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## Tocotrienol Rich Fraction Prevents Fenitrothion Induced Pancreatic Damage by Restoring Antioxidant Status

Siti Balkis Budin, Cha Ming Han, Putri Ayu Jayusman and Izatus Shima Taib  
Department of Biomedical Science, School of Diagnostic and Applied Health Sciences,  
Faculty of Health Sciences, Universiti Kebangsaan Malaysia,  
Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia

**Abstract:** Fenitrothion (FNT) is extensively used as pesticide and may induce oxidative stress in various organs. Tocotrienol, a form of vitamin E found in palm oil, reduces oxidative impairments in pathological conditions. This study aims to investigate the effects of palm oil tocotrienol rich fraction (TRF) on fenitrothion-induced oxidative damage in rat pancreas. Forty male Sprague-Dawley rats were divided into four groups: control group, FNT group, TRF group and FNT+TRF group. Regimens FNT (20 mg kg<sup>-1</sup> b.wt.) and TRF (200 mg kg<sup>-1</sup> b.wt.) were force-fed for 28 consecutive days with control group only receiving corn oil. Chronic administration of fenitrothion significantly ( $p < 0.05$ ) induced oxidative damage in pancreas of rats with elevated malondialdehyde and protein carbonyl level. Depletion of glutathione and significant ( $p < 0.05$ ) reduction in antioxidant enzyme activities in pancreas homogenate additionally suggested induction of oxidative stress. Despite these changes in pancreas of intoxicated rats, no significant ( $p < 0.05$ ) changes in blood glucose and pancreas histology were observed. Co-administration of FNT with TRF alleviated these oxidative changes and significantly ( $p < 0.05$ ) restored antioxidant status. Enzymatic activities of Superoxide Dismutase (SOD) and Catalase (CAT) were normalized. In conclusion, tocotrienol rich fraction of palm oil prevents fenitrothion-induced pancreatic oxidative damage in rats.

**Key words:** Organophosphate, antioxidants, pancreas, lipid peroxidation, blood glucose

### INTRODUCTION

The usage of organophosphate as pesticide in agriculture practices is very common since the 20th century (Kamath *et al.*, 2008). Organophosphates such as Fenitrothion (FNT) act as toxin by irreversibly inhibit the action of acetylcholinesterase, thus causing accumulation of neurotransmitter acetyl choline at the neuronal synapse (Katz, 2010). Like other organophosphates, FNT toxicity stimulates nicotinic and muscarinic acetyl choline receptor thus causing symptoms such as muscle contraction, muscle weakness, loss of consciousness.

Besides that, FNT can also induce oxidative stress in various organs such as liver and kidney (El-Shenawy, 2010; Elhalwagy *et al.*, 2008). FNT produces free radicals by enzymatic actions which metabolize FNT to reactive intermediate compound such as fenitrooxon. Organophosphate toxicity also causes ATP depletion in cells which then inhibits electron transport chain. The inhibition of electron transport chain will result in release of electron and leads to ROS production and oxidative stress (Milatovic *et al.*, 2006). Increased oxidative stress

may lead to lipid peroxidation, protein oxidation and DNA damage (Scandalios, 2005). Eventually, oxidative stress will cause cell death and organ damage.

Vitamin E which consist of two forms: tocopherol and tocotrienol can be differentiated by the molecular structure of side chain. Tocopherol has trimethyl-tridecyl side chain while tocotrienol has unsaturated isoprenoid side chain (Zingg, 2007). Both forms of vitamin E act as antioxidant by donating hydrogen atom of hydroxyl group at chromanol ring to Reactive Oxygen Species (ROS) and neutralize it (Brigelius-Fohle, 2006). Furthermore, studies have shown that tocotrienol has higher antioxidant activity compare to tocopherol (Maniam *et al.*, 2008). Palm oil is a main source for tocotrienol, consisting significant amount of  $\alpha$ -tocotrienol (29%) and  $\gamma$ -tocotrienol (28%). Besides that,  $\alpha$ -tocopherol (28%) can also be found in palm oil (Sundram *et al.*, 2003). Therefore, palm oil derived Tocotrienol Rich Fraction (TRF) contains high amount of tocotrienol and tocopherol thus has high antioxidant activities. TRF was proven to reduce oxidative stress in various organs such as brain (Tiwari *et al.*, 2009) and pancreas (Budin *et al.*, 2011).

