# Effects of palm oil tocotrienol-rich fraction on biochemical and morphological alterations of liver in fenitrothion-treated rats

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**Abstract**: Indiscriminate application of organophosphate (OP) pesticides has led to environmental pollution and severe health problems. The aim of the present study was to evaluate the effect of palm oil tocotrienol-rich fraction (TRF) on biochemical and morphological changes of the liver in rats treated with fenitrothion (FNT), a type of OP pesticide. A total of 28 male Sprague-Dawley rats were divided into four groups; control group, TRF-supplemented group, FNT-treated group and TRF+FNT group. TRF (200 mg/kg) was supplemented 30 minutes prior to FNT (20 mg/kg) administration, both orally for 28 consecutive days. Following 28 days of treatment, plasma biochemical changes and liver morphology were evaluated. The body and absolute liver weights were significantly elevated in TRF+FNT group compared to FNT group. TRF administration significantly decreased the total protein level and restored the activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in TRF + FNT group. In contrast, total bilirubin level, γ-glutamyltranferase (GGT) and cholinesterase activity in TRF + FNT group did not significantly differ from FNT group. Administration of TRF also prevented FNT-induced morphological changes of liver as observed by electron microscope. In conclusion, TRF supplementation showed potential protective effect towards biochemical and ultrastructural changes in liver induced by FNT.

**Keywords**: Organophosphate, fenitrothion, tocotrienol-rich fraction, liver damage.

## INTRODUCTION

Organophosphate (OP) pesticides are widely used in agriculture, industry and public health due to its high insecticidal activity, less environmental persistence and moderate toxicity (Tripathi and Srivastav, 2010). However, the extensive use of OP pesticides and their easy accessibility caused an increase risk of long-term and acute occupational health problems globally (Bhatti *et al*., 2010).

OP pesticides were known to cause inhibition of acetylcholinesterase (AChE) in target tissues which leads to the accumulation of acetylcholine (ACh) in synaptic junction and subsequent cholinergic crisis (Ojha *et al*., 2011). Apart from its inhibitory effect on AChE, toxicity of OP pesticide has been attributed to generation of reactive oxygen species (ROS) causing oxidative damage to various membranous cell components (Goel *et al*., 2005). Fenitrothion (O, O-dimethyl O-(3-methyl-4-nitrophenyl)-phosphorothioate), a broad spectrum organophosphorus compound has been used throughout the world, including Malaysia. In Malaysia, fenitrothion (FNT) is either used alone or in combination with other pesticides to enhance the quality of agriculture products and to control the spread of vector-borne disease (Knet and Seng, 2003; Nazni *et al*., 2005).

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Research in animal study has reported, FNT is extensively absorbed from the gastrointestinal tract following an oral administration and this pesticide mainly deposited in the blood and liver (FAO/WHO, 2001). Previous research has shown that FNT can induce significant biochemical, structural and functional alterations in the spleen (Li *et al*., 2010), reproductive systems (Turner *et al*., 2002), liver and kidney (Afshar *et al*., 2008). Liver is the most crucial organ involved in the biotransformation and detoxification of various xenobiotics, including environmental pollutants (Levi, 2000). Acute or chronic exposure of toxicants affects all the major function of the liver (Jaeschke, 2008). Disturbance in the liver enzymes and damage of hepatocytes were reported following exposure of FNT (Kumar *et al*., 1993; Elhalwagy *et al*., 2008). The FNT-induced hepatotoxicity is believed due to overproduction of reactive oxygen species (ROS) which results in the membrane damage of various tissues (Goel *et al*., 2005).

