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Effect of Tocotrienol Rich Fraction (TRF) on synaptogenic molecules in aging *Caenorhabdis elegans*

Goon Jo Aan*, Ambhighavathe Siva Sarmugam and Norwahidah Abdul Karim

Department of Biochemistry, Universiti Kebangsaan Malaysia Medical Center, Jalan Yaacob Latif, Bandar Tun Razak, Cheras, 56000 Kuala Lumpur, Malaysia.

***Correspondence Info:**
Goon Jo Aan (Ph.D),
Department of Biochemistry,
Universiti Kebangsaan Malaysia Medical Center,
Jalan Yaacob Latif,
Bandar Tun Razak, Cheras,
56000 Kuala Lumpur, Malaysia.
Tel : +603-91459544/91459558
Fax : +603-91459546
E-mail : joaan@ukm.edu.my

Abstract

Objective: This study was performed to determine the effect of tocotrienol rich fraction (TRF) on the expression of synaptogenic genes and proteins in aging *C. elegans*.

Method: Age synchronized DP132 strain worms were divided into two groups; control and treatment where worms were grown in nematode growth medium enriched with 50 µg/ml TRF. The RNA and proteins expressions of SYD-2, SAD-1 and AEX-3 were determined at day 2, 8 and 13 by q-RTPCR and western blot respectively.

Results: The RNA and proteins expressions of SYD-2, SAD-1 and AEX-3 decreased significantly with age in *C.elegans*. Interestingly, TRF treatment successfully increased the expression of these molecules from day 2 to day 8 which is known to be the reproductive phase of *C.elegans* but showed limited effect on the expression of these proteins at day 13.

Conclusion: TRF enhances the expression of molecules involved in synaptic function of young *C.elegans*.

Keywords: tocotrienol; synapse; aging; *C. elegans*

1.Introduction

Regional cognitive decline is one of the arising concerns in developing countries as the number of elderly people with biological brain aging is expected to rise[1]. Cognition reflects the brain's function mainly in memory and psychomotor function[2][3]. Previous reports have suggested that neural spine densities and neurogenesis in hippocampus, which plays a major role in learning and memory, undergo changes in strength with age[4][5][6]. However, the formation and plasticity of spines have been shown to be more essential for cognitive function because long-lasting changes in synapse impair its plasticity leading to impaired neuronal function[7][8]. Thus, dysfunction in signal transduction of synapses may be the key incident in the early stage of cognitive decline.

Alternative approaches have been emerging to improve cognition. Antioxidants such as vitamin E have been postulated to be pivotal as it is able to protect the brain from oxidative stress[9][10][11]. Currently tocotrienols are gaining much attention due to their potent antioxidant and neuroprotective properties. Presence of this unsaturated side chain allows tocotrienols to penetrate into tissues efficiently thus enables tocotrienols to have far reaching and efficient free radical scavenging properties compared to tocopherols[12][13]. The importance of synapse plasticity in cognition and advancing effects of tocotrienol in neuroprotection

brought to our interest to determine the modulatory effect of tocotrienol rich fraction (TRF) on the expression of RNAs and proteins of synaptogenic molecules in aging. *Caenorhabditis elegans* was the model of choice as it has a simple nervous system comprising of precisely 302 neurons wired by 7000 synapses, where each neuron can be individually identified[13]. The neurons have invariably positioned cell bodies and axonal tracts connected by thousands of electrical and chemical synapses between motor and chemical neurons[15]. Besides that, the worm has a complete sequenced genome which enables RNA interference and gene knockout advancement[16].

2.Materials and method

2.1 Nematode strain and worm synchronization

The transgenic *C.elegans* strain DP132 used in this experiment was obtained from Caenorhabditis Genetik Center (CGC), America. The nematodes were maintained at 20ºC on nematode growth media (NGM) with E.coli OP50 as a food source. Gravid adult worms were washed with M9 buffer in 15 ml falcon tubes. Lysis buffer was added into the worm suspension and vortexed at a high speed for 4 minutes followed by centrifugation at 1000 rpm for 1 minute. The pellet was washed three times with M9 buffer by centrifugation at 1000 rpm for 1 minute. To synchronize the age of worms, the pellet containing eggs were suspended in NGM media without OP50 and incubated overnight at 20ºC to yield higher number of worms at stage L1.

