International Journal of Biomedical and Advance Research
ISSN: 2229-3809 (Online); 2455-0558 (Print)
Journal DOI: 10.7439/ijbar
CODEN: IJBABN

Original Research Article

Tocotrienol Rich Fraction supplementation increased the antioxidant enzymes activities in skeletal and heart muscle of aging mice

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Abstract

Aging is initiated from free radical reactions which interrupted the antioxidant/prooxidant balance and consequently causing aging-related disease. Tocotrienol Rich Fraction (TRF) has been reported as a potent antioxidant against oxidative damage by increasing antioxidant enzymes levels in many organs such as brain, bones and blood but not in muscle. This study was carried out to evaluate the effect of long term TRF supplementation on antioxidant status in skeletal and heart muscle of aging mice. Mice were divided into 3 groups, control without treatment (CWT), refined, bleach and deodorization (RBD) oil control and TRF supplementation groups. 13.5 months supplementation was done on 5 months-old rats (young mice) until they reach 18.5 months old (old mice). The activity of antioxidant enzymes, i.e. superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GRx) in skeletal and heart muscles were measured. TRF supplementation increased SOD, CAT and GPx activities significantly in skeletal and heart muscles compared to both control groups. TRF supplementation increased the GRx activity significantly in skeletal and heart muscles compared to CWT but not RBD oil control group. In conclusion, long term TRF supplementation significantly increased the antioxidant enzymes activities in skeletal and heart muscles in aging mice.

Keywords: Aging, Antioxidant enzymes, Heart muscle, Oxidative stress, Skeletal muscle, Tocotrienol Rich Fraction.

1. Introduction

Aging is a physiological process that indicates the impairment of overall tissue function and response towards tissue damage.[1] According to the free radical theory of aging, reactive oxygen species (ROS) generated through mitochondria metabolism imposed deleterious effects towards the cells which would then lead to declination in the cell and organ functions and subsequently degenerative diseases and aging.[2, 3] Mitochondria produce less ATP and increase the production of reactive oxygen species (ROS) as by-products of aerobic metabolism in the aging tissues of the human and animals. The activities of free radical-scavenging enzymes are also altered in aging process which is due to the impairment of antioxidant enzymes activities.[4]

The activities and capacities of antioxidant systems of tissue cells are declined with age, leading to the gradual loss of prooxidant/antioxidant balance and accumulation of oxidative damage in the aging process. The concurrent age-related changes of these two systems result in the elevation of oxidative stress in aging tissues. Within a certain concentration range, ROS may induce stress response of the cells by altering expression of respiratory genes to uphold the energy metabolism to rescue the cell. However, beyond the threshold, ROS may cause a wide spectrum of oxidative damage to various cellular components to result in cell death or elicit apoptosis which contributes to age-related diseases.[4] Oxidative stress theory suggested that antioxidants might increase human lifespan since they could give the protection against oxidants.[5]

Antioxidant defense system comprises of enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and non-enzymatic antioxidants including vitamin A, vitamin C, vitamin E, ubiquinone and flavonoids. Antioxidants are molecules which interact with ROS and scavenge the free radicals preventing cellular damage and disease. Vitamin E is a potent naturally occurring lipid-soluble antioxidant possesses the ability to directly quench free radicals and function as a membrane stabilizer. It protects critical cellular structures against oxidative damage

from oxygen free radicals and reactive products of lipid peroxidation. The protective effect of vitamin E supplementation against oxidative stress induced by exercise has been reported in humans and rats.[6, 7, 8] It is also believed that antioxidant could intervened the processes of degenerative diseases which also responsible for the ageing phenomena. Reports also showed that vitamin E dietary supplementation have been shown to have unique properties which influence many pathways involved in age-related diseases such as cancer, [9, 10] cardiovascular [11, 12] and neurodegenerative disease.[13, 14]