The role of antioxidant in preventing oxidative damage in pathological conditions has extensively studied. Several pesticide toxicity studies have shown that vitamin E could ameliorate the toxic effects of OP pesticides (Ogutcu *et al*., 2008; Bhatti *et al*., 2010). Vitamin E is a lipid soluble antioxidant that can protects cellular membrane damage mediated by the radical chain-breaking action (Marchioli *et al*., 2001). Palm oil is one of the richest sources of vitamin E containing a complex mixture of tocopherol

and tocotrienol (Nesaretnam *et al*., 2007). Tocotrienol is highly found in palm oil compared to tocopherol, and therefore, it is known as palm oil tocotrienol-rich fraction (TRF). There are research reports on the potential effect of tocotrienol being a better antioxidant than α-tocopherol (Serbinova and Packer, 1994; Sen *et al*., 2007). Numerous health-related properties of tocotrienol have been identified, which includes its anticholesterolemic, anticancer, antihyperthensive, neuroprotective and antioxidative properties (Theriault *et al*., 1999). The use of antioxidants has been documented to prevent pesticide induced oxidative damage (Gokalp *et al*., 2003; Aly *et al*., 2010). Therefore, the main aim of the present study was to investigate the potential protective effects of palm oil TRF on biochemical and morphological alteration of liver in FNT-treated rat.

## MATERIALS AND METHODS

### *Chemicals*

Fenitrothion (Sumithion®), 99% purity was purchased from Supelco Analytical, USA. Palm oil tocotrienol rich fraction (Gold Tri.E® 70) was supplied by Sime Darby Malaysia,

### *Animals*

Adult male Sprague-Dawley rats (n=28), weighing 220-250 g were obtained from the Animal Laboratory Unit of Universiti Kebangsaan Malaysia. The animals were housed at the animal laboratory room in plastic cages, fed with a standard pellet and water *ad libitum*. The laboratory room was maintained at a temperature of 25ºC to 28ºC and exposed to a 12h light/dark cycle throughout the experiment. The rats were acclimatized for 1 week before the experimental period begins. All the experimental work involving the animals was performed according to the animal ethics guidelines with approval from UKM Animal Ethic Committee (UKMAEC) (FSKB/BIOMED/2010/BALKIS/14-JULY/311-AUGUST -2010-JULY-2011).

### *Experimental design*

A total of 28 male Sprague-Dawley rats were divided into four groups namely control group, TRF-supplemented group, FNT-treated group and TRF+FNT group. TRF and FNT were dissolved in corn oil, which acts as a vehicle. Rats in TRF+FNT groups were supplemented with TRF 30 minutes before the administration of FNT. Dose of FNT chosen in this study is according to previous study which showed 20 mg/kg induced biochemical and histopathological alterations without causing lethality to rats (Elhalwagy *et al*., 2008). Meanwhile, TRF dose was based on Budin *et al*., 2009 which showed that 200 mg/kg of TRF can reduce oxidative damage in rats. Following 28 days of treatment, the rats were anaesthetized with diethyl ether and cardiac puncture was performed to obtain the blood. The blood sample was collected into an EDTA tube and centrifuged at 3500 rpm for 15 minutes at 4ºC to separate the plasma. The rats were sacrificed, and the liver was dissected immediately for morphological evaluation. The plasma samples were stored at -40ºC for further biochemical analysis.

### *Toxicity evaluation*

Body weight of the rats was recorded, and the percentage of weight gain was calculated. Food and water intake of all experimental rats were monitored weekly. The absolute weight of the individual liver was recorded and the liver: body weight ratio was calculated. The clinical signs of toxicity with regard to the presence of tremor, piloerection, lacrimation and hypoactivity were observed daily throughout the experiment.

### *Biochemical assessment*

AChE activity in plasma was measured based on the method of Ellman *et al*., 1961.Plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyltransferase (GGT) enzyme activities and total bilirubin level were assessed using commercially available diagnostic kits (Biosystems, Spain) and analyzed using semi-automated laboratory bioanalyzer (BTS-350, Spain). Plasma total protein was determined according to the Bradford method using bovine serum albumin as standard (Bradford, 1976).