2.2 TRF treatment of *C.elegans*

Larva at stage 1 was transferred on to NGM media with and without 50 µg/ml TRF as control and treatment plates respectively. This concentration of TRF has previously been reported to increase the mean lifespan of *C.elegans*[17]. The transfer day was denoted as day 0. The nematodes were transferred to NGM media with 5-fluoro-2'-deoxyuridine (FUDR) (Sigma Aldrich, USA) on day 1 to prevent progeny production and treatment was continued until day 2, 8 and 13 where RNA and proteins expressions of synaptogenic molecules were determined.

2.3 Sample preparation

RNAs and proteins from the whole worm were extracted using trizol reagent (Ambion) based on the protocol by Molecular Research Centre Incorporated. Trizol reagent (10 ml) was added to 1 ml of washed worm pellet. The mixture was vortexed for 15 seconds and incubated at room temperature for 15 minutes to permit complete dissociation of nucleoprotein complexes. RNA in the supernatant was later precipitated while the phenol-chloroform organic layer was used for protein extraction procedure.

2.4 Western blot

Proteins (100µg/µl) were separated on 8% and 12% polyacrylamide gels and transferred to PVDF membranes (Life Technology, USA). The blots were incubated with 0.5% skim milk for 1 hour at room temperature and later incubated with anti SYD-2, anti-SAD-1 and anti-AEX-3 (working dilution 1:200, Santa Cruz) overnight at 4ºC. After washing with TPBS, the blots were incubated with secondary antibodies (working dilution 1:2000, Santa Cruz) for 1 hour at room temperature. Immunoreactive complexes were then visualized using ECL regent (Advansta, California). The housekeeping protein actin was used as an internal control (monoclonal antibody, working dilution 1:500, Santa Cruz). Protein expressions were quantified with mYimage analysis software.

2.5 Quantitative RT-PCR

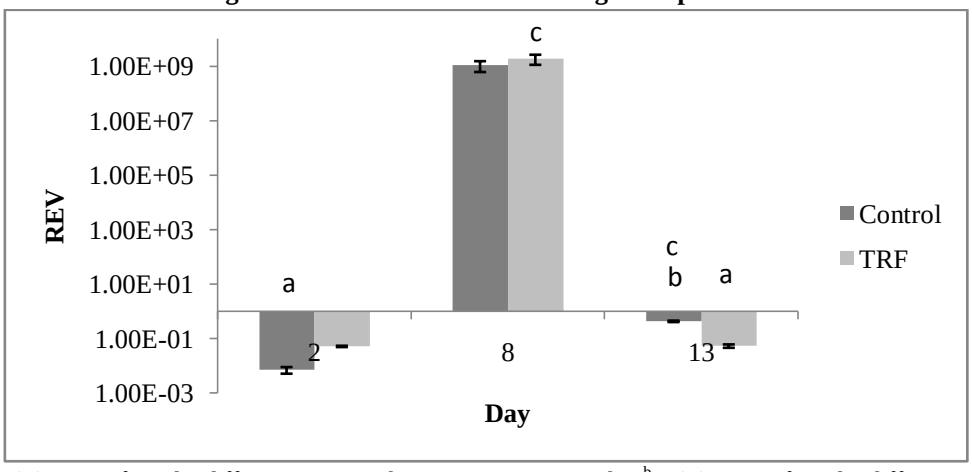
A two-step RT-PCR was employed to access relative changes in transgenic transcripts using iScript cDNA synthesis Kit and SYBR Green Supermix (Bio-Rad). The quality of extracted nucleic acid was assessed by Nano drop ND-100 where RNA in the range of 1.8-1.85 was used for further analysis. The samples were processed in triplicates and the relative ratios between treated worms and control genes were calculated.

2.6 Statistical analysis

Independent T- test was used to analyze all of the parameters. A $p<0.05$ value was considered statistically significant. The statistical analysis was conducted using SPSS 17.0 and all results were reported as min±S.D.