Vitamin E is an important antioxidant vitamin, playing an essential protective role against free radical damage.[15] Vitamin E comprises a group of substances belonging to 2 closely related families: tocopherols and tocotrienols, each existing in a number of isomeric forms: alpha, beta, gamma, and delta.[16] Administration of antioxidants such as vitamin E and overexpression of antioxidative enzymes, such as SOD, CAT and GRx, have in some cases increased the life expectancy of man.[5] Vitamin E dietary supplementation also showed significant effects on multiple risk factors for cardiovascular disease in baboons where 7 week supplementation of vitamin E resulted in significant increase of the total antioxidant status and lowering the oxidized LDL.[17]

In aging, muscle is one of the most affected organs that are less studied and the involvement of oxidative stress in muscle aging is not well understood. Previous researches were prone to investigate either the supplementation of tocopherol or the organs such as brain in conjunction with the neurodegenerative disease, but less were associated with the aging of muscle. Thus, there is an increasing interest on the effects of dietary antioxidants to encounter the role of oxidative stress on the aetiology of aging-related modifications in muscle. We are interested in investigating the effect of tocotrienol enriched vitamin E supplementation on age-associated antioxidant enzymes status in skeletal and heart muscles. In this study, Vitamin E (Tri E®) used was tocotrienol rich fraction (TRF) palm oil which contains 70% δ-tocotrienol and 30% α-tocopherol. From the previous aging studies, TRF is reported to have anti-aging properties on wide range of organs i.e. in skin [18], brain [19], and bone aging.[20]

2. Materials and methods

Experiments were conducted to examine the effects of TRF on antioxidant enzymes activity in aging mice after 13.5 months supplementation.

2.1 Animals

12 male mice C57BL/6 aged 5 months were housed at room temperature with 24 hours ventilation in three groups, two control groups: control without treatment (CWT) group and refined, bleach and deodorization (RBD) oil control group and one treatment group: TRF group. . The mice were fed 'rat chow' and drinking water made available ad libitum. Four mice were placed in each group which received following supplementation: CWT group was not received any supplementation, RBD oil l group was given 30mg/kg RBD oil and TRF group was given 30mg/kg TRF. 30 mg of TRF from Golden Hope Biogenic SDN BHD (Malaysia) was diluted in 1ml RBD oil from the same company. The diluted TRF was given orally to mice using oral gavage every day. Mice from control and treatment groups were supplemented continuously for 13.5 months starting from 5 months of age equivalent to 18.5 years old human until 18.5 months of age equivalent to 68.5 years old human. After supplementation period, the mice were sacrificed; the skeletal and heart muscles of mice were removed and weighed. This study was approved by the UKM Animal Ethics Committee (FP/BIOK/2008/NOOR/9-APR/223-APR-2008-NOV-2010).

2.2 Determination of antioxidant enzymes activity

For antioxidant enzyme activity analysis, tissues were homogenized in 1.15% KCl (g/5ml) and centrifuged at 8000rpm 9000gm using ultracentrifuge for 20 minutes in 4°C. Supernatant obtained were centrifuged at 35000rpm 105000gm using ultracentrifuge for 1 hour in 4°C to get the cytosol and it was kept at -80°C for further analysis.

Superoxide dismutase (SOD) activity was determined.[21] Absorbance was then measured at a wavelength of 560nm. One unit of SOD was defined as the amount of enzyme required to inhibit nitro tetrazolium reduction by 50% in per ml lysate. Enzyme activity was expressed as units per mg of protein (U/mg protein). Catalase (CAT) enzyme activity was determined.[22] The absorbance was measured at a wavelength of 240nm. One unit of catalase enzyme was defined as the amount of enzyme which liberates half the peroxide oxygen from H_2O_2 solution in 30s at room temperature. Enzyme activity was expressed as units per mg of protein (U/mg protein).

Glutathione peroxidase (GPx) assay was determined.[23] The conversion of NADPH to NADP+ was followed by measuring the change in O.D/min at 340nm. One unit of GPx was defined as the amount of enzyme required to oxidize 1 µmol NADPH/min per ml lysate or liver cytosol. Enzyme activity was expressed as miliunits per mg of protein (mU/mg protein). Determination of glutathione reductase (GRx) enzyme activity has found.[24] The principle is based on the presence of the enzyme, hydrogen will be transferred from reduced triphosphopyridine nucleotide (TPNH) to glutathione (GSH) and the reaction was measured at 340 nm.

