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Research Article
Effect of Tocotrienol-Rich Fraction on Superoxide Dismutase 3 Expression in Mice Liver

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Abstract

Background and Objective: Superoxide Dismutase (SOD) is responsible for the dismutation of superoxide radicals and protect cells from toxic impairment due to excessive oxygen exposure. Overexpression of the superoxide dismutase 3 isoenzyme (SOD3) had been suggested to prevent cancer growth. Tocotrienols are the more potent members of the vitamin E family. The objective of this study was to investigate the dose-dependent effect of Tocotrienol Rich Fraction (TRF) supplementation on liver SOD3 gene and protein expression. **Materials and Methods:** Thirty male ICR white mice were divided into five groups [control, TRF-treated groups (T200, T500, T1000) and BHA (the positive control)]. After 14 days of treatment, the animals were sacrificed. Liver RNA and protein were extracted for qPCR and Western blotting to determine SOD3 gene and protein expressions and analysed using one-way ANOVA and Student's t-test. **Results:** TRF oral administration significantly increased liver SOD3 gene and protein expressions dose-dependently, compared to controls. Liver SOD3 gene and protein expressions are significantly correlated with the doses of TRF administered. SOD3 gene expression is significantly highly correlated with SOD3 protein expression. **Conclusion:** In conclusion, TRF oral treatment for 14 days resulted in increased SOD3 gene and protein expression in mice liver in a dose-dependent manner, with the highest expression seen in mice treated with 1000 mg kg^{-1} TRF.

Key words: Superoxide dismutase 3, SOD3, liver, mice, tocotrienols, TRF, anti-ageing

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Eight analogues of vitamin E exist in nature and they are α, β, γ, δ tocopherols and α, β, γ, δ tocotrienols. Structurally, tocopherols have saturated side chains with no double bonds and can be found in large quantities in vegetable oils such as sunflower, wheat germ and canola oils. Tocotrienols have unsaturated side chains and with three double bonds and are abundantly found in palm oil, rice bran oil and annatto seeds. The unique structures of tocotrienols could explain their greater antioxidative potentials compared to tocopherols[1]. Palm oil obtained from the palm fruit of the *Elaeis guineensis* tree contains the most plentiful source of natural tocotrienols (70% tocotrienols and 30% tocopherols)[2,3]. The Tocotrienol-Rich Fraction (TRF) derived from the extraction process of palm oil that was utilized in our study mainly comprise isomers of tocotrienols. It had been revealed in previous studies that tocotrienols displayed antioxidant, anti-inflammatory, anti-ageing and anti-cancer properties[4-6].

In terms of elevating *in vivo* antioxidant potential, tocotrienols had been shown to increase the activity of antioxidant enzymes such as Superoxide Dismutase (SOD), NADPH: quinone oxidoreductase (NQO) and Glutathione Peroxidase (GPX) that neutralize free radicals[7]. The results of previous studies had also indicated that tocotrienols were able to induce the activity and/or expression of several phase II xenobiotic-metabolizing enzymes involved in cancer chemoprevention[8]. However, the detailed mechanisms in which tocotrienols increase the activities of these enzymes are still not well-understood. Phase II enzymes and antioxidant proteins are mainly regulated by a nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and are important for cellular defence by accelerating the removal of free radicals and toxic by-products[9]. As such, Superoxide Dismutase (SOD), also considered to be a phase II enzyme, is postulated to play a protective role against cancer and other diseases[10,11].

Superoxide Dismutase (SOD) is an antioxidant enzyme that mitigates the deleterious effects of several Reactive Oxygen Species (ROS), specifically the superoxide anion radicals. SOD is a highly efficient enzyme, where it catalyses the dismutation of two superoxide radicals to create Hydrogen Peroxide (H_2O_2) and oxygen. H_2O_2 is further converted to water and oxygen in a reaction catalyzed by catalase and peroxiredoxin.

