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Selective Uptake of Alpha-Tocotrienol and Improvement in Oxidative Status in Rat Brains Following Short- and Long-Term Intake of Tocotrienol Rich Fraction

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ABSTRACT

Introduction: Tocotrienol exerts neuroprotective effects resulting in an improved circulating oxidative status. However, accumulation of tocotrienol due to long-term intake may exert pro-oxidant effects. Thus the effects of short- and long-term supplementation of vitamin E tocotrienol rich fraction (TRF) on the parameters of oxidative status in rat brains were determined. **Methods**: *Wistar* rats aged 3 months were supplemented with TRF for 3 or 8 months. Control groups received equivolume of distilled water. Rats were sacrificed and brains harvested, weighed and homogenised. Supernatants were analysed for catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) activities, vitamin E and protein carbonyl. **Results**: A significant decline in the level of total vitamin E and its isomers with increasing age were found. TRF supplementation increased the level of total vitamin E with alpha-tocotrienol (ATT) being the major isomer raised. Glutathione peroxidase activity was also significantly increased in the long-term supplemented group compared to the short-term supplemented and control groups. The results also showed significantly higher superoxide dismutase activity ($p<0.001$), lower protein peroxidation ($p<0.05$) and heavier brain weights ($p<0.05$) in both supplemented groups but catalase activity remained unchanged. **Conclusion**: The study showed long- term TRF supplementation exerts positive effects on brain oxidative status

Keywords: Antioxidant, brain, long-term supplementation, oxidative stress, tocotrienol rich fraction (TRF)

INTRODUCTION

Ageing is the common risk factor for neuro-degenerative diseases **(**Lin & Beal, 2006). The consequences of these disorders are debilitating, especially in advanced stage with severe motor and cognitive impairments. One of the causes for the age-induced neuro-degeneration has been attributed to the accumulation of free radicals

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and hence increased oxidative stress (Harman, 1994). The brain is especially vulnerable to oxidative stress because of its high aerobic metabolic rate (Floyd & Hensley, 2002), rich polyunsaturated fatty acids (PUFA) content and lesser antioxidants levels compared to other organs (Rice-Evan & Burdon, 1993).

The intake of antioxidants may provide a means to protect the brain against age-induced free radical neural damage. The intake of both synthetic and natural antioxidants has been reported to have positive effects on the brain (Manju & James, 2010; Al-Omar *et al.,* 2006) and the cognitive functions (Chiu *et al.,* 2008). Vitamin E has also been reported to exert neuroprotective effects in cell culture studies (Musalmah *et al.,* 2006; Osakada *et al.,* 2004) with tocotrienols exerting stronger antioxidant (Mangialasche *et al.,* 2012) and more potent neuroprotective properties compared to tocopherols (Osakada *et al.,* 2004). Recently Mangialasche *et al.* (2012) reported low circulating levels of tocopherols and tocotrienols associated with a higher risk of developing mild cognitive impairment and Alzheimers disease. Although there are many studies reporting on improvement of oxidative stress status in circulation, there are fewer published reports on the modulation of oxidative status in the brain by vitamin E or tocotrienol rich fraction (TRF) specifically. Furthermore, vitamin E has also been reported to exert pro-oxidant effects when present at high concentrations and to be neurototoxic *in vitro* if tocotrienol was at high levels (Serbino & Packer, 1994). Therefore, it is important to establish whether continuous long term intake of tocotrienol rich fraction will exert detrimental effects. The present study was undertaken to examine the effects of short- and long-term supplementation of TRF on the oxidative status of the brain by measuring vitamin E isomers and antioxidant enzyme activities, as well as protein peroxidation products (protein carbonyl).

METHODS

This study was approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC). Twenty-four male *Wistar* rats aged 3 months weighing 200g ± 50g were housed individually in cages at room temperature 27°C ± 2°C with 12 hours of light and dark cycles. The rats were fed with the standard rat chow (Gold Coin, Malaysia) and tap water *ad libitum*.