### *Transmission electron microscopy examination*

The liver tissue was cut into 1 $mm^3$, fixed with 2.5% glutaraldehyde 0.1 N PBS at room temperature for 1 hour and post-fixed with osmium tetraoxide for 1 hour. Then the sample was dehydrated in ascending grade of acetone for 5 minutes each (70%, 90%, 100%, 100%) followed by 1:1 (aceton: resin) and finally embedded in epoxy resin. Ultrathin sections (90 nm) were prepared using ultramicrotome and stained with uranyl acetate and lead citrate. The sections were viewed and photographed on Tecnai G2 transmission electron microscope (FEI, USA) at 100 kV.

## STATISTICAL ANALYSIS

Data was analyzed using Statistical Package for Social Sciences (SPSS 17.0) and the results were expressed as mean ± standard error of mean (SEM). The significant difference between groups was calculated by using one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons.

## RESULTS

### *Evaluation of OP toxicity*

Following 28-days administration of FNT, rats began to develop the signs of FNT poisoning as early as 15 to 30 minutes after dosing and most of the symptoms lasted for 2 to 3 hours. The observed FNT toxicity signs include

tremor, piloerection, hypoactivity and lacrimation. The signs of poisoning were also observed in TRF+FNT group but in a lower degree of severity and a shorter duration (table 1).

**Table 1**: Sign of OP toxicity observe during 28 days of experimental period

| Group | Latency (h) | Sign of toxicity |
|---|---|---|
| Control | - | None |
| TRF | - | None |
| FNT | 0.25-3.0 | Hypoactivity, tremor, piloerection, lacrimation |
| TRF+FNT | 1.0-2.0 | Hypoactivity, tremor, piloerection |

None: No sign of toxicity observed during the observation period.

The food and water intake in FNT group was markedly decreased compared to the control and TRF group. TRF administration showed an improved food and water consumption in TRF+FNT group compared to FNT group alone. Each cage housed two rats and the calculation was made per rat. Therefore, the statistical analysis was impossible. The percentage of body weight gain was significantly reduced in FNT group compared to control and TRF groups ($p<0.001$). TRF administration able to increase the percentage of body weight gain significantly in TRF+FNT group than in FNT group. Results also showed that oral administration of FNT significantly reduced the absolute liver weight and relative liver weight when compared to control group ($p<0.05$). The administration of TRF to FNT-treated rats significantly increased the absolute liver weight in TRF+FNT group compared to FNT group. However, no significant changes were observed in relative liver weight (table 2).

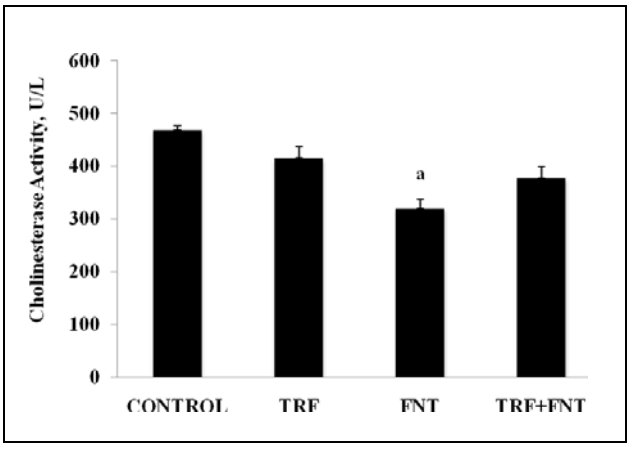


**Fig. 1**: Mean of AChE activity in plasma of all experimental groups.
The values are expressed as mean ± SEM. [a] $p<0.05$ compared to control and TRF groups.

***Biochemical indices***

The activity of liver enzymes (ALT, AST and GGT), the level of total bilirubin and total protein were significantly elevated while the activity of AChE was significantly reduced in FNT-treated group ($p<0.05$) compared to the control group (figs. 1-6). TRF supplementation significantly reduced the activity of ALT and AST and the levels of total protein in TRF+FNT group compared to FNT group. However, no significant difference was found for GGT activity, total bilirubin level and AChE activity for TRF+FNT group compared to FNT group alone.