**Corresponding Author:** Siti Balkis Budin, Department of Biomedical Science, School of Diagnostic and Applied Health Sciences, Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia Tel: +603-9289 7645

Pancreas has lower antioxidant enzyme activities and has high susceptibility to oxidative stress. Numerous studies have shown that organophosphate can cause oxidative stress in pancreas (Pourkhalili *et al.*, 2009; Kamath *et al.*, 2008). At the same time, various pathological conditions of the pancreas such as pancreatitis and diabetes mellitus have also linked to increased pancreatic oxidative stress (Chiang *et al.*, 2011). Few studies also suggested that TRF can reduce the oxidative damage in various organs including bone and pancreas (Maniam *et al.*, 2008; Budin *et al.*, 2009). However, no literature was found on antioxidant effect of TRF on organophosphate induced pancreatic oxidative damage.

Therefore, current study aims to evaluate the potential of TRF as antioxidant on FNT induced pancreatic oxidative damage and morphology changes in Sprague Dawley rats.

## MATERIALS AND METHODS

**Animals:** A total of 40 Sprague Dawley rats weighing 230-250 g were kept at ambient temperature of  $25\pm 2^{\circ}\text{C}$  and 12 h light/dark cycle and fed with food pellet and water *ad libitum*. The study was conducted from 2011 till 2012 under approval of Universiti Kebangsaan Malaysia Animal Ethics Committee.

**Experimental design:** The animals were equally divided into four groups: Control, FNT, TRF and FNT+TRF. Rats from control group were given corn oil daily while rats of TRF group were given  $200\text{ mg kg}^{-1}$  b.wt. of TRF daily. On the other hand, rats from FNT group were given  $20\text{ mg kg}^{-1}$  b.wt. of FNT daily. Lastly, rats of FNT+TRF group were given  $200\text{ mg kg}^{-1}$  b.wt. of TRF followed by  $20\text{ mg kg}^{-1}$  b.wt. of FNT after 30 min. All the treatments are given via oral gavage for 28 days consecutively. At the end of the period, the rats were fasted overnight. Blood was withdrawn from orbital sinus under light ether anesthesia and collected into tubes containing sodium fluoride for fasting blood glucose analysis. The animals were sacrificed and the pancreas was immediately excised.

**Preparation of pancreas homogenate:** The excised pancreas was rinsed with normal saline and cut into sections. Pancreatic section was homogenized in 1.15% potassium chloride solution using homogenizer (Ultra Turrax T25). The homogenates were centrifuged at 8,000 rpm for 20 min at  $4^{\circ}\text{C}$  and the supernatants were stored at  $-40^{\circ}\text{C}$  until further analysis.

**Biochemical analysis:** Fasting blood glucose level was measured using enzymatic glucose-oxidase kits (Biosystems, Barcelona, Spain). Total protein content in pancreas homogenate was measured using Bradford

(1976). Malondialdehyde (MDA) level of pancreas homogenate was determined using method by Ledwozyw *et al.* (1986). MDA in the sample reacts with thiobarbituric acid to form a pink complex which was measured maximally at 532 nm. Measurement of protein carbonyl (PC) level was done using method by Levine *et al.* (1990). In this method, carbonyl group of proteins reacted with 2,4 dinitrophenylhydrazine to form hydrozone which were measured spectrophotometrically at 370 nm.

Activity of Superoxide Dismutase (SOD) was determined using method by Beyer and Fridovich (1987) which measures the inhibition rate of formazan formation using spectrophotometer at 560 nm. Activity of another antioxidant enzyme, Catalase (CAT) was measured using method by Aebi (1984). The degradation rate of hydrogen peroxide was determined by measuring the decrease in absorbance at 240 nm per minute. Measurement of reduced Glutathione (GSH) level was carried out using method by Ellman (1959). GSH in the sample reacted with Ellman's reagent to form yellow colouration which was measured spectrophotometrically at 415 nm.

**Histological analysis:** The formalin-preserved pancreatic section was embedded in paraffin and cut into a tissue section of 3-5  $\mu\text{m}$  thickness. The tissue section was stained with hematoxylin and eosin (H and E) and observed under light microscope.

**Statistical analysis:** Statistical analysis was performed using Statistical Package for Social Sciences version 15.0. All results were expressed as Mean $\pm$ Standard Error of Mean (SEM). The normality distribution of data was verified by Shapiro-Wilk test. The difference in mean of biochemical parameters are compared using one way ANOVA followed by Turkey post hoc test. The differences between groups were considered significant at  $p<0.05$ .

## RESULTS

**Effect of TRF supplementation on blood glucose and pancreatic protein content:** Table 1 shows the fasting blood glucose level and total protein content in pancreas of experimental groups. No significant difference was observed in fasting blood glucose level between the groups however total protein content in pancreas of FNT group was relatively higher than all other groups.