Experimental design

The animals were divided randomly into two groups to receive either short (3 months) or long term (8 months) supplementation of TRF. Each group was further subdivided into two : control and supplemented groups. The control groups were given distilled water while the experimental groups were supplemented with equivolume of TRF (Golden Hope, Malaysia) at 200 mg / kg body weight via oral gavage. The TRF used in the study composed of 28.5% γ-tocotrienol (GTT), 22.9% δ-tocotrienol (DTT), 22.1% α-tocotrienol (ATT), 20.0% α-tocopherol (ATF) and 48.8 mg / g (6.5%) of β-tocotrienol (BTT). All rats were subsequently sacrificed under diethyl ether. Weighed brain tissues were then homogenised in ice-cold buffer (0.1M Tris-HCl of pH 7.5 at 4^{0}C) and centrifuged at 10,000 x g for 10 min at 4^{0}C. Finally, supernatants were analysed for concentration of total vitamin E and its isomers, catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities and protein carbonyl.

Vitamin E determination

Concentration of total vitamin E and its isomers: α-tocopherol (ATF), α-tocotrienol (ATT), γ-tocopherol (GTF), γ-tocotrienol (GTT) and δ-tocotrienol (DTT) was determined using reverse-phase high performance liquid chromatography (HPLC) with Fluorescent EM 330nm; EX 294 detector (Shimadzu, Japan). Supernatants of

homogenised brain (100 μl) were added to 10 mg / ml butylated hydroxyl toluene (BHT) in 95% ethanol to arrest auto-oxidation process. Protein was then precipitated with the addition of 500 μl 100% ethanol. After centrifugation at 3000 x g for 15 min at 18°C, pellets were discarded and 1.5 ml hexane (Merck, Germany) was added to the resultant supernatants. The mixtures were centrifuged again at the same speed and temperature for 15 min before harvesting the top layer. The vitamin E extracts were then dried using vacuum concentrator (Heto Lab Equipment, Denmark) and stored at -80°C before analysis with HPLC.

HPLC samples were prepared by diluting the supernatant at 1:100 dilutions with another HPLC grade-hexane. Peaks of samples obtained were compared with TRF standard and concentrations of total vitamin E, α-tocopherol (ATF), α-tocotrienol (ATT), γ-tocopherol (GTF), γ-tocotrienol (GTT) and δ-tocotrienol (DTT) in the rat brains from both control and experimental groups were calculated in μg / ml.

Antioxidant enzyme activity

The superoxide dismutase (SOD) activity was determined as a degree of inhibition of the reduction of Nitrotetrazolium Blue (NBT) according to the method described by Bayer & Fridovich (1987). Supernatant (0.02 ml) was added to the tubes containing 1.0 ml freshly-prepared mixed buffer solution containing 27.0 ml 50 mM phosphate buffer at pH 7.8, 1.5 ml 0.2 M L-methionine (Sigma, USA), 1.0 ml 1.72 mM NBT (Sigma, USA) and 0.75 ml Triton X-100 (Sigma, USA). Blank tube was prepared by substituting the supernatant with 0.02 ml 50 mM phosphate buffers. Reaction was initiated by the addition of 0.01 ml riboflavin solution (Sigma, USA). The tube was left under two lamps of Sylvania Grolux Fluorescence (Malaysia) of 20 watts for 7 min. Finally, the mixture solution was read at 560 nm using UV-160A spectrophotometer (Shimadzu, Japan). One unit of SOD activity corresponds to the enzyme concentration required to inhibit 50% of the reduction of NBT in 1 min under standard conditions. The activity of SOD was expressed in Unit / min / mg protein.

The glutathione peroxidase (GSH-Px) activity was determined as the amount of reduced glutathione (GSH) oxidised as described by Paglia & Valentine (1967). Briefly, 0.02 ml supernatant was added to 0.88 ml freshly-prepared mixed buffer solution which consisted of 0.1 ml 5 mM GSH (Sigma, USA), 0.1 ml GSH-Rd (Sigma, USA), 0.1 ml 8.4 mM NADPH (Sigma, USA), 0.01 ml 1.125 M sodium azide (NaN_3) (Searle Company, England) and 100 ml 50 mM phosphate buffer pH 7.0. Blank sample was prepared by replacing the supernatant with 0.02 ml 50 mM phosphate buffers. The reaction was started by the addition of 0.1 ml 2.2 mM hydrogen peroxide (H_2O_2) (Merck, Germany). The resulting solution was then read at 340 nm by kinetic mode using UV-160A spectrophotometer. The activity of GSH-Px was expressed in mUnit / min / mg protein.