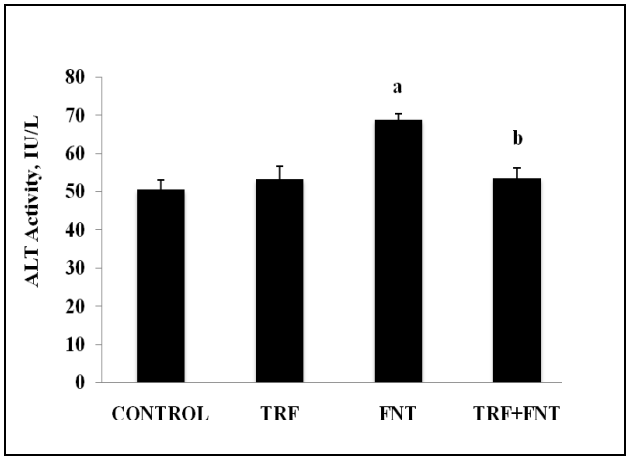


**Fig. 2**: Mean of ALT activity in plasma of all experimental groups.
The values are expressed as mean ± SEM. [a] $p<0.01$ compared to control and TRF groups. [b] $p<0.01$ compared to FNT group

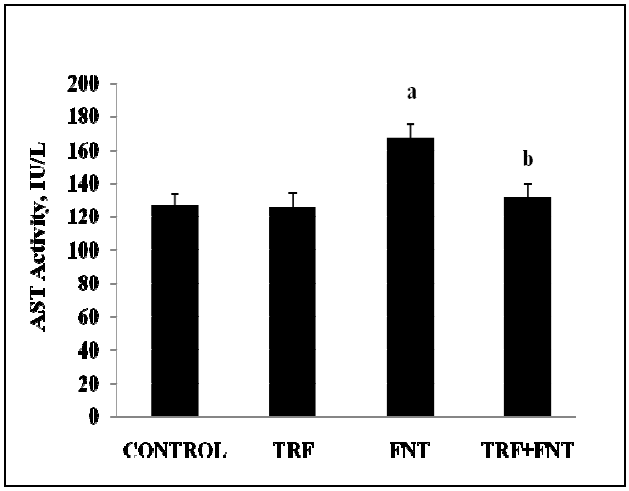


**Fig. 3**: Mean of AST activity in plasma of all experimental groups.
The values are expressed as mean ± SEM. [a]$p<0.01$ compared to control and TRF groups. [b]$p<0.01$compared to FNT group.

***Ultrastructural evaluation of the liver***

Transmission electron microscopy (TEM) findings confirmed the liver damage observed under the light microscope. Normal architecture of the hepatocyte in the control group was observed. Fig. 7 (A) showed an intact nucleus, a well-defined nuclear membrane and a normal chromatin structure. Mitochondria were abundant and

uniformly distributed in the hepatocyte. Pleats of endoplasmic reticulum were also prominent (fig. 7 B). Hepatocytes of TRF group were found similar to those in control group. FNT group hepatocyte showed a disruption of nuclear membrane and a condensed nuclear chromatin as presented in fig. 7 (C). Fig. 7 (D) showed marked swelling of mitochondria and loss of some mitochondrial cristae. Morphological changes of hepatocyte were improved in TRF+FNT group (fig. 7 E-F). The nucleus was intact but partial membranous disruption was noticed. The mitochondria were less swollen and elongated.