There are three isoforms of SOD that have been identified in mammalians, i.e.,cytoplasmic (SOD1), mitochondrial (SOD2) and extracellular (SOD3)[12]. SOD1 is a soluble cytoplasmic protein that exists as a homodimer that binds zinc and copper ions. SOD2 exists in the mitochondria in the form of homotetramer, where it binds the superoxide byproducts of oxidative phosphorylation and changes them to Hydrogen Peroxide (H_2O_2) and diatomic oxygen. SOD3 is secreted into the extracellular space and forms a glycosylated homotetramer connected to the extracellular matrix and cell surfaces through interaction with heparin sulfate proteoglycan and collagen[12]. Previous studies had revealed that SOD3 expression level is down regulated in breast and pancreatic carcinomas. Induced overexpression of SOD3 resulted in decreased growth and decreased metastasis of pancreatic cancer[13]. Increased expression of SOD3 was postulated to cause further inhibition of liver carcinogenesis[14]. It had been suggested in a previous study that SOD3 expression (but not SOD1 and SOD2) in rat mammary tissues was induced by the administration of antioxidants to experimental animals and the mechanism in which the antioxidants increased SOD3 expression in mammary tissues is through the Nrf2 pathway activation[10].

The effects of the vitamin E isomer γ-tocotrienol on the lung tissues of mice had been previously investigated[15,16]. In these studies, it was found that γ-tocotrienol administration increased SOD3 expression in mice lung tissues. Previous studies had also discovered that in mice treated with TRF, the activity of the SOD enzyme was increased. However, in these studies, the gene and protein expression profiles of liver SOD isoenzymes were not performed[17,18].

Therefore, the main objective of our study is to determine the dose-dependent effect of TRF oral administration on SOD3 gene and protein expression in mice livers.

MATERIALS AND METHODS

Study area: The study was carried out at the Animal Handling Facility, the Biochemistry Laboratory and the Molecular Laboratory in the Department of Pharmacology, Faculty of Medicine, Universiti Kebangsaan Malaysia, from October, 2014-December, 2015.

Materials: Tocotrienol-Rich Fraction (TRF) (trademarked as Gold TRI E 70™) was produced by the Sime Darby Corporation (located in Selangor, Malaysia). TRF is naturally made up of a mixture of alpha-tocotrienol (26%), beta-tocotrienol (4%), gamma-tocotrienol (32%), delta-tocotrienol (14%) and alpha-tocotrienol (24%). The chemical TRIzol was sourced from a company called Life Technologies (which is located in Carlsbad, California, USA). RNA reverse transcription kit for producing cDNA (iScript™), the kit used for qPCR assay (iQ™ SYBR Green supermix) and the thermal cycler used for PCR (MJMini™) are all bought from the Bio-Rad company located in Hercules, California, USA.

Primers for qPCR were obtained from a company called Vivantis Technologies which is located in the city of Oceanside, California, USA. The lysis buffer for protein extraction (RIPA lysis buffer) and the secondary antibody used for Western blotting (goat anti-rabbit IgG peroxidase) was bought from the Santa Cruz Biotechnology Company in the USA. The reagents used for detecting chemiluminescent signals in the Western blotting procedure were obtained from the Amersham Company located in Uppsala, Sweden. The membrane used for Western blotting (nitrocellulose) and the reagent used to visualize protein transfer in the membrane (Ponceau S dye) were bought from the Sigma-Aldrich company located in the town of Seelze, Germany. Primary polyclonal rabbit anti-mouse SOD3 antibody and Primary polyclonal rabbit anti-mouse β-actin antibody were purchased from Abcam Biotechnology (Cambridge, UK). All other chemicals were purchased from Sigma-Aldrich unless otherwise stated.

Animals and treatment: Male ICR white mice (25-30 g) were utilized in this study and were obtained from the Universiti Kebangsaan Malaysia Laboratory Animal Research Unit (LARU). The mice were kept in clean polypropylene cages in a ventilated room, with food and water available *ad libitum*. The food intake and body weight of mice were measured daily. Animals were treated with TRF (dissolved in corn oil) at three different increasing doses. The mice were divided into 5 groups. The first group consists of mice (n = 6) orally administered with the vehicle (corn oil) and was designated as the control group. The second group of mice (n = 6) were orally administered with 200 mg kg^{-1} TRF. The third group of mice (n = 6) were orally administered with 500 mg kg^{-1} TRF. The fourth group of mice (n = 6) were oral administered with 1000 mg kg^{-1} TRF. The fifth group of mice (n = 6) were orally administered with 100 mg kg^{-1} butylated hydroxyanisole (BHA) and was designated as the positive control group. All treatments were given for 14 consecutive days. On day 15, mice were sacrificed *via* cervical dislocation. Their livers were subsequently isolated, snapped frozen in liquid nitrogen and stored at -80°C until further use. All experimental procedures involving animals were approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC) with the approval number: PP/FAR/2011/AZMAN/22-MARCH/361-MAY-2011-MAY-2013.