Catalase (CAT) activity was measured as the amount of hydrogen peroxide (H_2O_2) consumed as described by Aebi (1984). Supernatant (0.05 ml) was added to 1.95 ml 50 mM phosphate buffer pH 7.0. Reaction was started by the addition of 1.0 ml of 30 mM H_2O_2 (Merck, Germany) The decrease in absorbance was measured at 240 nm by kinetic mode for 30 sec using UV-160A spectrophotometer (Shimadzu, Japan). The blank sample was run simultaneously with 1.0 ml 50 mM phosphate buffers instead of H_2O_2. One unit of CAT activity is defined as the decomposition of 1 μmol of H_2O_2 in one second. The activity of CAT was expressed in mUnit / sec / mg protein.

Protein carbonyl

Rat brain was homogenised in 1 ml 50 mM phosphate buffer, pH 6.7 containing 1 mM EDTA and then centrifuged at 10,000 x g for 10 min at 4°C. The resultant supernatant was analysed for protein carbonyl using

Cayman's Protein Carbonyl Assay kit (Cayman Chemical Company, Michigan, USA). The kit is based on the method described by Levine *et al.* (1994) using 2, 4-dinitrophenyl hydrazine (DNPH) reaction. Briefly, 200 μl supernatant was added to 800 μl DNPH in sample tube and 800 μl 2.5 M HCl in blank tube. Following incubation in the dark for 1 h at room temperature, 1 ml 20% trichloroacetic acid (TCA) was added to each tube to precipitate protein and then the tubes were incubated for a further 5 min at 4^0C. After centrifugation at 10,000 x g, 4^0C for 10 min, the supernatants were discarded and pellets were re-suspended in 1 ml 10% trichloroacetic acid (TCA), and then centrifuged at the same speed and temperature. The resultant pellets were subsequently washed and centrifuged three times with 1 ml 1:1(v/v) ethanol/ethyl acetate mixture to remove free reagents. After the final wash, the precipitated protein was re-dissolved in 500 μl guanidine hydrochloride solution and centrifuged to remove any left-over debris. Finally, 220 μl supernatants were transferred from sample and blank tubes to the 96-well plate. Absorbance at wavelength 370 nm was measured using a plate reader (Versamax, USA). In parallel, protein concentration was determined by adding 100 μl sample from the well to 900 μl guanidine hydrochloride in 1 ml of quartz cuvette, and the absorbance at 280 nm was determined using UV-160A spectrophotometer (Shimadzu, Japan). With the use of a molar absorption coefficient of 22 $mM^{-1}cm^{-1}$, the protein carbonyl content was calculated as nM DNPH incorporated (protein carbonyl) per 1 mg protein.

Statistical analysis

Results were expressed as means ± standard error of mean (SEM) and analyed using one-way ANOVA with the aid of Statistical Package for Social Sciences (SPSS) version 13.0. $p<0.05$ was accepted as statistically significant.

RESULTS

Vitamin E levels

The level of total vitamin E in the brain was significantly lower in older compared to young rats (Figure 1). With TRF supplementation for 8 months, the level of total vitamin E, ATT and GTT was significantly raised with ATT being the major isomer in the brains.

Antioxidant enzyme activity

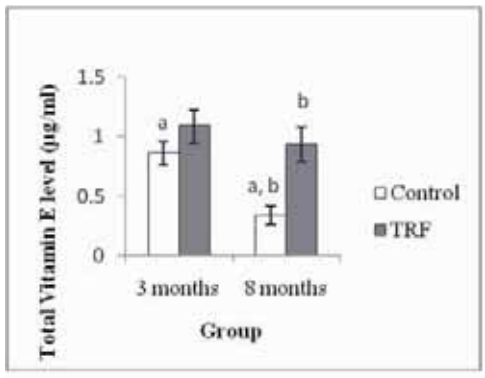
The brains of older rats had significantly higher GSH-Px activity compared to young rats (Figure 2b). However, there were no significant difference on SOD and CAT activities between the older and younger rats (Figures 2a and 2c respectively). TRF supplementation for 3 or 8 months significantly increased SOD activity. GSH-Px activity only increased significantly in the brains of 8-month supplemented group compared to control. TRF supplementation did not to have any effect in modulating the activity of CAT in the brain (Figure 2c).