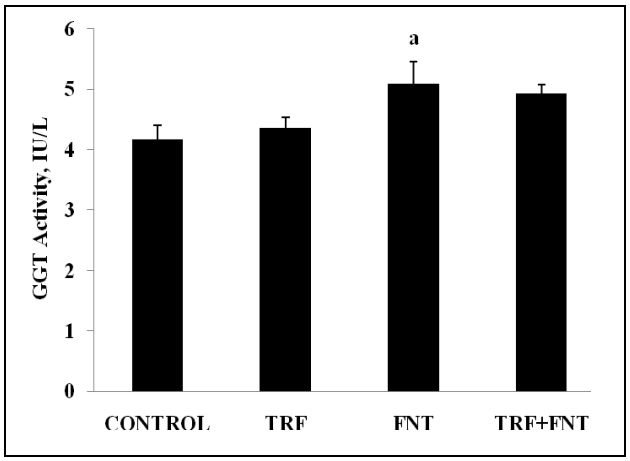


**Fig. 4**: Mean of GGT activity in plasma of all experimental groups.
The values are expressed as mean ± SEM. [a]p<0.05 compared to control and TRF groups.

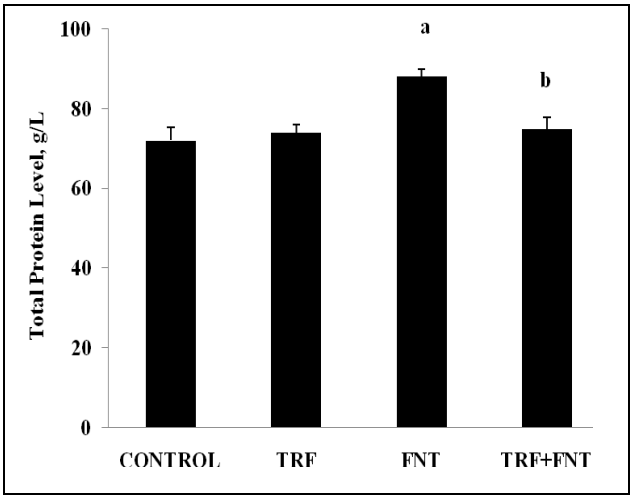


**Fig. 5**: Mean of total protein in plasma of all experimental groups.
The values are expressed as mean ± SEM. [a]p<0.001compared to control and TRF groups. [b]p<0.01 compared to FNT group.

## DISCUSSION

The main mechanism of OP pesticides toxicity is through the inhibition of AChE, which hydrolyze acetylcholine (ACh), a main neurotransmitter at central and peripheral nervous system (Costa, 2006). ACh accumulation leads to paralysis of cholinergic transmission in the central nervous system, autonomic ganglia, parasympathetic nerve endings, some sympathetic nerve endings and neuromuscular junction (Vidyasagar *et al*., 2004).

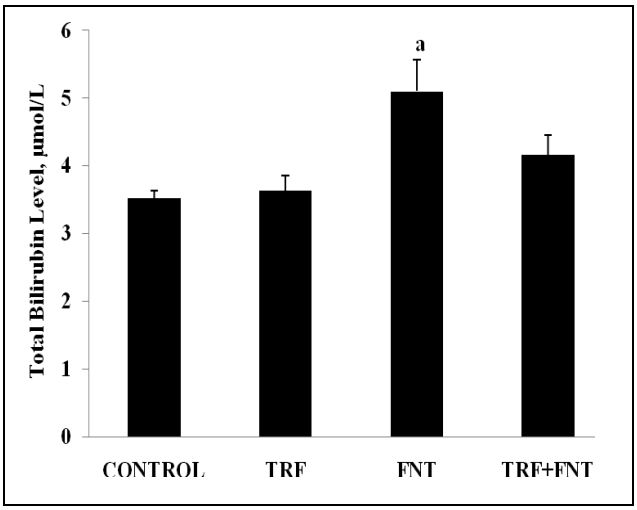


**Fig. 6**: Mean of total bilirubin in plasma of all experimental groups.
The values are expressed as mean ± SEM. [a]p<0.05 compared to control and TRF groups.