**Effect of TRF supplementation on lipid peroxidation and protein oxidation:** As shown in Fig. 1, pancreatic MDA level of FNT group is significantly higher ( $p<0.05$ ) as compared to control and TRF groups. On the other hand, FNT+TRF group has significantly lower ( $p<0.05$ ) MDA level compared to FNT group. Similar trend was

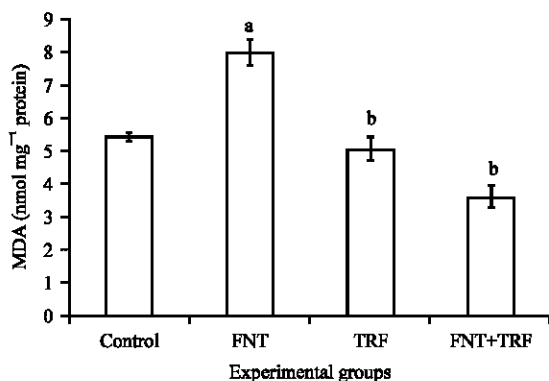


Fig. 1: Level of malondialdehyde (MDA) in pancreas homogenate by experimental groups (Mean±SEM), <sup>a,b</sup>Significantly different from control and FNT group ( $p<0.05$ ), respectively

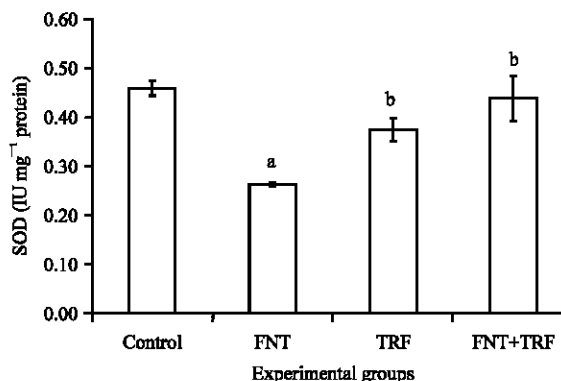


Fig. 3: Activity of superoxide dismutase (SOD) enzyme in pancreas homogenate by experimental groups (Mean±SEM), <sup>a,b</sup>Significantly different from control and FNT group ( $p<0.05$ ), respectively

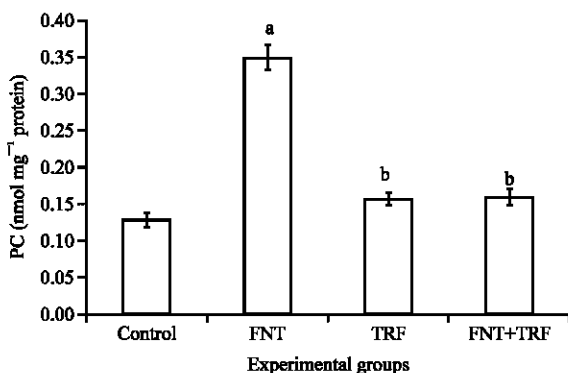


Fig. 2: Level of protein carbonyl (PC) in pancreas homogenate by experimental groups (Mean±SEM), <sup>a,b</sup>Significantly different from control and FNT group ( $p<0.05$ ), respectively

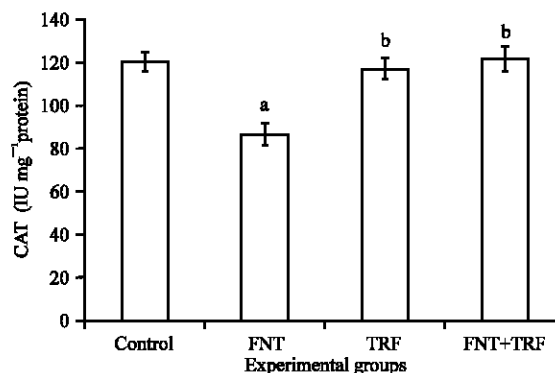


Fig. 4: Activity of catalase (CAT) enzyme in pancreas homogenate by experimental groups (Mean±SEM), <sup>a,b</sup>Significantly different from control and FNT group ( $p<0.05$ ), respectively

Table 1: Fasting blood glucose (FBG) level in plasma and total protein (TP) content in pancreas homogenate of experimental rats

Parameter	Experimental groups			
	Control	FNT	TRF	FNT+TRF
FBG (mol L <sup>-1</sup> )	8.26±0.49	8.35±0.51	7.65±0.41	8.48±0.55
TP (mg mL <sup>-1</sup> )	10.30±0.61	22.35±0.65 <sup>a</sup>	12.28±0.49 <sup>b</sup>	12.40±1.38 <sup>b</sup>

Values are as Mean±SEM, <sup>a,b</sup>Significantly different from control group and FNT group ( $p<0.05$ ), respectively

observed in pancreatic protein carbonyl level (Fig. 2) where FNT group has significantly higher ( $p<0.05$ ) carbonyl content than the control and TRF groups. Meanwhile, FNT+TRF group has significantly lower ( $p<0.05$ ) protein carbonyl level in pancreas homogenate than FNT group.