2.3 Statistical analysis

Data are presented as means ± standard deviations (SD). Data analysis was performed using Statistical Package for Social Sciences (SPSS 17.0) software and Microsoft Excel 2007. Statistical test used was ANOVA followed by Tukey' hsd for normally distributed data and Kruskal-Wallis and Mann-Whitney test for data that was not normally

distributed. Differences were considered significant when P-value was <0.05.

3. Results

In skeletal muscle, activity of SOD, CAT and GPx was found significantly higher ($p<0.05$) in TRF supplementation group as compared to CWT group and RBD oil group (Figure 1A, 1B, 1C). However, all of the above enzymes showed significantly higher in RBD oil group ($p<0.05$) as compared to CWT group. GRx activity was significantly higher ($p<0.05$) in TRF supplementation group as compared to CWT group but there were no significant difference between TRF and RBD oil groups (Figure 1D). There were also significantly higher ($p<0.05$) in GRx activity for RBD oil group as compared to CWT group in skeletal muscle of aging mice after supplementation.

In the heart muscle, the activity of SOD enzyme was significantly higher for TRF supplementation group compared to CWT group and RBD oil group (Figure 2A). However, this enzyme was found to be significantly higher in RBD oil group compared to CWT group. For CAT and GPx enzymes, the activity of both enzymes for TRF supplementation group were significantly higher compared to CWT group and RBD oil group (Figure 2B, 2C). However, there was no significant difference in both control groups. For GRx enzyme activity in heart, TRF supplementation group was significantly higher compared to CWT group but no significant difference with RBD oil group (Figure 2D). However, there was no significant difference between these two control groups for this enzyme.

Figure 1: Activities of antioxidant enzymes in skeletal muscle of aging mice supplemented with TRF

