)

The measurement of SOD activity in NRCM was determined using a colorimetric assay kit (Cayman Chemical Company, USA), based on the ability of SOD to catalyse the dismutation of superoxide anion generated during the xanthine/xanthine oxidase reaction, using a tetrazolium salt as an indicator. The assay was performed according to the instructions provided by the manufacturer.

DETERMINATION OF MITOCHONDRIAL MEMBRANE POTENTIAL (MMP, $\Delta\Psi_M$)

JC-1 staining was employed to assess mitochondrial membrane potential (MMP, $\Delta\Psi_m$,) a marker of mitochondrial oxidative phosphorylation activity, as previously described by Nowak et al. (2012). JC-1 is a lipophilic and cationic dye that permeates the plasma, as well as mitochondrial membranes of cells. A low JC-1 ratio indicates the presence of a low amount of the aggregated form of JC-1 in the mitochondria, which correlates with a high level of ROS. Fluorescence was determined by flow cytometry (FACSVerse; BD Biosciences, San Jose, CA), at the excitation of 488-nm argon-ion laser. JC-1 monomers (green) and J-aggregates were detected in FL1 (EM 525 nm) and FL2 (EM 590 nm) channels, respectively. $\Delta\Psi_m$ was presented as the ratio of the fluorescence intensity of J-aggregates to that of J-monomers. For observation, the same staining protocol was applied. The cells were then observed under a fluorescence microscope (EVOS FL digital inverted microscope, Thermo Fisher Scientific, USA).

DETERMINATION OF THE PERCENTAGE OF CELL DEATH

Annexin V-FITC apoptosis detection kit (BD Pharmigen, USA) was used for cell death analysis. The cells were washed with PBS three times and suspended in 100 µL of binding buffer. Staining was done with 5 µL of FITC-conjugated Annexin V and 10 µL of propidium iodide (PI), after which 400 µL of binding buffer was added as per the manufacturer's instructions. The percentages of both dyes were analysed by flow cytometry (FACSVerse, Becton-Dickinson, USA).

STATISTICAL ANALYSIS

Statistical analyses were performed using the SPSS 16.0 software (IBM, USA). Data are expressed as means ± standard deviations (mean ± SD) from three biological replicates performed in duplicate or triplicates. Comparison of treatment was analysed using one-way analysis of variance and secondary analysis for significance, with post-hoc Tukey's HSD or LSD. The results for all the tests were considered to be statistically significant if $p<0.05$.