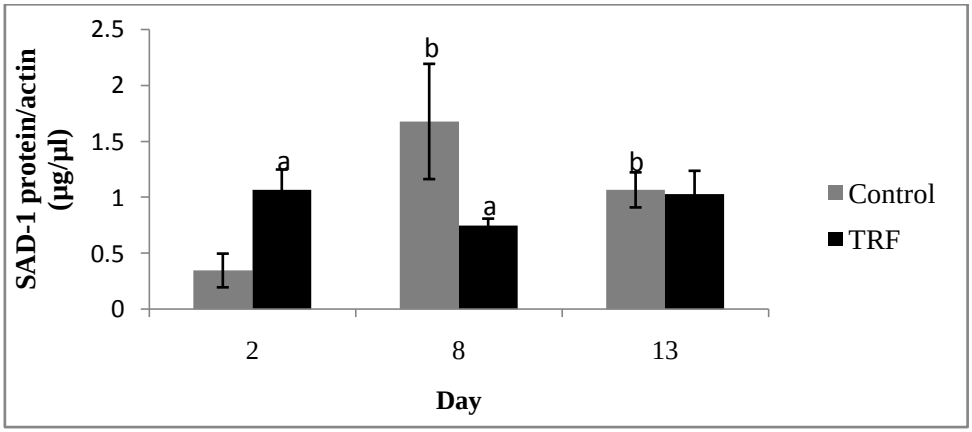
3.Results

Figure 1: Effect of TRF on SAD-1 gene expression



[a]p<0.05, significantly different compared to respective controls; [b]p<0.05, significantly different compared to control at day 2; [c]p<0.05, significantly different compared to control at day 8.

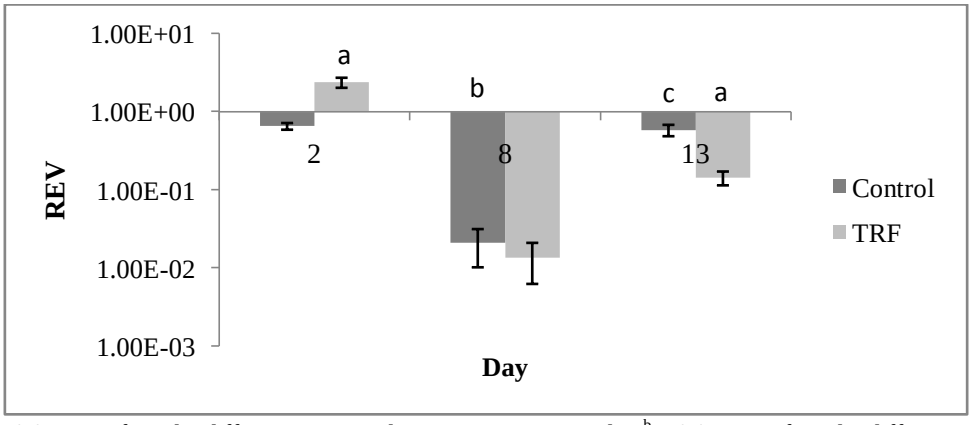
Figure 2: Effect of TRF on SAD-1 protein expression



[a]p<0.05, significantly different compared to respective controls; [b]p<0.05, significantly different compared to control at day 2.

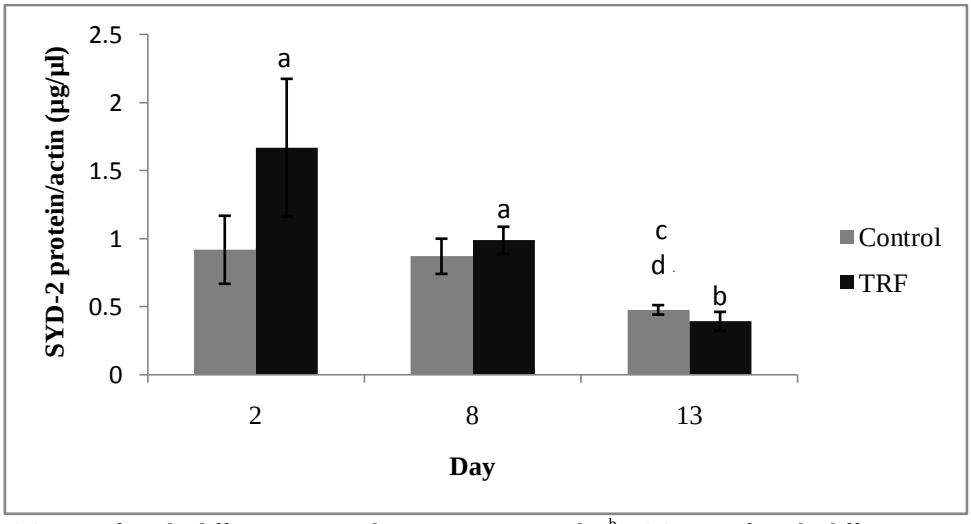
The expression of SAD-1 gene increased significantly at day 8 and declined at day 13 in line with the expression of its protein in control worms. TRF treatment significantly increased the expression of this gene at day 2 and 8 but remarkably reduced its expression at day 13 similar to the changes observed in control worms. At the protein level, TRF treatment increased the expression of SAD-1 protein significantly at day 2 but decreased its expression at day 13 corresponding to its gene expression. Interestingly, TRF treatment stimulated the expression of SAD-1 gene at day 8 ($p<0.05$) but the expression of this protein was significantly ($p<0.05$) depressed.