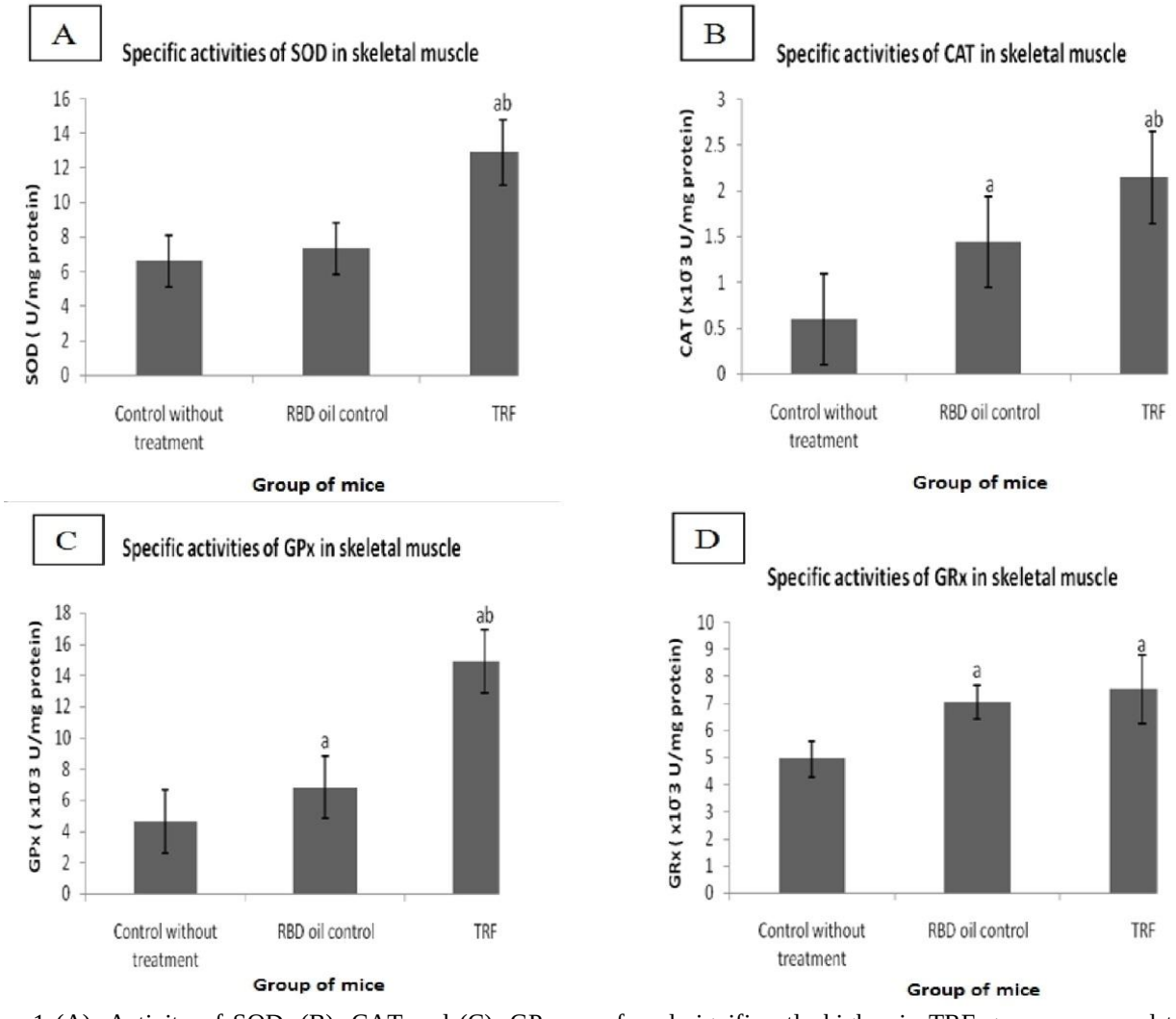

Figure 1 (A): Activity of SOD, (B): CAT and (C): GPx was found significantly higher in TRF group compared to control without treatment (CWT) and RBD oil group. (D): GRx activity was significantly higher in TRF group compared to CWT group but there was no significant difference between TRF and RBD oil group. All of the enzymes activities are significantly higher in RBD oil group as compared to CWT group.

a – $p<0.05$ significant difference as compared to CWT group

b – $p<0.05$ significant difference as compared to RBD oil group

Figure 2: Activities of antioxidant enzymes in heart of aging mice supplemented with TRF