Results and Discussion

EFFECTS OF H_2O_2 ON OXIDATIVE DAMAGE AND CELL DEATH

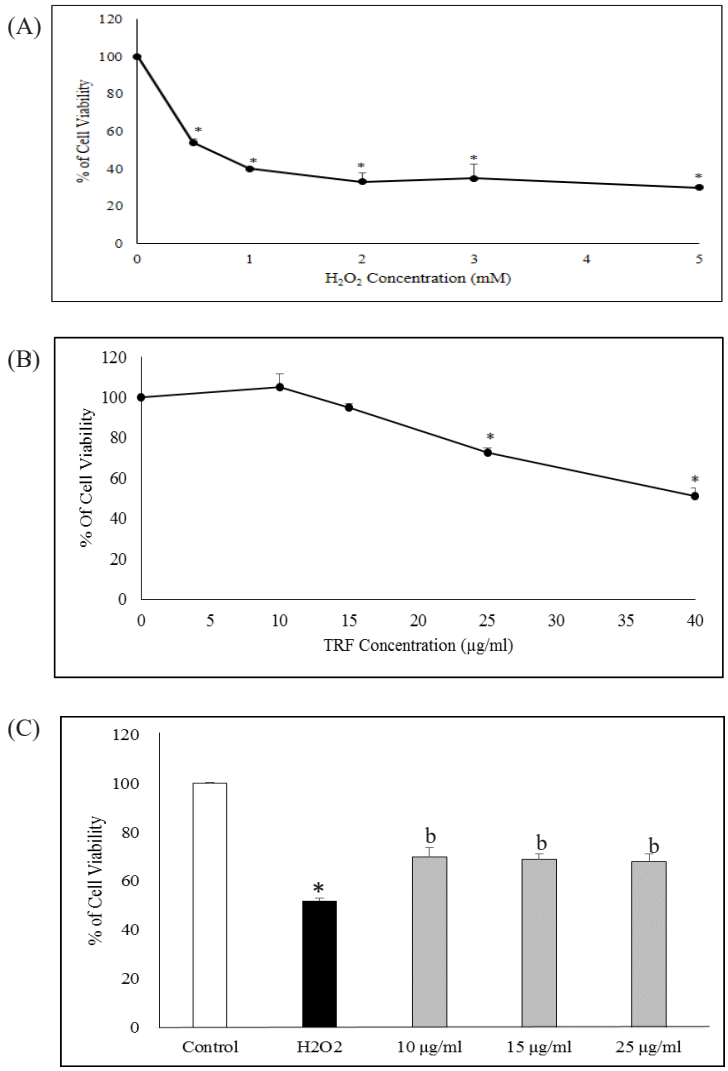
H_2O_2 reduces cell viability in a dose-dependent manner (0.5 to 5 mM), and at the concentration of 0.5 mM, exerts 50% of cell viability reduction (Figure 1(a)). Meanwhile, TRF reduces cell viability, starting from 25 to 40 µg/mL (Figure 1(b)). TRF concentration of 10 µL before the addition of H_2O_2 was chosen for the following experiment since the other higher doses of TRF gave the same effects (Figure 1(c)). In Figure 2, LDH activity is increased in NRCM induced by H_2O_2 compared to the control group ($p<0.05$). The leakage of LDH might be due to oxidative damage that usually corresponds to irreversible cardiomyocytes injury (Kourouma et al. 2015). It was also supported by the finding on the reduction of NRCM cell viability (Akyol et al. 2014). The increased LDH activity in the extracellular fluid is directly proportional to the increase in membrane lipid peroxidation due to ROS activity, indicating myocardial cell membrane damage (Hrelia et al. 2002).

Increased intensities of carboxy-H_2DCFDA and DHE-stains indicated high levels of ROS in H_2O_2-treated group compared to the control group ($p<0.05$; Figure 3). The high level of ROS may decrease SOD activity, shown in the H_2O_2-treated group compared to the control group ($p<0.05$; Figure 4). A study by Pinto et al. (2016) stated that the decreased activity of SOD3 increased tissue injury and apoptosis on cardiovascular ischaemia. As a consequence, depolarisation of the MMP ($\Delta\Psi_m$) and cell necrosis may occur. This is depicted by the increased intensity of the green fluorescence of J-monomers in JC-1 staining upon exposure of NRCM to H_2O_2 indicating mitochondrial depolarisation. JC-1 staining of NRCM exhibited a characteristic pattern of hypopolarised (green fluorescence of J-monomers) and hyperpolarised (red fluorescence of J-aggregates) mitochondria (Figure 5(a)). Figure 5(b) shows the ratio of JC-1 aggregates to JC-1 monomers in NRCM, which depicts the lower ratio of

the H_2O_2 group vis-à-vis the control ($p<0.05$). Previous studies have shown that the depolarisation of mitochondrial membranes led to energy depletion due to reduced ATP generation, which could eventually change the mode of cell death from apoptosis to necrosis (Nakamura et al. 2010).