RNA extraction: Total RNA from liver tissues was procured using TRIzol reagent, according to standard procedure. In summary, isopropyl alcohol (Sigma, USA) was added during each extraction step to precipitate the total RNA. The RNA pellet was then cleansed with 75% ethanol and dried before being solubilized in RNase-free water. Total RNA was kept at -80°C immediately after being extracted. The concentration and purity of the extracted RNA were obtained using NanoDrop spectrophotometer 2000c (Thermo Scientific, USA) at a wavelength of 260 nm (OD260). RNA with RNA integrity number (RIN) ranging from 7-10 and absorbance ratio of A260-A280 ranging from 1.5-2.0 was used for cDNA synthesis.

Reverse transcription: cDNA was synthesized from RNA using an iScript cDNA synthesis kit (Bio-Rad, USA) according to the manufacturer's instructions. In short, a volume (containing 1 μg) of total RNA from each sample was added to a mixture of 4 μL of 5X iScript reaction mix, 1 μL of iScript reverse transcriptase and a suitable volume of nuclease-free water (the final reaction mix volume is 20 μL). The final reaction mix was incubated at 25°C for 5 min, 42°C for 30 min and heated to 85°C for 5 min in a thermocycler (TC-412, Techne, Barloworld Scientific, UK). The cDNA was then used as a template for amplification by polymerase chain reaction (PCR).

Quantification of SOD3 gene expression by quantitative real-time PCR: Quantitative real-time PCR was performed using the MiniOpticon cycler (Bio-Rad, USA). The total reaction volume used was 20μL, comprising 1 μL of 10 μM forward primer and 1 μL of 10 μM reverse primer (500 nM final concentration of each primer), 10.0 μL of iQ™ SYBR® Green Supermix (2X) (Bio-Rad, USA), 6.0 μL of nuclease-free water and 2.0 μL of cDNA. Both forward and reverse primers for the genes of interest in this study were designed according to previous studies and synthesized by Vivantis Technologies (Oceanside, CA, USA). The primer sequences for our genes of interest are shown in Table 1.

The thermocycling conditions were initiated at 95°C for 30 sec, followed by 40 PCR cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 30 sec. At the end of each cycle, a melting curve (dissociation stage) analysis was performed to determine the specificity of the primers and the purity of the final PCR product. All measurements were performed in triplicate and NoTemplate Controls (NTC) were incorporated onto the same set of PCR tubes to test for contamination by any assay reagents. Threshold cycles were determined for each gene and quantification of templates was performed according to the relative standard curve method. The relative gene expression (DDCt) technique, as defined in the Applied Biosystems User Bulletin No. 2[21], was used to analyse the real-time PCR data.

Table 1: Primer sequence for SOD3 and GAPDH

Gene	Primer sequence	References
SOD3	F: 5′-CCTTCTTGTTCTACGGCTTGC-3′	Seta *et al.*[19]
	R: 5′-TCGCCTATCTTCTCAACCAGG-3′	
GADPH	F: 5′-GTGGAGTCTACTGGTGTCTTCA-3′	Kong *et al.*[20]
	R: 5′-TTGCTGACAATCTTGAGTGAGT-3′	

In this method, the SOD3 gene expression levels were normalized relatively against the expression levels of the housekeeping gene GAPDH in each assay runs. To validate the result of the melting curve analysis of the qPCR assay and also to determine the size of the PCR product, conventional agarose gel electrophoresis was further attempted. Electrophoresis was done using 1% agarose to obtain adequate band separation. For post-electrophoresis gel staining, a biosafe dye was utilised i.e., GelRed™ nucleic acid gel stain, which was produced by the Biotium Company located at Fremont, California, USA. The gels were immersed within the dye solution and stained for 30 min. The destaining step with distilled water was performed for 30 min. The visualization of the agarose gel bands was done under ultraviolet light using the FluorChem FC2 gel documentation system produced by the Alpha Innotech Company located in San Jose, California, USA.

Total protein extraction for western blotting: Liver protein extracts from each liver sample were produced by homogenising 100 mg of liver tissue in 500 mL RIPA lysis buffer. Every 1 mL of 1×RIPA lysis buffer was fortified with 10 µL phenylmethylsulfonyl fluoride (PMSF), 10 µL sodium orthovanadate and 10 µL protease inhibitor cocktail solution. The crude liver homogenates were subjected to centrifugation at a speed of 13,000×g for 30 min at 4°C. The resulting supernatants were collected and their concentrations were determined using the method of Lowry which utilised bovine serum albumin as protein standards[22].

