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Tocotrienol-Rich Fraction Supplementation Modulates Antioxidant Enzymes Activity and Reduces DNA Damage in APPswe/PS1dE9 Alzheimer's Disease Mouse Model

(Suplementasi Fraksi Kaya Tokotrienol Memodulasi Aktiviti Enzim Antioksidan dan Mengurangkan Kerosakan DNA pada APPswe/PS1dE9 Model Mencit Penyakit Alzheimer)

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ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by deterioration of the brain functions that result in impairment of memory, cognition and behavioural functions. Oxidative stress is well known to be one of the causative factors for AD. Thus this disease is potentially modulated by natural antioxidants such as vitamin E. The aim of this study was to evaluate the effect of tocotrienol-rich fraction (TRF) supplementation on antioxidant enzymes and DNA damage using APPswe/PS1dE9 transgenic mouse model of AD. Animals were supplemented with TRF (200 mg/kg) or alpha-tocopherol (αT) (200 mg/kg) for six months starting from nine months old. We found that superoxide dismutase (SOD) activity in AD mouse was decreased by supplementation of TRF and αT as compared with AD control mouse with no significant differences in glutathione peroxidise (GPx) activity in all groups. TRF supplementation significantly increased catalase (CAT) activity. The level of DNA damage of AD mouse shows significant decrease with supplementation of TRF and αT. In conclusion, TRF was able to modulate antioxidant enzymes activity and decreased the level of DNA damage of AD transgenic mouse model.

Keywords: Alzheimer's disease; oxidative status; tocotrienol-rich fraction

ABSTRAK

Penyakit Alzheimer (AD) adalah gangguan progresif neurodegeneratif yang boleh dicirikan dengan kemerosotan fungsi otak yang mengakibatkan kerosakan ingatan, kognitif dan fungsi tingkah laku. Tekanan oksidatif terkenal sebagai salah satu faktor penyebab AD. Oleh itu penyakit ini berpotensi dimodulasikan oleh antioksidan semula jadi seperti vitamin E. Kajian ini bertujuan menilai kesan suplementasi fraksi kaya tokotrienol (TRF) ke atas enzim antioksidan dan kerosakan DNA menggunakan APPswe/PS1dE9 model mencit transgenik AD. Model haiwan ini telah diberi suplementasi TRF (200 mg/kg) atau αT (200 mg/kg) selama enam bulan dari usia sembilan bulan. Keputusan menunjukkan aktiviti SOD menurun pada mencit AD yang telah disuplementasi dengan TRF dan αT berbanding dengan mencit AD kawalan. Tiada perbezaan signifikan dalam aktiviti GPx dalam semua kumpulan. Manakala mencit AD yang diberi suplementasi TRF menunjukkan peningkatan ketara dalam aktiviti CAT. Daripada segi kerosakan DNA, suplementasi TRF dan αT menunjukkan penurunan ketara pada mencit AD. Kesimpulannya, TRF berpotensi untuk memodulasi aktiviti enzim antioksidan dan mengurangkan tahap kerosakan DNA model mencit transgenik AD.

Kata kunci: Fraksi kaya tokotrienol; penyakit Alzheimer; status oksidatif

Introduction

Alzheimer's disease (AD) is a chronic neurodegenerative disorder that is manifested by global brain deterioration leading to progressive impairments of intellectual, cognition, behaviour and memory (Grand et al. 2011). The prevalence of AD has increased significantly for the past recent years impacting the healthcare system worldwide (Alzheimer's Disease International 2013). Oxidative stress has been strongly suggested to be one of the causative factors in AD. Oxidative stress is defined as an imbalance level of free radicals and antioxidant (Feng & Wang 2012; Moneim 2015). Brain is the most vulnerable tissue in the body to be targeted by the oxidative agents such as free radicals due to its high oxygen consumptions. Low level of antioxidants can lead to a cascade of oxidative events that further enhance the production of amyloid-β in AD brain (Butterfield et al. 2007; Smith et al. 2007). Previous studies have shown the association between oxidative stress and AD. Aβ peptide generated free radical peptides in a cell-free incubation (Hensley et al. 1994). Oxidative stress level was also found to be elevated in the hippocampus and cerebral cortex of AD mouse model (Lovell et al. 1995; Resende et al. 2008) and AD patients (De Leo et al. 1998; Resende et al. 2008; Rinaldi et al. 2003). Increased oxidative stress

in AD appeared to be due to increase production of free radicals level which leads to alteration of antioxidant enzymes activity and level including superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and endogenous antioxidants, namely glutathione, vitamins A, C and E, and carotenoids (Chauhan & Chauhan 2006; Zhao & Zhao 2013).