**Effect of TRF supplementation on antioxidant enzymes and glutathione:** In addition, FNT group has significantly lower ( $p<0.05$ ) SOD activity compared to control and TRF

groups (Fig. 3). However, supplementation with TRF has significantly increased ( $p<0.05$ ) SOD activity in FNT-intoxicated rats. Similarly, CAT activity of FNT group is also lower compare than control group (Fig. 4). Subsequently, FNT+TRF group has higher CAT activity than FNT group. Apart from these, FNT intoxication has significantly depleted GSH content in rat pancreas as compared to the control group (Fig. 5). Supplementation with TRF has restored GSH content to level of control group.

**Effect of TRF supplementation on pancreas in fenitrothion intoxicated rats:** Figure 6 shows the histology of pancreatic tissue from control group, FNT group, TRF group and FNT+TRF group respectively. No observable histological change was seen among all the groups. Healthy islet cells of Langerhans were seen without lymphocyte infiltration, fibrosis or amyloid substance.

## DISCUSSION

Oxidative stress has been studied for years as the underlying mechanism for organ damage in chronic organophosphate intoxication (Ogutcu *et al.*, 2008; Kalender *et al.*, 2005). In fact, lipid peroxidation was found to be a common denominator for membrane damage which often becomes reason for loss of membrane integrity in cells of intoxicated animals (Kalender *et al.*, 2007, 2010). Biotransformation of organophosphates in liver generates free radicals as by product in moderate

amount. However, their level is exaggerated during chronic toxicity due to increased cytochrome P450 activity in the liver. High level of free radicals promotes initiation and propagation of lipid peroxidation and protein damage.

In present study, chronic administration of FNT for 28 days has increased MDA level and PC level in pancreas of rats. MDA and PC content are indicative of lipid peroxidation and protein oxidation in the tissues studied. Accumulating MDA content in lipid bilayer membrane of pancreas can cause loss of its integrity and promote leakage of pancreatic enzymes into circulation (Abdollahi *et al.*, 2004). On the other hand, oxidation of protein further disrupts cellular metabolism by actively degrading key enzymes and regulating protein factors. Increased lipid peroxidation was also reported in other organs intoxicated with organophosphates (El-Shenawy, 2010; Elhalwagy *et al.*, 2008; Kaur *et al.*, 2007). Additionally, glutathione content was depleted in pancreas of the rats. Glutathione acts as endogenous antioxidant, efficiently detoxifying activated oxygen species or reducing lipid peroxides itself. Tissue glutathione concentration is a sensitive index that reflects its potential for detoxification (Kamath and Rajini, 2007). GSH stores get exhausted as it continues to detoxify free radicals. Concomitant depletion of GSH with increased MDA and PC level further proves the occurrence of oxidative stress.

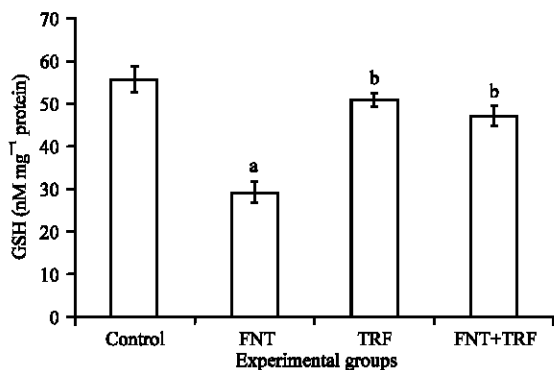


Fig. 5: Level of reduced GSH in pancreas homogenate by experimental groups (Mean±SEM), <sup>a,b</sup>Significantly different from control and FNT group ( $p < 0.05$ ), respectively