Figure 3: Effect of TRF on syd-2 gene expression



[a]p<0.05, significantly different compared to respective controls; [b]p<0.05, significantly different compared to control at day 2; [c]p<0.05, significantly different compared to control at day 8.

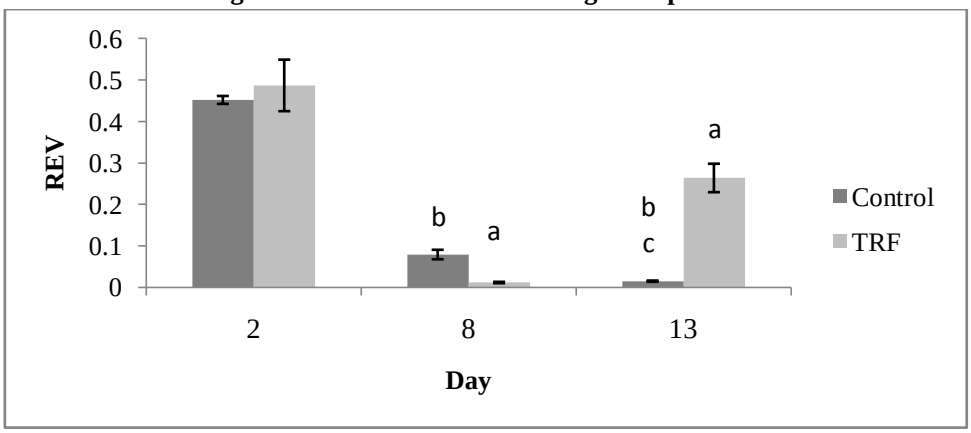
Figure 4: Effect of TRF on SYD-2 protein expression



[a]p<0.05, significantly different compared to respective controls; [b]p<0.05, significantly different compared to TRF treatment at day 2; [c]p<0.05, significantly different compared to control at day 2; [d]p<0.05, significantly different compared to control at day 8.

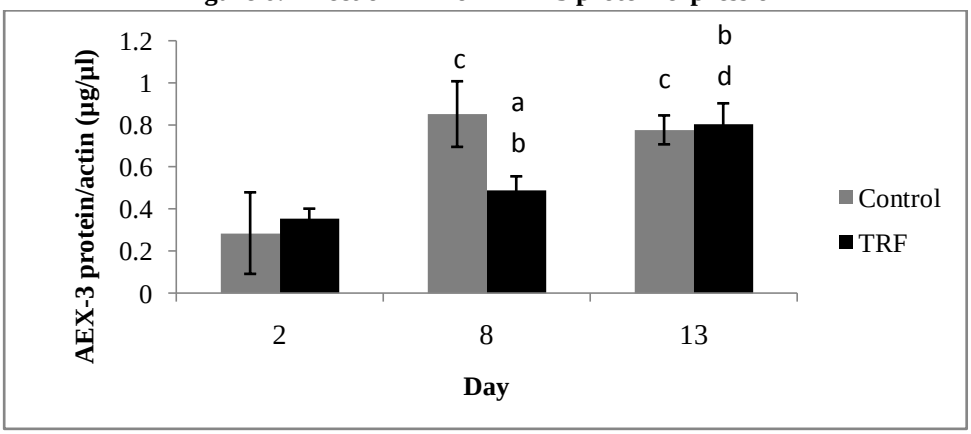
In control worms, the expression of syd-2 gene declined at day 8 (p<0.05) and increased (p<0.05) at day 13 with similar trend noted in TRF treated worms. However, the expression of syd-2 gene was significantly higher (p<0.05) in TRF treated worms as compared to the control at day 2. The expression of syd-2 protein also declined with age (p<0.05) regardless of TRF treatment. TRF treatment only stimulated SYD-2 protein expression (p<0.05) at day 2 which corresponds to its gene expression.

Figure 5: Effect of TRF on aex-3 gene expression



[a]p<0.05, significantly different compared to respective controls; [b]p<0.05 significantly different compared to control at day 2, [c]p<0.05, significantly different compared to control at day 8.

Figure 6: Effect of TRF on AEX-3 protein expression



[a]p<0.05, significantly different compared to control; [b]p<0.05, significantly different compared to TRF treatment at day 2; [c]p<0.05, significantly different compared to control at day 2; [d] p<0.05, significantly different compared to TRF treatment at day 8.