Vitamin E is classified into tocopherol and tocotrienol. Tocotrienols are structurally different from tocopherol by having three double bonds on their isoprenoid side chain instead of a saturated phytyl side chain (Sen et al. 2006). Tocotrienols have been shown to have more potent anticancer (Yap et al. 2008) and neuroprotective (Sen et al. 2000) properties. Different studies using mixtures of vitamin E, known as tocotrienol-rich fraction (TRF), is reported to possess high antioxidant contents (Chin et al. 2011; Taib et al. 2015), anticancer (Abdul Hafid et al. 2013; Hafid et al. 2010; Rahman et al. 2014; Srivastava & Gupta 2006; Zhang et al. 2015), and anti-ageing activities (Makpol et al. 2013, 2011). More recent findings have indicated that TRF significantly improved cognitive functions in aged rats (Nagapan et al. 2013; Taridi et al. 2014, 2011). Therefore the present work was aimed to evaluate the effect of TRF supplementation on blood antioxidant enzymes and DNA damage of APPswe/PS1dE9 transgenic mouse model of AD.

Materials and Methods

Chemicals

TRF (Golden Tri™ E 70, cat. #SB12112670) was purchased from Sime Darby (Kuala Lumpur, Malaysia) which consisted of 24% α-tocopherol (αT), 27% α-tocotrienol (αT3), 4% β-tocotrienol (βT3), 32% γ-tocotrienol (γT3) and 14% δ-tocotrienol (δT3) in every 1 gram of TRF. αT was purchased from Sigma-Aldrich (cat. #T3634; St. Louis, MO, USA) and vitamin E-stripped palm oil (also known as RBD Palm Olein IV 60) was supplied by the Malaysian Palm Oil Board (MPOB), Selangor, Malaysia.

Transgenic Mice

Wild type and double transgenic male mouse B6C3-Tg (APPswe, PS1dE9)85Dbo/Mmjax with C57BL/6J genetic background were purchased from The Jackson Laboratory (cat. #004462; Bar Harbor ME, USA). The AD mouse model expressed mutant amyloid precursor protein (Mo/HuAPP659swe) and mutant presenilin 1 (PS1dE9) which were associated with early-onset of AD (Jankowsky et al. 2001). All mice were placed in a specific-pathogen-free condition and maintained individually in polycarbonate cages on a 12:12 light-dark cycle with light started at 7:00 am and 24 h ventilation. All equipments and materials such as cages, corn cobs, food pellets and drink containers were sterilized using UV light and autoclaved before use. This study was approved by the UKM Animal Ethics Committee (UKMAEC, approval #FP/BIOK/2013/ZURINAH/15-MAY/509-MAY-2013-MAY-2016) and all animal works were conducted according to the national and international guidelines.

Genotyping

The mice genotype was confirmed by PCR. Tissues were obtained from ear punch and DNA was extracted using Puregene Core Kit A (cat.#8367396; Qiagen, Hilden, Germany). DNA extraction was performed according to manufacturer's protocol. Primer sequences were based on the study by Hong et al. (2013). *APPswe*: (forward) 5'-GACTGACCACTCGACCAGGTTCTG-3' and (reverse) 5'-CTTGTAAGTTGGATTCTCATATCCG-3'; for *PS1A:* (forward) 5'-AATAGAGAACGGCAGGAGCA-3' and (reverse) 5'-GCCATGAGGGCACTAATCAT-3'; and for *PS1B* (forward) 5'-CCTCTTTGTGACTATGTGGACTGATGTCGG-3' and (reverse) 5'-GTGGATAACCCCTCCCCCAGCCTAGACC-3'. PCR mixture consisted of 10× PCR Buffer, 15 mM $MgCl_2$, 10 mMdNTPs mix, 5 U/μL AmpliTaq gold (all from Applied Biosystems, Waltham, MA, USA), 5 mM primer each and 10-20 ng DNA in a total volume of 25 μL. PCR amplification was performed in the iCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA) with an initial denaturation at 95°C for 10 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 1 min; and a final extension at 72°C for 8 min. PCR products were confirmed with 2% agarose gel electrophoresis.