In the present study, signs of toxicity observed such as hypoactivity, tremor, piloerection and lacrimation were observed in FNT-treated rats. All these symptoms are the indicators of acute cholinergic OP toxicity due to AChE inhibition. Accumulation of ACh at the muscarinic site causes an increase in secretions, which results in lacrimation. Meanwhile, excess ACh at nicotinic site causes muscle fasciculations (tremor), piloeraction and a flaccid muscle (Kamanyire and Karalliedde, 2004). These observations were consistent with the biochemical results which showed FNT caused significant inhibition of AChE in plasma. Administration of TRF to FNT rats caused fewer severe sign of toxicity. However, AChE activity was not significantly increased in TRF+FNT groups indicating TRF supplementation could not completely reverse AChE inhibition by FNT. Therefore, the symptoms of OP toxicity were still observed in TRF+FNT rats.

Body weight changes, absolute and relative organ weights are important measures in the evaluation of target organ toxicity (Crissman *et al*., 2004). Changes in body, organ weight and organ-to-weight ratio could be due to the toxic effects of xenobiotics, which results in organ injury (Wang *et al*., 2007). In this study, subacute FNT administration produced toxicity in rats as shown by the significant decrease in body weight, absolute and relative liver weight. The decreased of body weight in OP intoxicated rats may be due to their inability to feed and drink as a consequence of muscle weakness (Agrawal and

Sharma, 2010). Therefore, the food and water intake of the FNT group was found to be less than the control group. According to Cattley and Popp (2002), acute or chronic liver injuries could result in the loss of hepatocytes. The reduction in absolute liver weight may be explained by the liver cells' loss due to FNT-induced liver damage. Supplementation of TRF caused significant improvement in body weight gain and absolute liver weight indicating the weight loss observed in OP pesticide toxicity may be due to the combination of cholinergic stress and liver damage as reported by previous research (Saafi *et al*., 2011).