Western blotting: The Western immunoblotting method was performed for the detection of SOD3 protein. Initially, the liver protein extract (100 µg) must be separated first using sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresis. In this study, 15% SDS-PAGE gels were used for better protein separation. After the electrophoresis step had completed, the process of transferring the separated proteins contained within the gel onto the nitrocellulose membrane was performed using the wet transfer method. The membrane was then incubated for 20 min at room temperature in a blocking solution (150 mM NaCl, 3 mM KCl, 25 mM Tris, 0.1% (v/v) tween-20 and 10% non-fat milk powder (pH 7.4)). After blocking, the membrane was incubated with primary polyclonal rabbit anti-mouse SOD3 antibody and primary polyclonal rabbit anti-mouse β-actin antibody for 1 hr at room temperature. Subsequently, incubation with a peroxidase-conjugated goat anti-rabbit IgG secondary antibody was carried out for another hour at room temperature. Protein bands were visualized using the enhanced chemiluminescence method according to the manufacturer's instructions (Amersham, Uppsala, Sweden). The intensity of the protein bands was quantified, relative to the signals obtained for actin, using ImageJ software.

Statistical analysis: Data are presented as mean±standard error of the mean (SEM). Significant differences between mean values of multiple groups were determined using one-way ANOVA and Student's t-test. The SPSS software (version 22) was used to statistically analyze the data obtained in this study. The results were deemed to be statistically significant when the p-value is less than 0.05 ($p<0.05$).

RESULTS

Body weight and food intake after TRF treatment: To determine if different doses of TRF (200, 500 and 1000 mg kg^{-1}) influenced the weight and intake of food in mice, those parameters were measured and recorded every two days until the mice were sacrificed on day 15. In mice administered orally with 1000 mg kg^{-1} TRF, their weight and intake of food decreased slightly after 14 days of treatment compared to the other groups (Table 2). The weight and food intake of mice administered orally with 200 and 500 mg kg^{-1} TRF did not differ much from the mice in the control group. However, the differences in weight and intake of food between all the various experimental groups at the end of the treatment period were not statistically significant (Table 2). BHA treatment itself did not induce any significant difference in those parameters and the results did not vary prominently from those of the control and TRF-treated groups.

Liver SOD3 gene expression: To analyze the effect of TRF administration on SOD3 gene expression, mice were treated orally with different doses of TRF (200, 500 and 1000 mg kg^{-1}) in the presence of vehicle-treated control mice (fed corn oil) and a positive control group administered BHA (100 mg kg^{-1}) for 14 days. After 14 days of treatment, the mice were killed, their livers isolated and SOD3 gene expression in the liver was measured using quantitative real-time PCR (qPCR). As shown in Fig. 1, TRF at concentrations of 200, 500 and 1000 mg kg^{-1} resulted in a significant dose-dependent increase in the fold change of SOD3 gene expression levels (1.6-, 2.7- and 4.0-fold

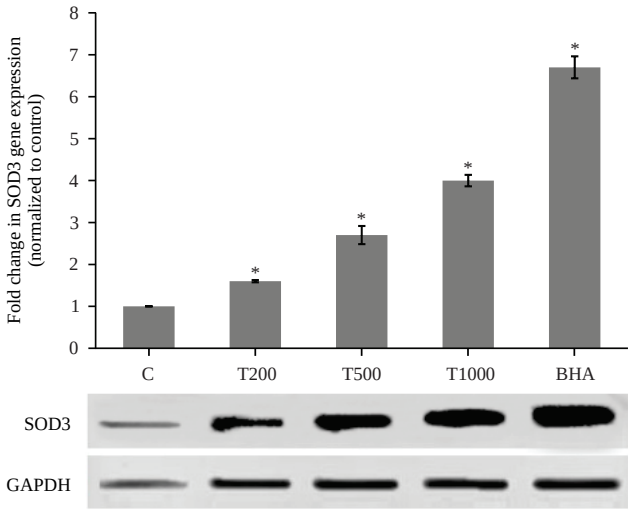

Fig. 1: Effect of different doses of TRF on SOD3 gene expression in the livers of mice, evaluated using real-time PCR (qPCR)