Aex-3 gene expression decreased with age in contrast to its protein expression where a significant increased AEX-3 level was noted at day 8 in the control worms ($p<0.05$). Although TRF treatment suppressed the gene expression at day 8 ($p<0.05$) as compared to day 2, the gene expression increased significantly ($p<0.05$) at day 13. The trend of protein expression was similar to that of aex-3 gene expression in TRF treated worms.

4. Discussion

Tocotrienols have unsaturated isoprenoid side-chains with double bonds in the 3', 7' and 11' positions that occur naturally in esterified forms[18]. It has been found to have strong neuroprotective properties, independent of their antioxidant activities[19]. In this study, TRF treatment effectively increased the expression of SAD-1, SYD-2 and AEX-3 proteins which are involved in synaptic information transmission.

SAD-1 protein interacts with Nab-1/Neurabin that is involved in polarized trafficking of presynaptic components into axons[20]. At day 2, TRF treatment doubled the expression of SAD-1 gene as well as its protein which leads to enhanced polarization of neurons and synaptic organization. However, the expression of SAD-1 protein decreased by day 8 with TRF treatment. This occurred most likely because overexpression of SAD-1 causes premature termination of axons leading to activation of autophagy or ubiquitin mediated proteasome degradation of the SAD-1 protein Post translational modifications too may play a part in decreased expression of this protein as protein turnover occurs to enable the protein pool to be refreshed with newly synthesized proteins by removal of existing or damaged protein molecules[22].

Another possible factor that may cause decreased protein expression at day 8 is the shift of SAD-1 function to synaptic assembly at the later stages of synapse formation since its activity is dispensable for the maintenance of synapse[23]. In *C.elegans*, SAD-1 kinase localizes at synaptic sites along the axon and is necessary for axon polarization and synapse formation mediated by STRD-1/ STRADα, PAR-4/LKBI and AMPK protein complex[24]. Binding of SAD-1 with NAB-1/Neurabin enhances the polarity of the synapse. NAB-1 synaptic localization was found to decrease in adult state[21]. This corresponds to decreased expression of SAD-1 at day 8. Although the SAD-1 gene was over expressed at day 8, it did not affect the synthesis of SAD-1 protein. This may be due to transcriptional stability during cell cycle that permits timely adjustments to changes in growth conditions[25].

Although TRF treatment caused decreased syd-2 gene expression at day 8, there was an insignificant change in the expression of this protein. This could be due to the regulation of syd-2 expression by its binding molecules for proper functioning of the neurons where syd-2 is positively regulated by syd-1 and negatively regulated by RSY-1[21]. RAB3 promotes synaptic targeting of vesicles by mediating tethering of synaptic vesicles to active zone components in the presence of AEX-3 which functions as a guanidine exchange factor for small GTPase Rab3. The increased expression of AEX-3 with TRF treatment at day 2 might have stimulated the hydrolysis of Rab3-GTP by Rab3-GAP hydrolase that is involved in synaptic homeostasis thus resulting in decreased AEX-3 expression towards day 8 of treatment[26].

The significant increased synaptogenic gene and protein expression upon two days of TRF treatment is thought to be mediated by the TOR pathway which has been found to modulate cell growth and proliferation by affecting protein turnover through translation, autophagy, ribosome biogenesis and transcription of ribosomal genes[27]. TOR responds to nutrients and functions in two different complexes, TORC1 and TORC2. TORC1 receives signals from growth factors and nutrients and interacts with DAF-15/Raptor as well as Rag GTPases in the regulation of longevity apart from mediating the expression of transcriptional factors such as DAF-16/FOXO and SKN-1/Nrf [28][29]. According to a recent study, genes in mTOR pathway were up-regulated from L4 to day 6 of adulthood in *C.elegans* and down-regulated from day 6 to day 15 which corresponds to decreased synapse associated protein expression found in aging worms in this study[30].

Although TRF treatment managed to increase the expression of synaptogenic proteins, this can only be seen in the early ages of *C.elegans* known as the reproductive phase. Increased expression of these proteins may enhance synaptic transmission leading to cognitive improvement because tocotrienol uptake in fetal brain has been reported to be high and it promoted long lasting learning ability by influencing the brain development[31]. Based on our results, it can be concluded that TRF treatment increases the expression of proteins involved in synaptic function. However, further research on the relation between cognitive function and expression of more

synapotogenic and mTOR complex molecules would reveal better understanding on the influence of TRF on synapse plasticity.

Acknowledgement

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