Protein carbonyl contents

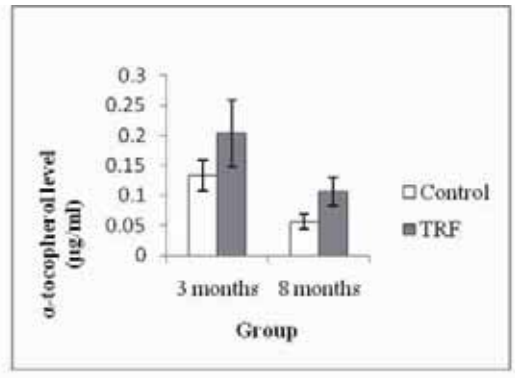
There was no significant difference in the amount of protein carbonyl between young and older groups (Figure 3). TRF supplementation for 3 or 8 months significantly reduced the level of protein carbonyl ($p<0.05$).

DISCUSSION

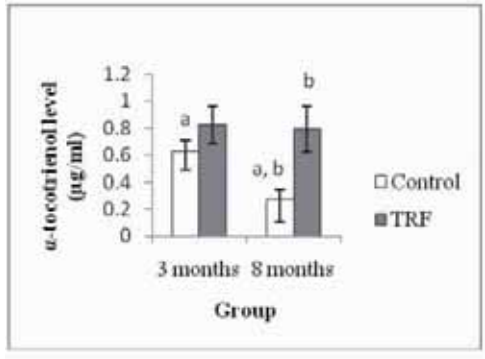
In vivo animal studies have shown that administration of vitamin E protects the brain from oxidative stress induced by mechanical restrain, lipopolysaccharide and ageing (Kashif, Zaidi & Banu, 2004); Kheir-Eldin *et al.,* 2000; Jolitha, Subramanyam & Devi (2006). In these studies, vitamin E was given for a short duration of 2-6 hours, 1 week and 30 days respectively. Although there is scant literature on the effect of acute