Following uptake, the liver is the first organ exposed to

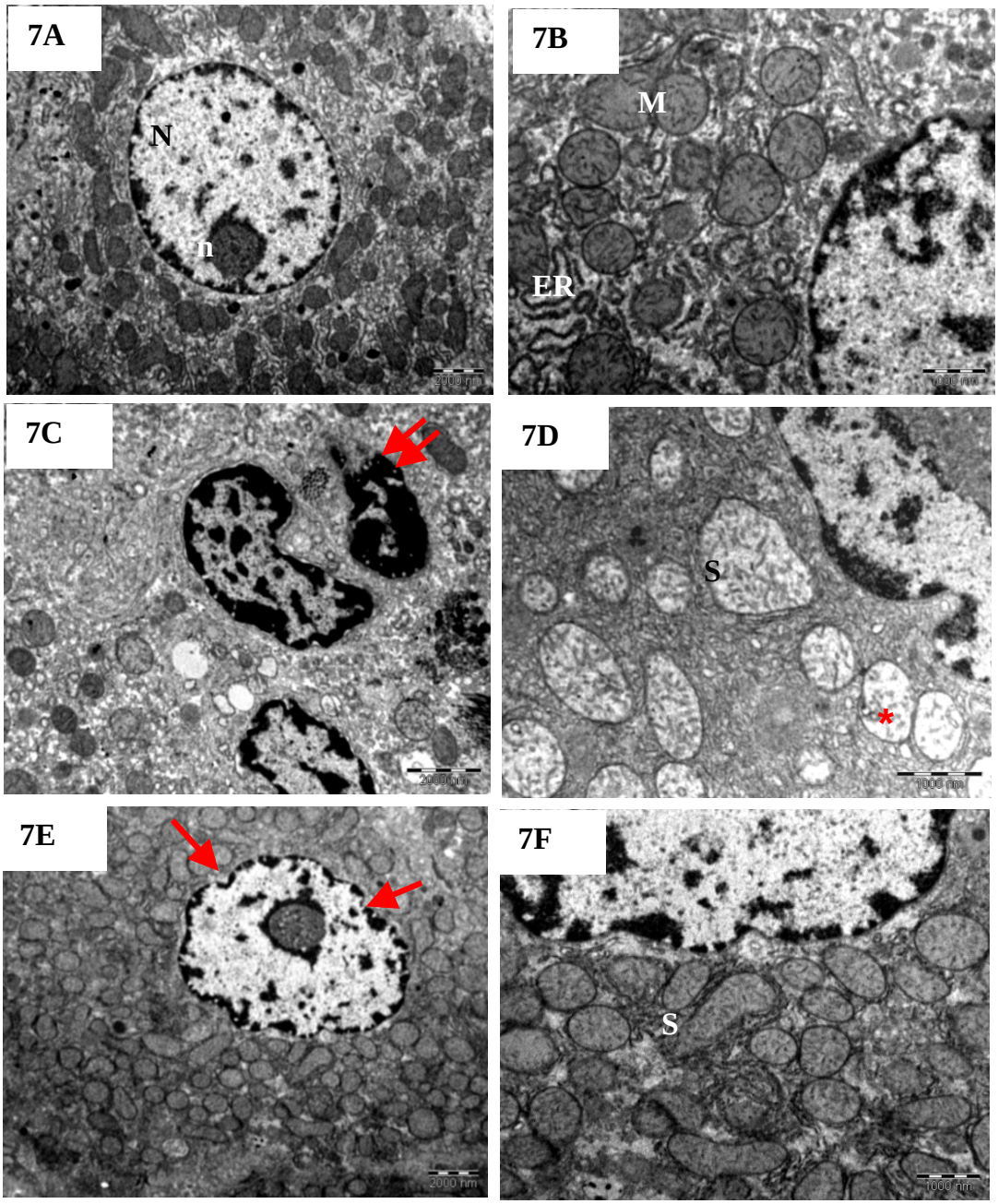


**Fig. 7**: Electron micrograph of hepatocyte in control rat (A-B) showing an intact nucleus (N), nucleolus (n) and continous nuclear membrane. Mitochondria (M) were scattered around the nucleus and pleats of endoplasmic reticulum (ER) were prominent. Electron micrograph of hepatocyte in FNT intoxicated rat (C-D) showing the disruption of nuclear membrane (double arrow) and condensed chromatin structure. Mitochondria were swollen (S) and some of the mitochondrial cristae were lost (asterisk). Electron micrograph of hepatocyte in TRF+FNT treated rat (E-F) showing an intact nucleus and partially disrupted nuclear membrane (arrow). Elongated and mild swelling of mitochondria (S) were observed.

**Table 2**: Body weight, liver weight and relative liver weight of experimental rats

| Parameters and treatments | Control | TRF | FNT | TRF+FNT |
|---|---|---|---|---|
| Body weight | | | | |
| Initial (g) | 232.17±10.72 | 232.14±13.10 | 231.00±9.93 | 231.67±5.16 |
| Final (g) | 354.3±9.97 | 343.5±21.42 | 269.28±10.63 | 300.00±21.89 |
| Weight Gain (%) | 34.42±3.97 | 32.35±3.05 | 14.21±1.53 [a] | 22.3±6.57[a,b] |
| Absolute liver weight (g) | 9.78±1.02 | 9.51±1.86 | 6.49±0.67 [a] | 7.6±0.82[a,b] |
| Relative liver weight (g) | 2.82 ± 0.17 | 2.83±0.43 | 2.35±0.18 [a] | 2.53±0.20 |
| Food intake (g/day/rat) | 21.95 | 21.16 | 17.26 | 19.27 |
| Water intake (mL/day/rat) | 37.17 | 37.82 | 28.78 | 33.79 |

The values are expressed as means ± SD. [a] $p<0.05$ compared to control and TRF groups. [b] $p<0.05$ compared to FNT group.