Mice were treated with 200, 500 and 1000 mg kg^{-1} TRF for 14 days. Data are presented as Mean±SEM. Amplified products were also visualized by agarose gel electrophoresis and gene expression was confirmed by the identification of the appropriate bands. GAPDH served as the reference gene. T200: TRF at a dose of 200 mg kg^{-1}, T500: TRF at a dose of 500 mg kg^{-1}, T1000: TRF at a dose of 1000 mg kg^{-1}, C: Control mice, BHA: Positive control group was given butylated hydroxyanisole (100 mg kg^{-1}). Asterisk (*) indicates a statistically significant difference from the control group (p<0.05)

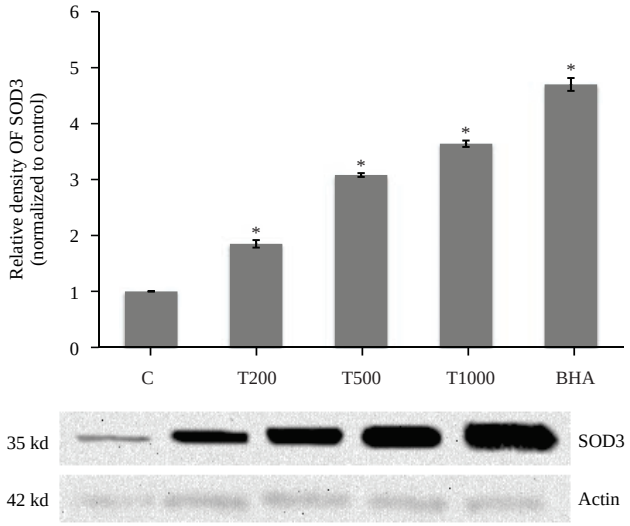

Fig. 2: Effect of different doses of TRF on SOD3 liver protein expression. Mice were treated with 200, 500 and 1000 mg kg^{-1} TRF for 14 days

Their livers were then harvested and SOD3 protein expression levels were determined by Western blotting. The intensity of protein bands was quantified relative to the signals obtained for actin using ImageJ software and was normalized to control. The graph represents the average optical density (±SEM) of bands from three different experiments. Asterisk (*) indicates a statistically significant difference from the control group (p<0.05). T200: TRF at a dose of 200 mg kg^{-1}, T500: TRF at a dose of 500 mg kg^{-1}, T1000: TRF at a dose of 1000 mg kg^{-1}, C: Control mice, BHA: Positive control group was given butylated hydroxyanisole (100 mg kg^{-1})

Table 2: Body weight and food intake of control and treated mice

	Body weight (b.wt.)		
Groups	Day 1 (g)	Day 14 (g)	Food intake (gb. wt.$^{-1}$/day)
Control	28.67±2.13	33.33±2.27	4.50±1.11
T200	27.67±1.96	32.33±2.11	4.37±1.85
T500	28.33±1.25	30.33±1.84	4.17±1.92
T1000	31.67±1.17	30.33±2.18	2.09±1.16
BHA	30.00±1.32	30.67±1.43	4.93±1.23

T200, T500, T1000: Groups of mice administered TRF orally at a daily dose of 200, 500 and 1000 mg kg^{-1} b.wt., respectively. Control: Control mice, BHA: A positive control group that was given butylated hydroxyanisole (100 mg kg^{-1}). Values are given as Mean±SEM (n = 6 for each group). No statistical significance was found between control and treated groups (ANOVA)

respectively, as compared to controls; p<0.05). Mice that were treated with 100 mg kg^{-1}BHA also displayed significant increases in SOD3 gene expression levels, compared to control mice (6.7-fold; p<0.05) (Fig. 1).

Liver SOD3 protein expression: Oral treatment with TRF at concentrations of 200, 500 and 1000 mg kg^{-1} to mice for 14 days significantly elevated liver SOD3 protein expression levels by 1.9-, 3.1- and 3.6-fold respectively, compared to controls (p<0.05). After 14 days, mice treated orally with BHA (100 mg kg^{-1}) for 14 days showed the highest increase in SOD3 protein expression levels (4.7-fold; p<0.05), compared to controls (Fig. 2).

The observed effect of SOD3 protein expression levels is in agreement with the observed effect on gene expression levels, in which the lowest expression was observed at the dose of 200 mg kg^{-1} TRF and the highest expression was observed at the dose of 1000 mg kg^{-1} TRF. However, the highest expression of all was observed when mice were administered BHA at the dose of 100 mg kg^{-1}.