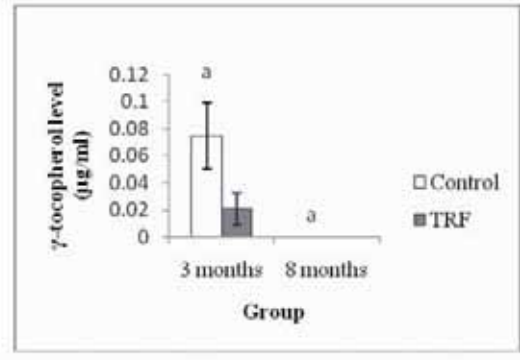
(a) Total vitamin E level



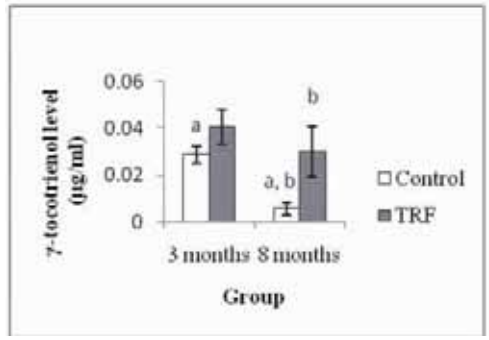
(b) α-tocopherol (ATF) level



(c) α-tocotrienol (ATT) level

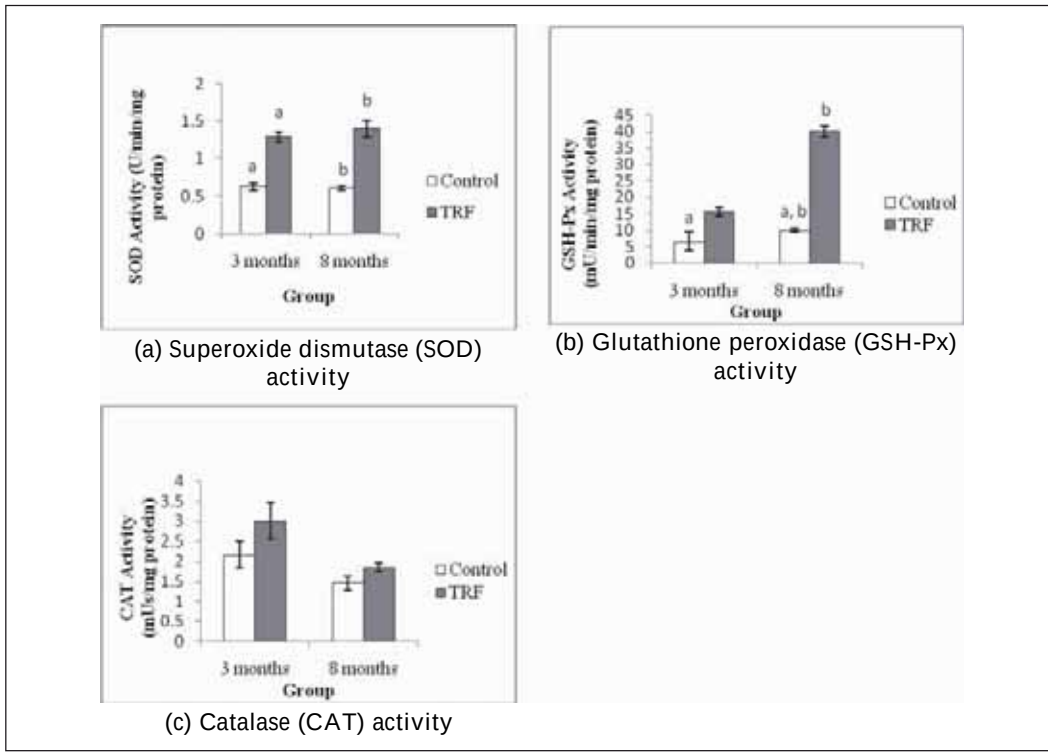


(d) γ-tocopherol (GTF) level



(e) γ-tocotrienol (GTT) level

Figure 1: Level of vitamin E and its isomers in the rat brains with and without TRF supplementation. Vitamin E levels were determined using reverse-phase HPLC. Brains of older rats contained significantly lower levels of total vitamin E, ATT, GTT and GTF. TRF supplementation for 8 months significantly increased the level of total vitamin E, ATT and GTT, with ATT being the major isomer. Data is presented as means ± SEM (n=6). Similar alphabets, a and b, denote significant difference between the groups (p<0.05).



(a) Superoxide dismutase (SOD) activity

(b) Glutathione peroxidase (GSH-Px) activity

(c) Catalase (CAT) activity

Figure 2. Activities of antioxidant enzymes in the rat brains with and without TRF supplementation. Aging significantly increased GSH-Px activity. TRF supplementation significantly increased SOD activity (p<0.001) while only the 8- month supplementation increased GSH-PX activity (p<0.001). TRF supplementation had no effect on CAT activity. Data is presented as mean ± SEM (n=6). Similar alphabets, a and b, denote significant difference between the groups (p<0.05).

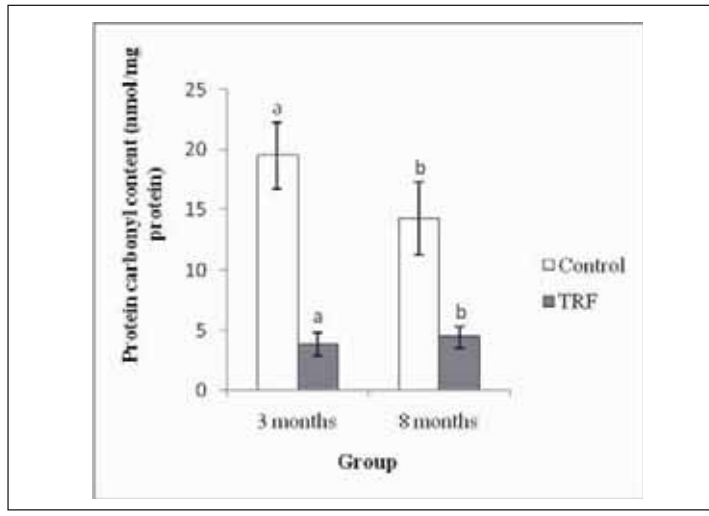


Figure3: Protein carbonyl contents in rat brains with and without TRF supplementation. There was no significant difference in the level of protein carbonyl between young and older groups. TRF supplementation significantly reduced the level of protein carbonyl. Data is presented as mean ± SEM (n=6). Similar alphabets, a and b, denote significant difference between the groups (p<0.05).

administration of vitamin E on the age-induced oxidative status parameters in the brain, there are lesser reports on the effect of chronic long term intake. Therefore, in the present study, we examined the effect of long-term intake of vitamin E tocotrienol rich fraction on age-induced oxidative status in the rat brain.