significant concentrations of various xenobiotics including environmental pollutants (Roganovic-Zafirova and Jordanova, 1998). Due to large blood supply, high concentration of xenobiotic-metabolizing enzymes and production of reactive metabolites by the oxidative reaction, liver is susceptible to target organ toxicity (Levi, 2000). Plasma enzymes, such as ALT, AST and GGT are mainly monitored for the evaluation of liver damaged (Ozer *et al*., 2008). Previous studies have shown that OP pesticides may cause the release of hepatic enzymes into blood circulation (Ogutcu *et al*., 2008). In this study, plasma enzymatic activities of ALT, AST and GGT were increased in FNT group as compared to control group. These results indicate the increased plasma enzyme activities are associated with hepatocyte degeneration. In this study, supplementation of TRF could prevent the liver damage caused by FNT as revealed by the remarkable decrease in the activities of plasma ALT and AST.

Administration of FNT caused a significant rise in plasma total bilirubin level and total protein. Bilirubin which is a product of haemoglobin degradation is a marker of hepatobiliary injury (Ozer *et al*., 2008). The increase of total bilirubin in plasma is attributable to the impairment of hepatocellular function in acute or subacute hepatic necrosis (Latner, 1975). Although it was statistically insignificance, TRF could reduce the total bilirubin level in TRF+FNT group. In the initial stage of tissue reaction following OP pesticide exposure, growth protein may be stimulated (Puga and La Regina Rodrigues, 1974) and acute phase protein will be generated (Tizzard, 1996) causing an in increase in total protein level. Previous studies also reported that high-protein level is reflected by the increase in globulin level (Mansour and Mossa, 2010). In this study, there was remarkable decrease in total protein level in FNT rats supplemented with TRF.

The shift in the balance of ROS and the antioxidant level has been reported to cause oxidative damage. Therefore, antioxidants are intimately involved in the prevention of cellular damage. Remarkable improvement of hepatic function in OP intoxicated were also found in rats supplemented with ginger (Farag *et al*., 2010), green tea (Elhalwagy *et al*., 2008) and date palm fruit (Saafi *et al*., 2011). It is possible that the hepatoprotective effect is due to their antioxidant activity. Being a part of total antioxidant systems, non-enzymatic antioxidant such as vitamin E and C could alleviate oxidative damage (Uzunhisarcikli and Kalender, 2011).

Due to their lipid solubility, vitamin E could intercalate into cell membrane and effectively scavenge the chain-propagating peroxyl radicals. Palm oil TRF has been identified as an excellent antioxidant which is able to suppress ROS production more efficiently (Mutalib *et al*., 2003). The higher antioxidant property is contributed by their uniform distribution in the lipid bilayer, efficient interaction of the chromanol ring with lipid radicals and higher recycling efficiency from chromanoxyl radicals (Watson and Preedy, 2009). Therefore, in the present study, most of the biochemical indices showed improvement due to the administration of TRF to FNT-intoxicated rats.

In this study, FNT intoxication results in ultrastructural changes of the liver such as swelling of mitochondria, damage to hepatocytes nuclear membrane and loss of nuclear integrity (Kumar *et al*., 1993). The swelling of mitochondria is indicative of the increase in energy required for the cells to overcome the toxic effects of OP pesticides (Kalender *et al*., 2005). Meanwhile the changes in nuclear shape and chromatin condensation are the characteristic of cell death due to oxidative damage (Sharma *et al*., 2005). Supplementation of TRF could attenuate the ultrastructural changes of the liver. The morphological alterations are consistent with the biochemical changes that have been initially observed. These findings suggested that TRF could compensate or repair the oxidative damage in the liver due to the harmful effects of FNT.

## CONCLUSION

The results of the present data showed that there were changes in biochemical indices, and the liver morphology was altered following subacute administration of FNT.

Although, supplementation of TRF could ameliorate total protein level, ALT and AST activity, the total bilirubin level and the activity of AChE and GGT were incompletely reversed. This open the door for future research in FNT toxicity which can be combated with palm oil TRF supplementation.

## ACKNOWLEDGEMENTS

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