Supplementation

Mice were divided into 4 different groups (n=8 per group): Wild type mouse; APPswe/PS1dE9 transgenic mouse without supplementation as control; APPswe/PS1dE9 transgenic mouse supplemented with TRF; and APPswe/PS1dE9 transgenic mouse supplemented with αT. All mice received six months supplementation from nine months old. APPswe/PS1dE9 transgenic mouse were supplemented by oral gavage with TRF (200 mg/kg) or αT (200 mg/kg), while the wild type and APPswe/PS1dE9 transgenic mouse without supplementation were administered with distilled water.

Samples Collection and Preparation

Upon the completion of supplementation period, blood samples were collected through cardiac puncture using 1 mL syringe and 25G needles that was readily coated with heparin. Blood was centrifuged and erythrocytes were collected. The erythrocytes were lysed with ice-cold HPLC-grade water. The erythrocyte lysate were centrifuged and the supernatant was collected and used for SOD, GPx and CAT assays.

Antioxidant Enzyme Assays

SOD activity was determined using Superoxide Dismutase Assay Kit (cat. #706002; Cayman Chemical Company, Ann Arbor, MI, USA). GPx activity was determined using Glutathione Peroxidase Assay Kit (cat. #703102; Cayman Chemical Company). CAT activity was determined using Catalase Assay Kit (cat. #707002; Cayman Chemical Company). All assays were performed according to the manufacturer's protocol. Absorbance was determined using microplate reader (Infinite® 200 PRO and Magellan 7.0 Data Analysis Software, Tecan, Maennedorf, Switzerland).

Comet Assay

The comet assay was performed to measure the level of DNA strand breaks in cells. Fresh mice blood was obtained from the tail. The assay was conducted according to the method that was described previously (Singh et al. 1988).

Statistical Analysis

Statistical analyses were performed using student's unpaired t-tests for single comparisons. Differences were considered to be significant at $p<0.05$ level using GraphPad Prism® 5 (Version 5.01, GraphPad Software, Inc., USA).

Results

Figure 1 shows the genotyping result for *APPswe* and *PS1dE9* genes. Only mice with both APPswe and PS1dE9 transgenes were used as transgenic mouse model of AD.

The effect of TRF supplementation on transgenic mouse model of AD was assessed by measuring the activity of antioxidant enzymes in the erythrocyte and the level of DNA damage from the whole blood. SOD activity was significantly decreased ($p<0.05$) in AD mouse supplemented with TRF (113.97 ± 9.23 U/mL) and AD mouse supplemented with αT (125.38 ± 9.80 U/mL) when compared with non-supplemented AD mouse (181.03 ± 11.42 U/mL) (Figure 2(a)). The activity of GPx showed no significant difference among all groups (Figure 2(b)). CAT activity was increased significantly ($p<0.05$) in AD mouse following TRF supplementation (10.55 ± 1.08 μmol/min/mL) compared with wild type mouse (6.55 ± 1.08 μmol/min/mL) and non-supplemented AD mouse (6.67 ± 0.67 μmol/min/mL) (Figure 2(c)).

DNA damage was determined by scoring the DNA based on its tail's pattern. DNA was graded from score 0 to 4 with no damage (score 0), mild to moderate damage (score 1 and 2) and extensive damage (score 3 and 4) (Figure 3(a)). DNA damage level was significantly increased ($p<0.05$) in non-supplemented AD mouse (14.04 ± 1.475%) compared to wild type mouse (9.96 ± 1.15%). Meanwhile, the increased DNA damage in the AD mouse was decreased significantly by TRF (3.47 ± 0.35%) and αT (3.07 ± 0.43%) supplementation (Figure 3(b)).