Data from the present study showed that vitamin E levels in the brain decreased with age. This may probably be due to a decrease in intestinal absorption, transportation and / or uptake by neurons. Age also induced an increase in the activity of the antioxidant enzyme, GSH-Px, probably as a compensatory mechanism (Fraser *et al.,* 2005). At 11 months (equivalent to middle aged humans), the animals in the present study showed that oxidative damage in terms of protein peroxidation level was not significantly different from young rats suggesting that the compensatory mechanisms by the brain endogenous antioxidants are still sufficient to maintain favourable oxidative status level. This result is in agreement with that reported by Goto *et al.,* (1999) in male F344 rats. However it should be pointed out that in rat hepatocytes and in human fibroblasts, the amount of protein carbonyl is reported to increase progressively with age (Starke-Reed & Oliver, 1989).

TRF supplementation increased vitamin E level in the brain. Continuous supplementation for 8 months increased the total vitamin E level similar to the levels in the younger rats. Among the vitamin E isomers, ATT was noted to be the major isomer present in the brain. The present study also noted that although ATT only formed a third of the total composition of TRF, it was the major isomer raised in the brain. One probable reason for the preference for ATT may be due to the unsaturated side chain of ATT which allows for better penetration into tissue with saturated fatty layer (Suzuki *et al.,* 1993). Furthermore, the ATT in TRF was of free rather than esterified form – the membrane of brain cells have limited esterase activity and thus may limit the uptake of the esterified tocotrienol (Sen *et al.,* 1999). The preferential uptake of neurons for ATT is interesting and may probably correlate to the observation of it being the most potent neuroprotective isomer *in vitro* (Osakada *et al.,* 2004). ATT was reported to exert its neuroprotective effect via inhibition of glutamate-induced activation of phospholipase A2 (Khanna *et al.,* 2010). It also modulates the mediators of arachidonic acid metabolism, which is one of the most abundant polyunsaturated fatty acids in the central nervous system (Sen, Rink & Khanna (2010). More studies should be carried out with ATT since there are very few reports of its actions in the literature.

TRF supplementation significantly increased SOD and GSH-Px activities in the brain. Such findings have also been reported in the rat plasma (Taridi *et al.,* 2011). However, the present result showed that vitamin E supplementation did not affect CAT activity significantly. Both GSH-PX and CAT are enzymes which reduce the peroxides produced from the reaction catalysed by SOD. Thus the increased level of GSH-PX may be sufficient to meet the needs of the brain in this situation. The increased levels of SOD and GSH-PX resulted in reduced protein carbonyl level in the brain even after 3 months of supplementation. Similar observations have been reported in nematodes *Caenorhabditis elegans* (Adachi & Ishii, 2000) and in rat brain mitochondria (Kamat & Devasagayam, 1995).

The present findings indicate that long term (8 months) supplementation of TRF improved vitamin E levels, increased antioxidant enzymes levels and reduced protein carbonyl content in the brain. This improvement in oxidative status in the brain may prove beneficial to prevent or delay age-related cognitive and motor performance decline. Gamma- tocotrienol for example, has been shown to protect against telomere shortening in human fibroblasts during

ageing (Makpol *et al.,* 2010). The present finding did not observe adverse effects of vitamin E as was previously reported in neuron cells *in vitro* (Then *et al.,* 2009). It is interesting to note that reports on vitamin E toxicity have only been observed using cells in culture (Tafazoli , Wright & O'Brien (2005). This could reflect the small amount of vitamin E absorbed from the diet and / or some mechanism regulating its absorption, transport and uptake by cells.

CONCLUSION

In conclusion, long term intake of TRF maintains vitamin E levels in the brain against age-induced decline. The brain has a preferential uptake for vitamin E ATT. This is associated with an improvement in the oxidative status of the brain.

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