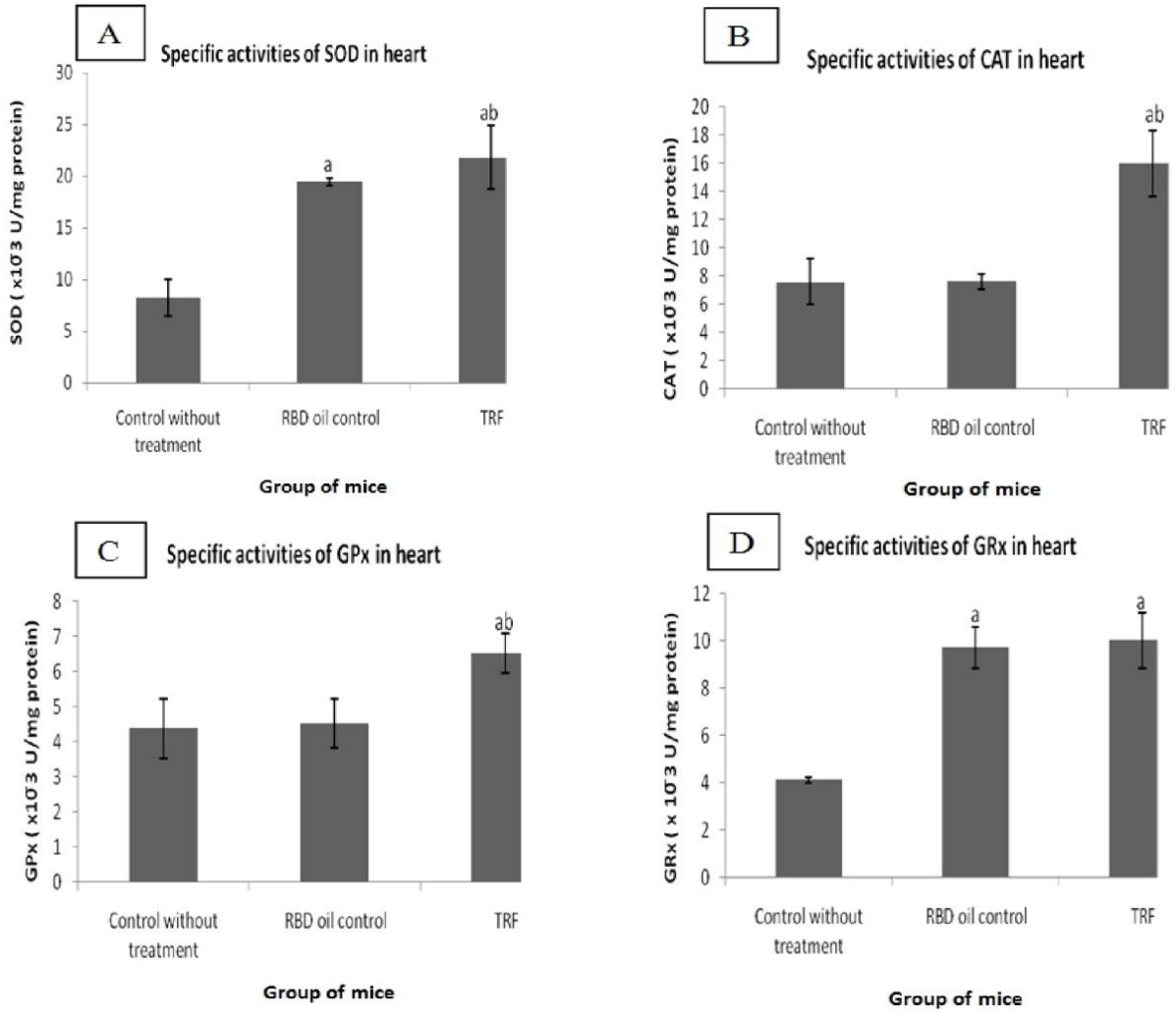

Figure 2 (A): The activity of SOD enzyme was significantly higher for TRF group compared to CWT group and RBD oil group. RBD oil group also showed a significantly higher of SOD activity compared to CWT group. (B): CAT and (C): GPx activities for TRF group were significantly higher compared to CWT group and RBD oil group but no significant difference in both control groups. (D): GRx activity of TRF group was significantly higher compared to CWT treatment group but no significant difference with RBD oil group and there was no significant difference between these two control groups for this enzyme.

a – $p<0.05$ significant difference as compared to CWT group

b – $p<0.05$ significant difference as compared to RBD oil group

4. Discussion

Sarcopenia, the progressive loss of skeletal muscle mass, strength and function with the increasing age, has now become a significant issue among the gerontologist since the year 2010 as it imposed serious health consequences in terms of frailty, immobility and morbidity.[25] Previous studies suggested the involvement of oxidative stress on the aging-related skeletal muscle deteriorations as the highly oxygen-consuming skeletal muscle is prone to oxidative-stress-induced aging.[26, 27] A variety of markers, such as thiobarbituric reactive substances (TBARS), urinary 8-hydroxy-deoxyguanine secretion (8-OHDG), malondialdehyde (MDA), 4-hydroxyalkenals (HAE), and protein carbonyls were measured to evaluate the presence of oxidative stress damage towards aged skeletal muscle. Aging has increased the presence of MDA, 4-HAE, 8-OHDG, protein carbonyls and H_2O_2 content and decreased the GSH/GSSG ratio in the skeletal muscle of aged rats concurrently.[27-29] Thus, we postulated that aging in skeletal muscle was associated with the increasing oxidative damage and the involvement of free radicals. Various interventions such as exercise, growth hormone and testosterone have been proposed to improve the condition of skeletal muscle during aging.