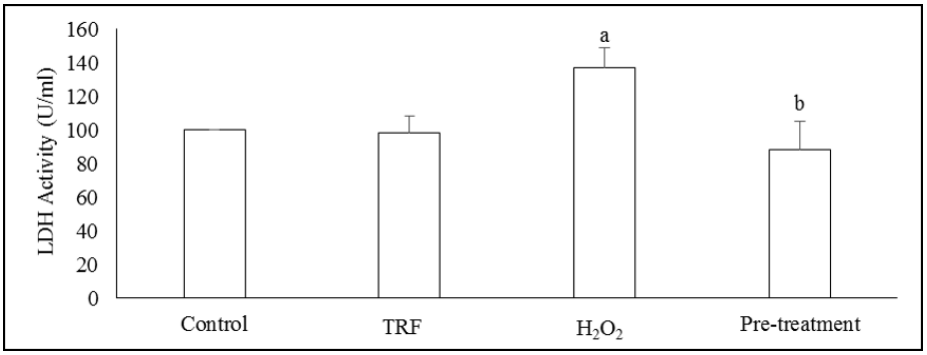
The NRCM cell death was shown by the increased the percentage of cell necrosis (PI^+) following H_2O_2 induction; thus, reducing the percentage in early and late apoptosis compared to the control group ($p<0.05$; Figure 6). ROS are very reactive and unstable. ROS attack cellular biomolecules such as DNA, lipid, and protein; thus giving rise to oxidative damage (Birben et al. 2012; Sahhugi et al. 2014). ROS include superoxide, hydroperoxyl, hydroxyl radicals, and H_2O_2 which is non-radical but is still classified as a ROS because of its high oxidative reactivity (Dröge 2002). ROS are generated both intracellularly and extracellularly (Zhang et al. 2016). Intracellular ROS are predominantly produced during the activation of the mitochondrial respiratory chain (Brand et al. 2004). ROS-induced damage to the mitochondrial membrane lipids disrupts the integrity and permeability of the membrane, apart from causing depolarising alterations in the membrane potential (Lane et al. 2015). It leads to cell membrane injury and leakage of cellular contents into the cytoplasm (Zhang et al. 2018).

In this study, H_2O_2 was used to induce oxidative damage in NRCM. This has been widely implicated in cellular oxidative damage studies and the progression of cardiovascular diseases such as atherosclerosis, hypertension, heart failure, and myocardial infarction (Sugamura & Keaney 2011; Zarkasi et al. 2019). Low antioxidant availability in cardiomyocytes subjects them to oxidative damage.



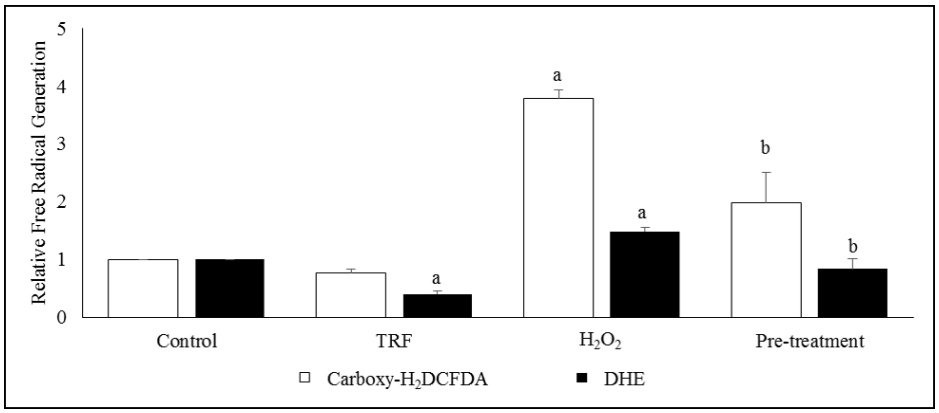
Data were expressed as mean ± SD from three independent experiments performed in triplicates (n=3). * indicates a significant difference compared to control group ($p<0.05$) and [b] indicates a significant difference compared to H_2O_2 group ($p<0.05$)