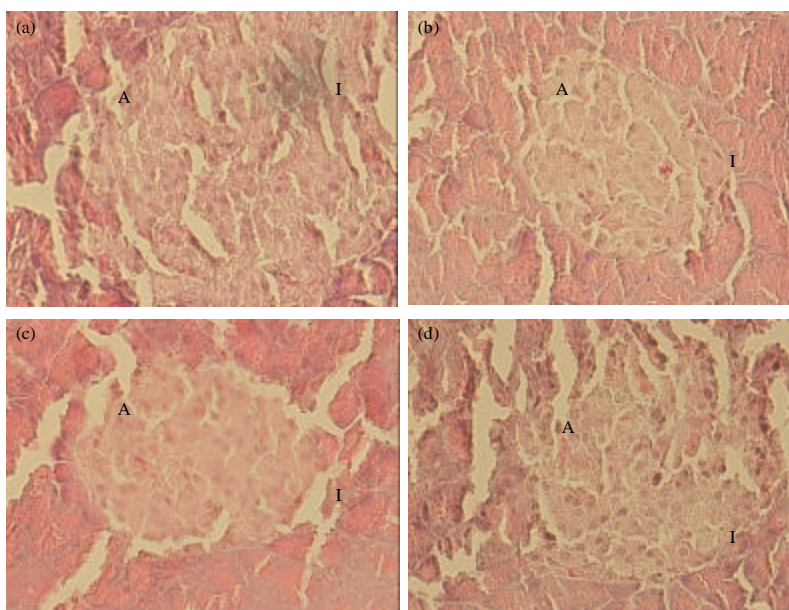


Fig. 6(a-d): Histological section of pancreas from (a) Control, (b) FNT, (c) TRF and (d) FNT+TRF groups using H and E staining, 40x magnification, A: Acini, I: Islet of Langerhans

Antioxidant enzymes, Superoxide Dismutase (SOD) and Catalase (CAT) presumably make first line of the defense. Fenitrothion intoxication also inhibited SOD and CAT activities in rat pancreas in this study. In agreement with our results, Kamath *et al.* (2008) found that dimethoate induced pancreatic oxidative damage by altering enzyme activities of SOD and CAT. SOD acts as 'incomplete antioxidant' by removing superoxide radicals. During fenitrothion intoxication, superoxide radicals are generated extensively. Continuous scavenging activity of SOD results in their exhaustion (Shittu *et al.*, 2012) which also maintains superoxide content at high concentration. High superoxide content was shown to inhibit CAT activity (Kono and Fridovich, 1982). Besides, changes in phospholipids and fatty acid due to lipid peroxidation modulate membrane fluidity and influences enzymatic activity as well as functionality of receptors in plasma membrane (Nasuti *et al.*, 2003).

Dietary antioxidants play a crucial role in preventing oxidative stress induced tissue damage and studies support protective effect of vitamin E against organophosphate induced oxidative impairments (Ogutcu *et al.*, 2008; Oral *et al.*, 2006). Experimental studies have also revealed that free radical scavenging activity of tocotrienols is higher than tocopherols due to their better distribution in cell membranes (Tiwari *et al.*, 2009). Present study shows that 200 mg kg<sup>-1</sup> b.wt. of TRF given orally for 28 days decreases pancreatic MDA level and PC level in FNT+TRF group. TRF is a potent inhibitor of lipid peroxidation and protein oxidation owing to an efficient interaction of the chromanol ring with lipid radicals and a high recycling efficiency from chromanoxyl radicals (Serbinova *et al.*, 1991).

Apart from these, tocotrienols are up taken into cells of pancreas at a higher rate (Saito *et al.*, 2004), due to which they were able to scavenge abundant free radicals that has permeate membrane and enter cytosolic portions. The reason for this property relies on its chemical structure. Tocotrienol has a total of five double bonds in its structure making it highly unsaturated (Tiwari *et al.*, 2009). Thus, it can efficiently penetrate into the saturated fatty layers of tissues (Suzuki *et al.*, 1993). High scavenging activity of tocotrienol depletes reactive oxygen species in cells and explains the maintenance of endogenous glutathione level and antioxidant enzyme activities. The antioxidant enzyme activities were not inhibited and normalized at similar level to control group. Increases antioxidant enzyme activity and glutathione level indicates that TRF can restore the antioxidant status of pancreatic cells.

Despite findings on oxidative stress, no significant difference in fasting blood glucose level was seen among all four groups. The pancreatic oxidative stress induced

by FNT was insufficient to cause changes in glucose homeostasis. In a previous study by Rezg *et al.* (2006), malathion given intra-gastrically for 32 days similarly did not affect the fasting blood glucose level of rats. Concurrently with pancreatic histology analysis shows no observable microscopic difference among all groups. Thus, the oxidative stress induced by FNT in current study is also insufficient to cause microscopic changes in pancreas tissue.

## CONCLUSION

In conclusion, we provide evidence supporting that antioxidant property of TRF may be responsible for preventing fenitrothion-induced oxidative damage in rat pancreas by restoring antioxidant enzyme activities and glutathione content. TRF supplementation was able to lower the lipid peroxidation and protein oxidation to further protect the cells.

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