Skeletal muscle possess an array of defence mechanism includes the enzymatic and non-enzymatic antioxidants in order to counteract the free radical damages. The antioxidant enzymes SOD, CAT and GPx worked in a network in order to eliminate the ROS. SOD dismutase the superoxide radical ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2),

followed by the decomposition of H_2O_2 to water by the GPx and CAT which work in parallel.[30] GRx involved in the reduction of glutathione disulfide (GSSG) to glutathione (GSH) in the expense of nicotinamide adenine dinucleotide phosphate (NADPH). The decreased activity of these antioxidant enzymes in the skeletal muscle of aged rats are reported previously.[28] However, our findings revealed that the supplementation of TRF increased the activities of these enzymes in the aged skeletal muscle of mice. TRF prevent the propagation of peroxyl radical and further preventing free radical chain reaction, leading to the protection from oxidative damage. Asides from itself acting as non-enzymatic antioxidant, present study revealed the involvement of TRF to activate an array of antioxidant enzymes in the skeletal muscle in order to neutralize the increasing ROS during aging. Increment of activity of GRx, one of the GSH metabolizing enzymes upon 13.5 months supplementation of TRF suggested the replenishment of GSH which decreased with age in the skeletal muscle.[28] GSH protects the cells from oxidative damage via directly scavenging radicals as well as serving as electron donors for GSH-dependent antioxidant enzymes, such as GPx and GRx.[29, 31] In short, there is a possibility where the effects of TRF on the antioxidants enzymes SOD, CAT, GPx and GRx in the skeletal muscle are interrelate.

To our knowledge, previous studies involving antioxidant towards the aged skeletal muscle were mainly vitamin E (α-tocopherol), vitamin D and vitamin C. The reported increase of activity of SOD and CAT in the skeletal muscle of aged mice is in agreement with a study carried out using dietary combination of vitamin E (α-tocopherol) and vitamin C.[29] However, the combination of vitamin E (α-tocopherol) (30 000 mg/kg) and vitamin C reduced the activity of GPx in the skeletal muscle of aged rats (30 months of age) which is contrary to our findings using TRF as our target supplementation.[29] Moreover, combination of vitamin E (300 IU/kg) with others nutrients including vitamin A, zinc and selenium did not exert alteration on the antioxidant capacity, SOD activity and oxidative damage of the skeletal muscle of aged rats.[29] Instead, the findings reported the improvement of total glutathione in liver and reduced protein oxidative damage in the liver and spleen of the aged rats supplemented with the combination therapy. The contrast on the activity of antioxidant activity may suggest the differences in the mechanisms modulated by combination therapy of vitamin E (α-tocopherol) with other nutrients and our target supplementation which is solely tocotrienol rich fraction (TRF). Besides that, dose and duration of supplementation may contribute to the differences in the activity of antioxidant enzymes in the skeletal muscle of aged mice.

Aging-related deteriorations in the cardiovascular contribute to the incidence of atherosclerosis, stroke, congestive heart failure and finally death. Previous study reported that cardiovascular aging is associated with the accumulation of peroxidation of lipid and protein.[33] Moreover, the activity of antioxidant enzymes SOD, CAT, GPx and GRx were reduced during aging.[34] Various interventions involving antioxidant supplementation such as green tea extraction, β-carotene, vitamin C and vitamin E has been postulated to overwhelm the involvement of ROS in the aging of heart.[35] Interestingly, our present study shows that supplementation of TRF increased the activity of antioxidant enzymes SOD, CAT, GPx and GRx in the heart muscle of aged mice. The increased activity of cardiac antioxidant enzymes via TRF was in line with the effects of green tea extract towards the aging heart in the rats.[34]