Discussion

Multiple lines of evidence have shown that oxidative stress plays a critical role in the initiation and progression of AD (Wang et al. 2014). Oxidative stress can increase the production and aggregation of Aβ and promote

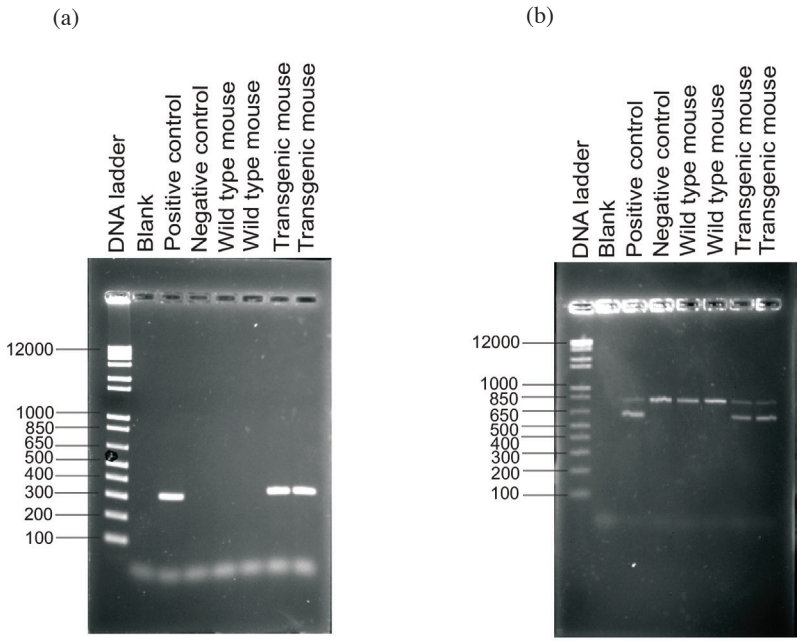
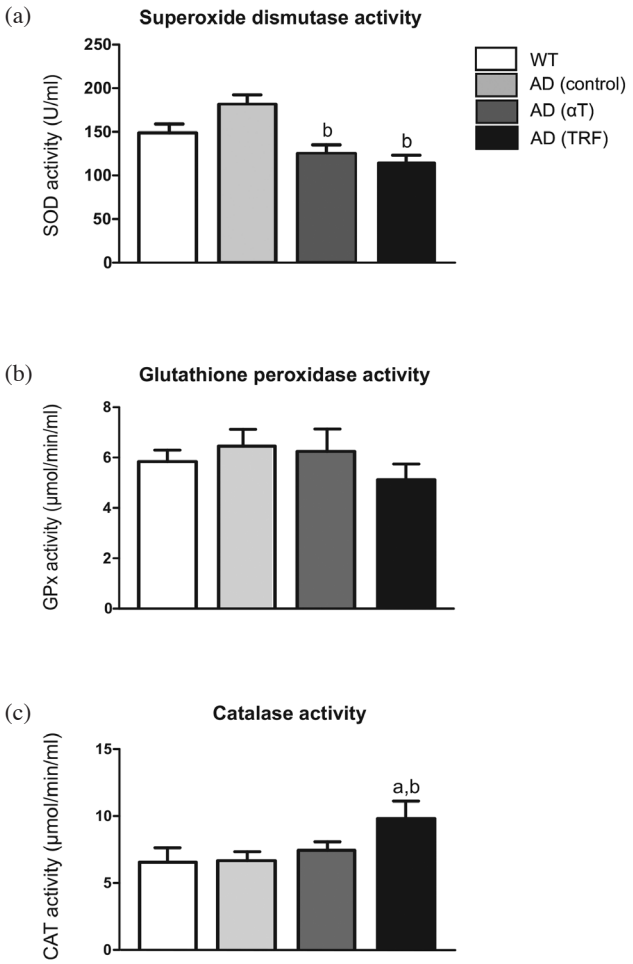


FIGURE 1. Representative gels show the genotyping of (a) APPswe and (b) PS1dE9 genes. Transgenic mouse is confirmed by the presence of APPswe gene band at 350bp and PS1dE9 gene at 608bp and 799bp

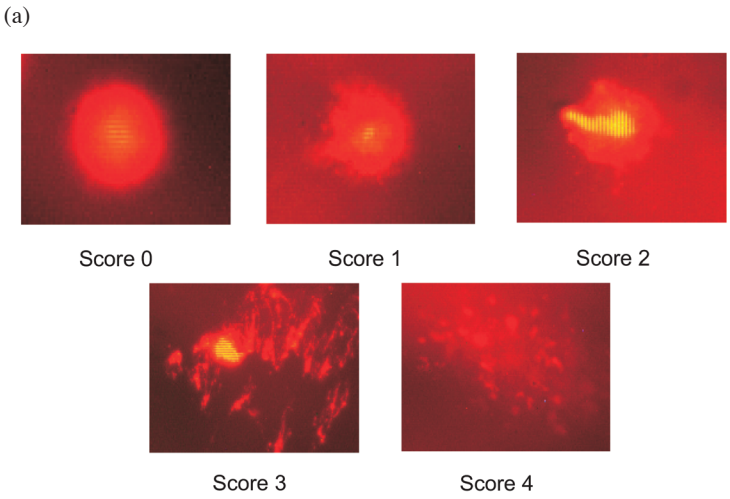


a $p<0.05$ compared with WT (control);
b $p<0.05$ compared with AD (control);
c $p<0.05$ compared with AD (αT);
d $p<0.05$ compared with AD (TRF)