Pattern of SOD3 gene and protein expressions and their relationship with regards to the TRF doses administered: The SOD3 gene expression levels (normalized to control animal values) in mice liver was found to be highly correlated (R^2 = 0.99) and linearly associated (p<0.01) with the dose of TRF administered (Fig. 3). This suggests that an increase in the TRF dose administered to mice would cause an associated increase in SOD3 gene expression in the liver.

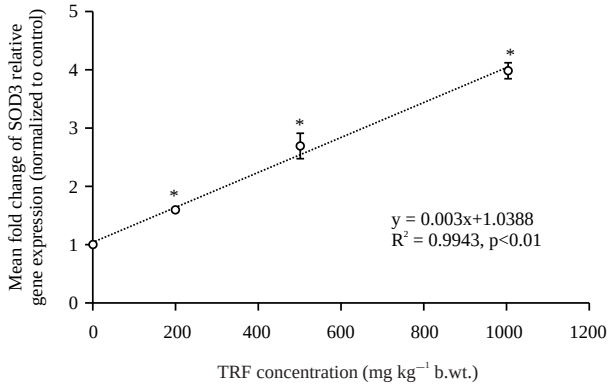

Fig. 3: TRF induced SOD3 gene expression *in vivo* dose-dependently in a highly correlated manner and a significantly linear fashion

TRF in increasing doses (200, 500 and 1000 mg kg^{-1}) was administered orally to mice for 14 days. On day 15 mice were sacrificed, their livers harvested and SOD3 gene expression levels were determined by quantitative real-time PCR (qPCR). The SOD3 gene expressions were quantified relative to GAPDH housekeeping gene expressions and was normalized to the control animal values. The mean values of the fold increase in relative SOD3 gene expression levels were then plotted against the dose of TRF administered and the trendline of the plots was obtained. Asterisk (*) indicates a statistically significant difference from the control group ($p<0.05$)

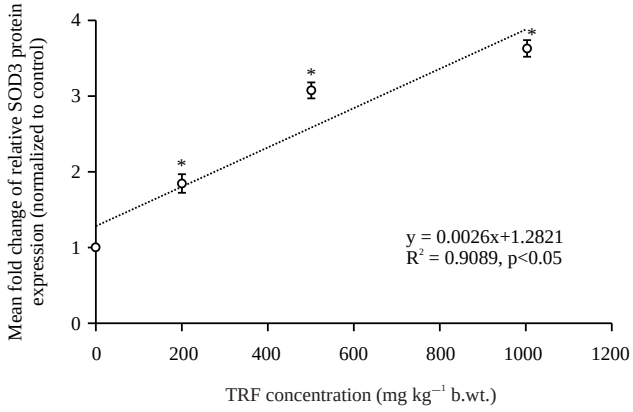

Fig. 4: TRF induced SOD3 protein expression *in vivo* dose-dependently in a correlated manner and a significantly linear fashion

TRF in increasing doses (200, 500 and 1000 mg kg^{-1}) was administered orally to mice for 14 days. On day 15 mice were sacrificed, their livers harvested and SOD3 protein expression levels were determined by Western blotting. The intensity of protein bands was quantified relative to the signals obtained for actin using ImageJ software and was normalized to control animal values. The mean values of the fold increase in relative SOD3 protein expression levels were then plotted against the dose of TRF administered and the trendline of the plots was obtained. Asterisk (*) indicates a statistically significant difference from the control group ($p<0.05$)

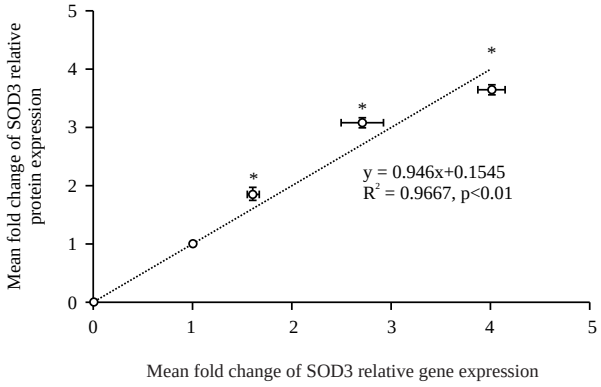

Fig. 5: SOD3 gene expression levels were highly correlated with the SOD3 protein expression levels in a significant linear relationship in the liver

Values for gene expression was plotted against the values of protein expression at the exact doses of TRF administered to mice and the trendline of the plots was obtained. Asterisk (*) indicates a statistically significant difference from the control group ($p<0.05$)

The SOD3 protein expression levels (calculated as relative density against control animal values) in mice liver was also found to be correlated ($R^2 = 0.90$) and linearly associated ($p<0.05$) with the dose of TRF administered (Fig. 4). This suggests that an increase in the dose of TRF administered to mice would similarly cause a concomitant increase in liver SOD3 protein expression.