Endothelial dysfunction in aging is mainly due to overproduction of ROS and the oxidative stress have severe impact on the development of coronary artery disease and stroke in the elderly.[36] Hypertension and diabetes are closely associated with the endothelial dysfunction.[33] Budin *et al* (2009) reported that orally administration of TRF (200mg/kg/day) for 8 weeks reduced the blood glucose levels, MDA and 4-hydroxynonenal (4-HNE) content and the activity of SOD in the aorta of streptozotocin-induced diabetic rats and concurrently improved dyslipidemia.[34] On contrary, Shirpoor *et al* (2009) reported 6 weeks TRF supplementation increased the activity of SOD and CAT and reduced the level of oxidation stress (8-isoprostane and protein carbonyl) in the heart of diabetic rats. Even though some findings reported the effects of TRF on the activity of SOD in contrast with our findings, TRF in whole exert a positive effect in maintaining the vascular wall integrity in the oxidative stress-induced diabetic mice. The difference in the findings may due to the age of animal model, difference of doses and duration of treatment.[35]

TRF supplementation may exert different effects on aging, depending on the organ investigated. In contrast to our findings, previous study demonstrated that 2 months of TRF supplementation (30 mg/ kg body weight) decreased the activity of SOD in the plasma of aging mice, but without any effect on the activity of CAT and GPx in the plasma of aging C57BL/6 inbred mice.[37] Similarly, 6 months of TRF supplementation reduced the activity of SOD, but increased the activity of GPx without affecting the activity of CAT in the plasma of aged human subjects.[30] These results were contradicted with our measurements of antioxidant enzymes' activities in the skeletal and heart muscle of aged rats. Such inconsistency in the reported literature may be due to the subject variation, dose and duration of TRF treatment, targeted organ, methodology and the environmental factors.

On the other hand, some studies have shown that TRF supplementation increase the antioxidant enzymes' activity in aging rats which similar to our findings. In our study, it is observed that the long term supplementation of TRF to the young 5 month-old mice until they reach 18.5 month-old aged mice, increased the activity of SOD, CAT,

GPx and GRx in the skeletal and heart muscles respectively. This was in line with results by a study where 8 months of TRF supplementation protects against oxidative DNA damage and improve cognitive functions of brain aging rats while in the same time increased the activity of antioxidant enzymes (SOD, CAT and GPx) in the blood of the rats.[19] Hence, one may proposed that TRF supplementation imposed different effects on activity of antioxidant enzymes in different organs.

Interestingly, RBD palm oil as the vehicle control in this study exhibited positive effects on the activity of the antioxidant enzyme in the skeletal and heart muscles of the aged mice. RBD palm oil is produced through the processing of crude palm oil involving removal of a significant amount of free fatty acid, carotene and tocopherols.[41, 42] RBD palm oil consists of low level of fatty acids, total vitamin E, α-tocopherol, phytosterols and carotenoids in relatives to red palm oil.[42] Our findings reported that in the skeletal muscle of aged rats, RBD palm oil increased the activity of antioxidant enzymes except the SOD enzyme in relative to the control group. In addition, RBD palm oil raised the activity of antioxidant enzymes SOD and GRx in the heart muscle of aged mice without any significant effect on the activity of enzyme CAT and GPx. Based on the findings, we hypothesized that the traces of vitamin E may have adequate effect on the activity of antioxidant enzymes in the skeletal and heart muscles of aged mice. However, as a whole, TRF supplementation exerted better and higher antioxidant enzymes' activities in the skeletal and heart muscles of aged mice except the GRx enzyme. In conclusion, TRF supplementation was able to increase endogenous antioxidant enzymes in skeletal and heart muscles which function to protect tissues and organs from oxidative damage which contribute to sarcopenia and cardiovascular disease in aging thus increase the longevity.

Acknowledgements

This study was financially supported by Universiti Kebangsaan Malaysia Research University Grant (UKM-GUP-SK-07-21-199).

Conflict of interest

The authors declare that they have no conflict of interests.

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