FIGURE 1. Cytotoxicity of H_2O_2 and cell viability percentage on NRCM treated with palm TRF and palm TRF+H_2O_2on NRCM: (a) effect of different concentration of H_2O_2 (0.5-5 mM) on cell viability, (b) palm TRF concentration on NRCM cell viability at 24 h of incubation time, and (c) the effect of three different concentrations of TRF on NRCM prior to addition of H_2O_2



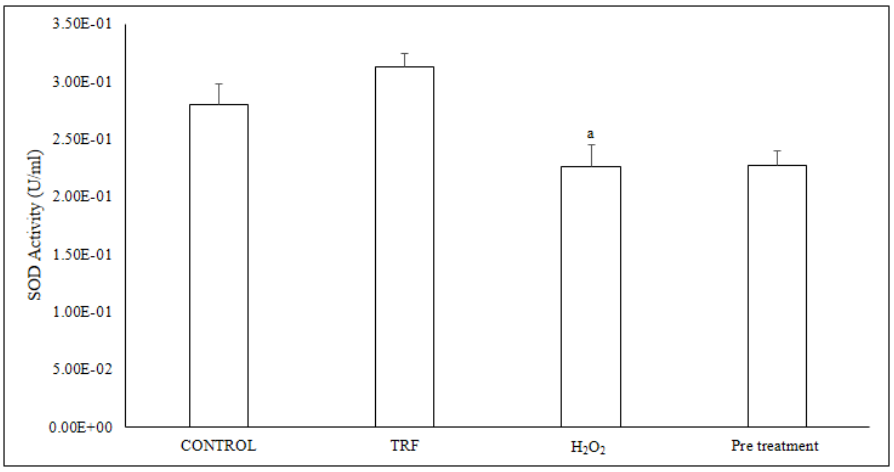
Data were expressed as mean ± SD (n = 3). [a] indicates a significant difference compared to control ($p<0.05$) and [b] indicates a significant difference compared to H_2O_2 group ($p<0.05$)

FIGURE 2. Effect of palm TRF on LDH activity of NRCM induced with H_2O_2



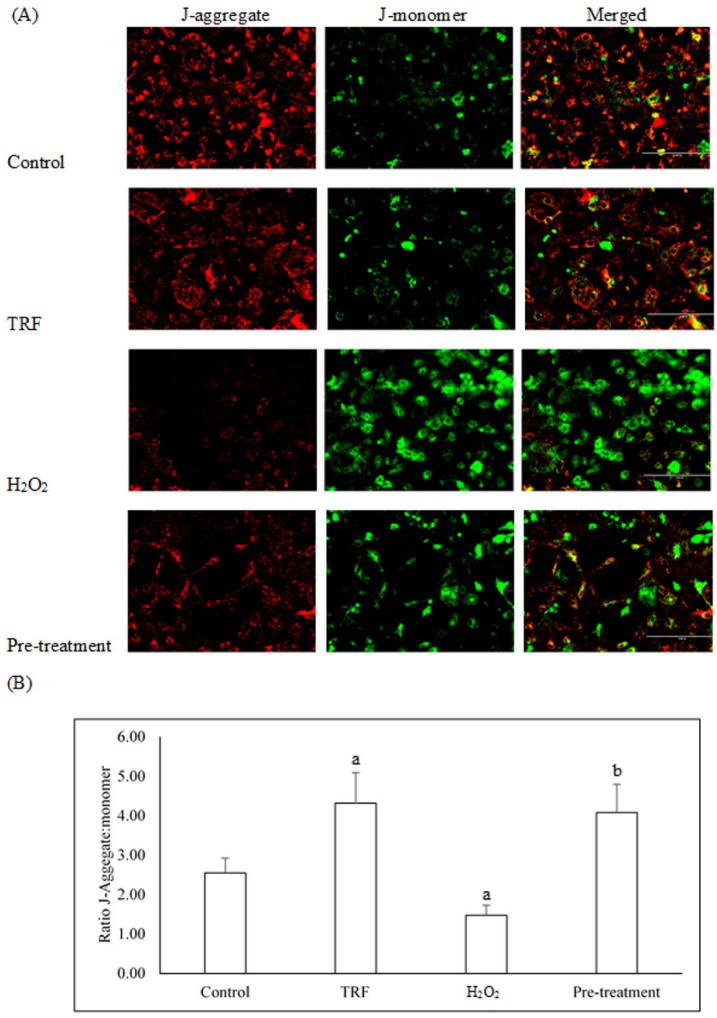
Data were expressed as mean ± SD from three independent experiments performed in triplicates (n=3). [a] indicates a significant difference compared to control ($p<0.05$) and [b] indicates a significant difference compared to H_2O_2 group ($p<0.05$)