FIGURE 2. Effects of TRF and αT supplementation on (a) SOD activity, (b) GPx activity and (c) CAT activity in mice erythrocytes
Data is presented in mean ± SEM

phosphorylation of tau protein, the hallmark of AD (Zhao & Zhao 2013). Aβ-induced oxidative imbalance may increase the levels of by-products such as malondialdehyde, carbonyl, 8-hydroxyldeoxyguanosine and 8-hydroxylguanosine that lead to oxidation of lipid, protein, DNA and RNA. Defects in antioxidant defence mechanisms may cause increased oxidative stress and further facilitated Aβ depositions in transgenic mice with APP mutation (Zhao & Zhao 2013). APPswe/PS1dE9 mouse is an established model of AD that presented with Aβ deposition as early as 6 months old with abundant plaques mainly found in the hippocampus and cortex (Jankowsky et al. 2004; Lesuisse et al. 2001). Swedish mutations (K595N/M596L) increased the amount of Aβ produced by favouring processing through the beta-secretase pathway that is well known can induce oxidative stress. Study performed by Zhang et al. (2012) found an elevation of MDA and protein carbonyl, while decreased level of antioxidant enzymes such as SOD and GSH-px in 3.5 months old APPswe/PS1dE9 mouse compared to WT mouse. The antioxidant activities of tocotrienols have been linked to improve cognitive performance in animal studies. Supplementation of mixed tocotrienols (α-, β- and γ-tocotrienols) managed to reduce cognitive deficit in streptozotocin-induced diabetic rats by decreasing the level of brain oxidative stress (Kuhad et al. 2009). Meanwhile Taridi et al. (2014) have shown that TRF supplementation for 3 months was able to reverse the age-related cognitive impairments in aged rats.

SOD is an important part of cellular antioxidant defence system which catalyses the dismutation of superoxide anion to hydrogen peroxide and oxygen (Rodriguez et al. 2004). Hydrogen peroxide was then decomposed to water by GPx or CAT. In the absence of antioxidant enzymes, hydrogen peroxide is converted to hydroxyl radical that causes oxidative damage to cellular macromolecules. Our results showed that AD mouse without vitamin E



a $p<0.05$ compared with WT (control);
b $p<0.05$ compared with AD (control);
c $p<0.05$ compared with AD (αT);
d $p<0.05$ compared with AD (TRF)

FIGURE 3. (a) Examples of comet images from whole blood stained with ethidium bromide. DNA was graded from score 0 to 4 based on the severity of the damage and (b) Effects of TRF or αT supplementation on the level of DNA damage in AD mice
Data is presented in mean ± SEM

supplementation demonstrated a significant increase in the level of SOD activity compared to the wild type mice. The high level of SOD activity in AD mouse may be due to high level of oxidative stress thus triggers the antioxidant defence mechanisms. The increased in SOD activity in AD mouse was attenuated by supplementation of TRF and αT, suggesting that both vitamins act as potent antioxidant to scavenge free radicals which spare the activity of SOD in AD mouse. Consistent with our findings, De Leo et al. (1998) found that SOD activity was elevated in erythrocytes of AD patients. SOD activity was also increased in brain cortex of 3×Tg-AD transgenic mouse model of AD (Resende et al. 2008). On the other hand, meta-analysis by Schrag et al. (2013) found that SOD activity was unaffected in the erythrocyte of AD patients compared to age-matched controls. In contrast, SOD activity was lower in the frontal and temporal cortex of the brain (Marcus et al. 1998), in plasma and erythrocyte of AD patients compared with age-matched controls (Rinaldi et al. 2003). The effects of TRF on SOD activity in the pathogenesis and progression of AD were largely unknown. Other studies in human and ageing models showed that TRF has the ability to modulate the activity of SOD. TRF supplementation decreased SOD activity in senescent human diploid fibroblasts (Makpol et al. 2013), healthy adults with age above 50 years (Chin et al. 2011) and aged mice (Aliahmat et al. 2012). These findings point to a notion that antioxidant activity of TRF was likely accounted for the compensated decrease of SOD activity (Makpol et al. 2013) which potentially provide protection against oxidative stress in AD.