The results also showed that the increase in liver SOD3 gene expression was highly correlated ($R^2 = 0.97$) and linearly associated ($p<0.01$) with the increase in liver SOD3 protein expression at each TRF dose administered (Fig. 5). This suggests that an increase in SOD3 gene level would likely cause a similar increase in SOD3 protein level in mice liver at every TRF dose given to mice.

DISCUSSION

Detoxification and metabolism of chemicals such as drugs and xenobiotics occur mainly in the liver. Phase I and phase II enzymes in the liver are responsible for the metabolism, biotrans formation and detoxification of drugs, toxic chemicals and their harmful derivatives.

This study focuses on SOD3, a phase II enzyme that is found in mice liver[14]. To the best of our knowledge, this is the first study investigating the effect of increasing doses of TRF on SOD3 expression in the liver. Through our experiments, we attempted to determine whether TRF was able to induce a dose-dependent effect on SOD3 gene and protein expressions

in mice livers. The results indeed indicated that TRF significantly increased SOD3 gene and protein expressions in the liver and thus potentially enhanced the antioxidant and chemopreventive effects in the liver for protection against oxidative stress and harmful xenobiotics.

The cytochrome P450 superfamily of enzymes, which are classified under phase I enzymes, are mostly found in the endoplasmic reticulum of hepatocytes and catalyze various xenobiotic biotransformations[23]. Apart from phase I enzymes, the liver is abundant with phase II enzymes which are involved in antioxidant defence and detoxification of electrophilic entities. An example of a phase II enzyme is SOD3[24]. Superoxide Dismutases (SODs) are a family of enzymes that catalyze the conversion of superoxide anion to hydrogen peroxide. Three SOD isoenzymes are known to exist and they are SOD1, SOD2 and SOD3. SOD3 is expressed in several human tissues including lungs and blood vessels[25]. SOD3 scavenges superoxide free radicals and might therefore play an important role in combatting oxidative and toxic insults[26]. Antioxidants (e.g. vitamin E) can modify the basal and inducible SOD3 expression through the Nrf2 pathway[10]. It is hypothesized that TRF upregulates SOD3 expression in the liver through the Nrf2 signalling pathway. Thus, the focus of the current study is to determine the effect of increasing TRF doses on SOD3 expression in mice livers.

The present study demonstrated the dose-dependent effects of TRF (200, 500 and 1000 mg kg^{-1}) on SOD3 gene and protein expression levels, employing qPCR and Western blot analysis, in the liver tissues of mice. The results obtained showed that TRF positively induced the gene and protein expression levels of SOD3 in a dose-dependent manner, with the highest expression seen in mice treated with 1000 mg kg^{-1}. Our findings showed that SOD3 expression was significantly induced by all doses of TRF administered, with the highest level observed by when TRF was administered at a dose of 1000 mg kg^{-1}, followed by 500 and 200 mg kg^{-1} respectively. However, the highest observed level of SOD3 expression induced by TRF was still below the expression levels induced by BHA treatment. Since BHA is the classical inducer of phase II enzymes, it could be assumed that the increased antioxidant activities observed after tocotrienols treatment in previous animals studies might be partly mediated through the increased expression of phase II genes and proteins[17,27-29]. The results of our study clearly showed that TRF administered at increasing doses resulted in a significant dose-dependent increase in SOD3 gene and protein expression levels. Although previous studies had shown the activity of SOD was increased in the liver of TRF-treated mice, the expression profile of liver SOD was not performed[17,18]. Our study is the first to indicate that administration of TRF (or tocotrienols to be exact) can increase SOD3 gene and protein expression levels concomitantly and dose-dependently in mice livers.