FIGURE 3. Effect of palm TRF on H_2O_2-induced ROS production in NRCM



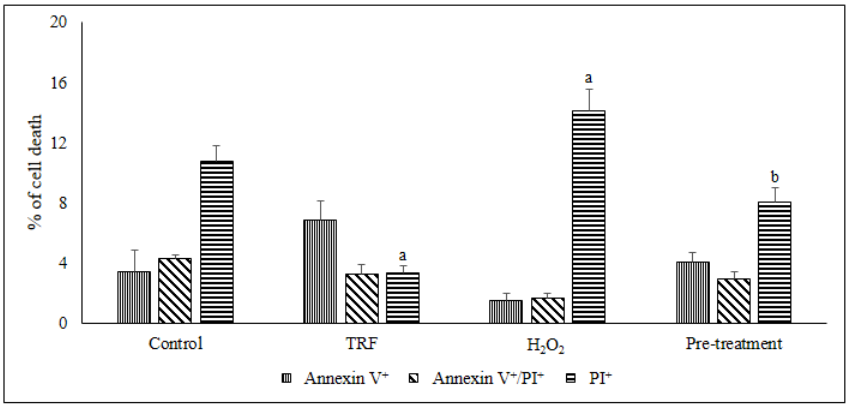
Data were expressed as mean ± SD (n = 3). [a] indicates a significant difference compared to control group ($p<0.05$)

FIGURE 4. Effect of palm TRF on SOD activity of NRCM induced with H_2O_2



Data were expressed as mean ± SD, from four independent experiments performed in duplicates (n=4). [a] indicates a significant difference compared to control ($p<0.05$) and [b] indicates a significant difference compared to H_2O_2 group ($p<0.05$)

FIGURE 5. Mitochondrial membrane potential changes: (a) the effect of TRF on mitochondrial membrane potential using microscopic observation by JC-1 staining, and (b) the ratio of JC-1 aggregate to JC-1 monomer of NRCM



Data were expressed as mean ± SD from three independent experiment performed in triplicates (n=3) with [a] indicates a significant difference compared to control group ($p<0.05$) and [b] indicates a significant difference compared to H_2O_2 group ($p<0.05$). Annexin V^+ indicates early apoptosis, Annexin V^+/PI^+ indicates late apoptosis and cell death, PI^+ indicates cell necrosis

FIGURE 6. Percentage of NRCM cell death treated with palm TRF and H_2O_2

EFFECTS OF PALM TRF ON OXIDATIVE DAMAGE AND CELL DEATH

Vitamin E, specifically tocotrienol, has been widely studied for their antioxidant properties that protect cells from oxidative damage and death (Wu et al. 2010). Its antioxidant effect was indicated by increased cell viability of NRCM in palm TRF prior to the addition of H_2O_2 compared to H_2O_2 (Figure 1(c)). The percentage of cell viability remained consistent from 10 to 25 µg/mL of palm TRF prior to the addition of H_2O_2. Hence, the lowest concentration of 10 µg/mL palm TRF was chosen for the following experiment.