Previous studies on the activity of GPx in AD patients and animal models were rather controversial. Our results indicated that wild type and AD mouse had no difference in the level of erythrocyte GPx activity with or without TRF supplementation. Other studies reported that GPx activity was unchanged in erythrocyte (Perrin et al. 1990) and in frontal, temporal and cerebellar cortex (Marcus et al. 1998) of AD patients. Aliahmat et al. (2012) found no significant changes in aged mouse with supplementation of TRF. However, GPx activity was elevated in the hippocampus, amygdala and piriform cortex (Lovell et al. 1995) and serum (Padurariu et al. 2010) of AD patients and in the

cortex of 3×Tg-AD transgenic mouse model of AD (Resende et al. 2008) while GPx activity was decreased in plasma of AD patients (Rinaldi et al. 2003). Despite the actual effect of TRF on GPx activity in AD remains unclear, other studies have shown that TRF increased GPx activity in senescent human dermal fibroblasts (Makpol et al. 2013), healthy older adults (Chin et al. 2011) and aged rats (Taridi et al. 2014).

Our data showed that CAT activity was unaffected by APPswe and PS1dE9 mutations in the AD mouse model. A meta-analysis has shown that blood CAT activity was unchanged in AD patients (Schrag et al. 2013). Others have shown CAT activity was higher in erythrocyte (Perrin et al. 1990) and in hippocampus (Lovell et al. 1995), while decreased in temporal cortex (Marcus et al. 1998) of AD patients. Our study found that TRF supplementation significantly increased CAT activity in AD mouse model. Other studies have shown that CAT activity was unaffected in HDFs treated with TRF (Makpol et al. 2013), in erythrocyte of aged rats supplemented with TRF (Aliahmat et al. 2012) and in healthy elderly adults supplemented with TRF (Chin et al. 2011). Although this was opposed to our findings, work by Taridi et al. (2014) showed TRF supplementation increased CAT activity in erythrocyte of aged rats. Overexpression of CAT in CAT/APP double transgenic mouse was able to increase lifespan, reduced Aβ deposition and brain oxidative DNA damage level (Mao et al. 2012), supporting the theory that TRF was able to provide protection against oxidative stress in AD.

Oxidative stress associated with AD appears to be associated with DNA strand breaks found in brain tissues of AD subjects (Feng & Wang 2012). Our data indicated that the level of DNA damage in the AD control mouse was significantly higher as compared to wild type mouse. This was in accordance to other findings where the level of DNA strand breaks was found to be elevated in cortex (Anderson et al. 1996; Mullaart et al. 1990), while 8-OHdG level was increased in lymphocyte (Mecocci et al. 2002) and brain (Lyras et al. 1997) of AD patients. APPswe/PS1dE9 transgenic mouse model overexpressed amyloid-β thus induces high level of DNA damage (Jiao et al. 2012). Our study found that TRF and αT supplementation significantly reduced the level of DNA damage in AD mouse. This is supported by the findings by Nakashima et al. (2004) that supplementation of αT was able to decrease the level of oxidised DNA. TRF was reported to reduce DNA damage in human study and animal models. Supplementation of TRF has successfully reduced blood DNA damage in aged rats (Taridi et al. 2011). Increased DNA damage in rats that undergo exercise training protocol was reversed by the supplementation of TRF (Abd Hamid et al. 2011). TRF has also been shown to reduce DNA damage in the leukocyte of healthy older adults (Chin et al. 2008).

Conclusion

In conclusion, TRF has the ability to modulate antioxidants system of an AD transgenic mouse model. The ability of TRF to suppress the SOD activity and reduce the level of DNA damage as well as increase CAT activity highlight the potential of TRF for future alternative treatment for Alzheimer's disease. Further studies have to be carried out to determine the effect of TRF supplementation on cognitive performance and brain oxidative status in AD mouse which warrants better understanding on the role of TRF in the pathogenesis of AD and its progression.

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