In our study, we used an animal (mice) model and we looked specifically at the effect of TRF in the liver. Other studies which utilized the mice model, but on different organs, had yielded similar results. A previous *in vitro* study had investigated the effect of vitamin E isoform γ-tocotrienol on BALB/C mice which were induced with asthma. It was found that treatment with γ-tocotrienol increased total SOD activity up to control levels in the lungs of asthma-induced mice in a dose-dependent manner. The gene expressions of SOD2 and SOD3 (but not SOD1) in lung tissues were also upregulated back to normal levels in these asthmatic mice[15]. In another study, BALB/C mice were chronically challenged with cigarette smoke to induce Chronic Obstructive Pulmonary Disease (COPD). Administration of γ-tocotrienol to these mice restored the antioxidant enzyme activity of SOD to control levels. The gene expressions of SOD1 and SOD3 (but not SOD2) were decreased in the lungs of COPD-induced mice. Treatment with γ-tocotrienol restored SOD1 gene expression back to normal levels. However, the SOD2 and SOD3 gene expression levels in the lungs of these mice significantly exceeded control mice values after being treated with γ-tocotrienol[16]. An *in vitro* study involving human retinal pigment epithelial cells that were pretreated with α-tocopherol showed that there was a reduction in ROS generation, an increase in Nrf2 expression and induction of many phase II enzymes, including SOD[30]. Contrastingly, the results of another study in mice indicated that the antioxidant effects of tocopherols are not mediated through Nrf2[31].

In our study, TRF was able to increase liver SOD3 levels in normal adult white mice. The antioxidant activities of tocotrienols (which are the major components of TRF) have been linked to Nrf2 activation in previous animal studies. It was shown that the γ-tocotrienol vitamin E isomer was able to significantly induce nuclear translocation and accumulation of Nrf2 in the lung tissues of mice. Interestingly, the more common α-tocopherol isomer was unable to cause significant accumulation and translocation of Nrf2 in the nucleus of mice lung tissues, compared to control animals[15,16], further indicating that the tocotrienols are more potent than the tocopherols. However, the exact mechanism by which TRF (or tocotrienols) induce SOD3 expression in mice livers is still not

fully understood. Since SOD3 is a phase II enzyme and phase II enzymes are regulated by Nrf2, it is suggested that tocotrienols (either directly or indirectly through their by-products) can dissociate the Nrf2/Keap1 complex, allowing Nrf2 to translocate to the nucleus and increase the expression of phase II enzymes (including SOD3) in the liver cells. To further strengthen this assumption, it was discovered that the 5′-untranslated region (5′-UTR) region of the SOD3 gene contains the Antioxidant Response Element (ARE), which is the binding site for Nrf2[32,33]. However, further studies need to be done to confirm this mechanism.

CONCLUSION

Increased expression of SOD3 in the liver could potentially protect the liver from oxidative stress. Excessive oxidative and chemical stress to the liver could potentially cause acute/chronic liver damage and carcinogenesis. To prevent such ailments to occur, it would be wise to implement the certain chemopreventive measure in our daily life. Overexpression of various isoforms SOD(especially SOD3) by safer and natural pharmacological means could potentially be the new therapeutic target for liver degenerative diseases. Tocotrienols, as potential nutraceuticals, were nontoxic to laboratory animals even at high doses. In our study, TRF was able to increase the expression of SOD3 in a dose-dependent manner. Therefore, the recommended therapeutic dose of TRF which is effective in the prevention or treatment of liver diseases in humans should be aggressively investigated. Consuming TRF in the form of daily supplements or using palm oil as dietary modification could therefore be affordable chemoprotective measures in the long run. Further studies are needed to conclusively support the effectiveness of these measures.

SIGNIFICANCE STATEMENT

The Tocotrienol-Rich Fraction (TRF) is the most common source of tocotrienols to be used as a health supplement. This study indicated that the administration of TRF significantly upregulated SOD3 gene and protein expression levels concomitantly and dose-dependently in mice livers. It is most probable that TRF upregulates SOD3 expression in the liver through the Nrf2 signalling pathway since BHA (the classical inducer of phase II enzymes and also an activator of the Nrf2 pathway) also increased SOD3 expression in this study. The apparent increase in antioxidant activities observed after tocotrienols treatment in previous studies might be due to the increased expression of phase II genes and proteins caused by Nrf2 activation. Thus, it is recommended that TRF (and tocotrienols in general) could potentially be used as a liver chemopreventive agent (liver tonic) to improve liver health in humans, thus offering protection against liver toxicity and cancer.

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