The reduction of cell viability in palm TRF treatment before H_2O_2 induction indicated protection against oxidative damage. It is further supported by the significant decrease of LDH activity (Figure 2; $p<0.05$), and the intensities of both carboxy-H_2DCFDA and DHE-stains stains compared to the H_2O_2-treated group (Figure 3). Furthermore, the cells treated with palm TRF alone also exhibited decreased DHE stain intensity vis-à-vis the control. In this study, pre-treatment of palm TRF successfully reduced the LDH activity and ROS level in the H_2O_2 group, but no changes in the SOD activity is observed (Figure 4). This suggested that the protection of TRF might occur directly by reducing ROS production (Nazrun et al. 2008) without the modulation of SOD activity. Dieterich et al. (2000) reported no changes were detected in the SOD and GPx expression of the heart tissue of patients with heart failure, and these mitochondrial antioxidant enzymes may not involve in the adaptive response to oxidative stress. Palm TRF consists of 70% tocotrienols and 30% tocopherols (Sambanthamurthi et al. 2000). Whereby tocotrienol has greater antioxidant activity than tocopherol (Ali & Woodman 2015). Meanwhile, studies have also reported that tocopherols exert a cardioprotective effect in their ability to protect mitochondria from oxidative stress (Wang et al. 2016). TRF is a well-known scavenger of ROS and it acts by donating electrons to free radicals; thus, inhibiting chain initiation and propagation (Sharma et al. 2012). Therefore, the declining level of ROS in the pre-treatment group could be due to the scavenging activity of TRF. In addition, TRF could act as membrane stabiliser by maintaining membrane integrity, thereby restricting the leakage of LDH (Howard et al. 2011).

The MMP ($\Delta\Psi_m$) is preserved in control groups, as well as in the TRF treatment and TRF pre-treated groups. It is illustrated by the higher intensity of red fluorescence than the green fluorescence (Figure 5(a)). This demonstrates the protective effect of palm TRF on NRCM. The MMP is significantly increased ($p<0.05$) in the pre-treatment with TRF, as observed by the ratio of JC-1 aggregates to JC-1 monomers in NRCM (Figure 5(b)). Previous studies have shown that γ-tocotrienol protects mitochondria from oxidative stress (Nowak et al. 2012), which in turn reduces the occurrence of cell death especially necrosis (Miura et al. 2010). The possible mechanism of γ-tocotrienol protection against cell death is by preventing the opening of mitochondria permeability transition pore and components of the respiratory chain.

This led to the retained ATP production and the prevention of cell death (Nowak et al. 2012; Wang et al. 2016). In this study, the pre-treatment with palm TRF reduced the percentage of necrotic cells (PI^+) significantly compared to the H_2O_2 group ($p<0.05$; Figure 6), and more cells at detected in early and late apoptosis. Myocardial tissue damage during ischemia initiates apoptosis and necrosis; and further lead to nuclear and mitochondrial DNA release, as well as increased purine metabolism (Casey et al. 2007; Fauconnier et al. 2011). Zarkasi et al. (2020) reported that the TRF cardioprotective mechanism in rats with isoprenaline-induced myocardial infarction might be related to the activation of the purine salvage pathway to regenerate new substrates for DNA synthesis. Palm TRF might have an anti-necrotic effect towards NRCM and further investigation needs to be performed to look for the possible mechanism.

H_2O_2 was shown to induce depolarisation of the mitochondrial membrane and increase the occurrence of cell necrosis which was prevented by palm TRF. Previous studies have shown that γ-tocotrienol protects mitochondria from oxidative stress (Nowak et al. 2012), which in turn reduces the occurrence of cell death especially necrosis (Miura et al. 2010). The possible mechanism of γ-tocotrienol preventing cell death is by avoiding the opening of mitochondria permeability transition pore and components of the respiratory chain. Therefore, ATP production is maintained, preventing cell death (Nowak et al. 2012; Wang et al. 2016).

CONCLUSION

This study has demonstrated the protective effect of palm TRF that could protect cells from H_2O_2-induced mitochondrial injury and cell death, which was reflected by the decline in endogenous ROS production.

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