**Tocotrienol-Rich Fraction of Palm Oil Reduced Pancreatic Damage and Oxidative Stress in Streptozotocin-Induced Diabetic Rats**

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Abstract: Increased free radicals production and decreased of antioxidant defense system are common complications encountered in diabetes mellitus (DM). Tocotrienol-rich fraction of palm oil (TRF) is an antioxidant that is rich in tocotrienol and tocopherol. The main aim of the present study was to determine the effect of TRF against oxidative stress in pancreas of diabetic rats. Forty male Sprague Dawley rats were divided into four groups: non-diabetic (NDM), non-diabetic supplemented TRF (NDM+TRF), diabetic rats and diabetic rats supplemented TRF (diabetic+TRF). Diabetes was induced through a single intravenous injection of streptozotocin at the dose of 45 mg/kg and TRF was administered orally and daily at the dose of 200 mg/kg for 28 days. At the end of four weeks study, TRF supplementation reduced hyperglycemic state and prevented the severe damage to the islet cells of pancreas. Supplemented TRF also significantly reduced (p<0.05) the status of oxidative stress of pancreas as evidenced by reduction in the level of malondialdehyde (MDA) and protein carbonyl than those diabetic rats alone. Meanwhile, improved antioxidant defense system was also observed in diabetic group supplemented with TRF which was demonstrated by significantly higher (p<0.05) levels of glutathione (GSH) as well as superoxide dismutase (SOD) activities as compared to diabetes rats without TRF supplementation. In conclusion, TRF was able to reduce pancreatic damage and oxidative stress in streptozotocin-induced diabetic rats and the process was probably mediated by its antioxidant effects.

Key words: Antioxidants, type 1 diabetes mellitus, lipid peroxidation, protein oxidation, histology.

**INTRODUCTION**

Type 1 Diabetes Mellitus is a chronic disease characterized by pancreas failure to secrete sufficient insulin, leads to failure in glucose homeostasis and eventually hyperglycemia (WHO, 1999). Hyperglycemia has put diabetic patients at great risk of developing serious complications in long time course. During hyperglycemic state, free radicals are highly produced through increased glucose oxidation, formation of advanced glycation end product, protein kinase C activation and hexosamine pathway (Brownlee, 2001; Evans, et al., 2002). The increase in free radicals causes disturbance in antioxidant defense mechanism systems such as vitamin A, C, and E and enzymatic antioxidants such as superoxide dismutase which eventually leads to the damage of cells (Maritim, et al., 2003).

TRF is derived from palm oil source and it consists of mainly tocotrienol and tocopherol which provide high antioxidant activity (Weber, et al., 1997). TRF can reduce oxidative stress in many pathological conditions (Serbinova et al., 1992; Kamat and Devasagayam, 1995). Previous study showed that TRF reduced the oxidative stress and diabetes related complications by inhibiting the production of free radical in diabetic animals (Musalmah, et al., 2001; Budin, et al., 2009). Furthermore, Suzuki, et al., (1993) reported that tocotrienol is more potent as antioxidant than tocopherol and this finding was further supported by Abdul Mutalib, et al., (2003) whose study showed that tocotrienol is more effective in preventing cell lipid peroxidation caused by oxidative stress compared to tocopherol.

The islet cells of pancreas are susceptible to oxidative damage due to the low levels of antioxidant such as superoxide dismutase (SOD), glutathione and catalase (Lenzen et al., 1996). Recent research had found that the high reactivity of free radicals will produce toxic effect towards the islet cells of pancreas (Bagri, et al., 2009). Although studies had provided evidences for the beneficial role of TRF in various pathological conditions, the effects of TRF in pancreatic damage and oxidative stress in experimental diabetes still remains unclear. Hence, this study was conducted to determine the antioxidant effect of TRF on diabetes-induced pancreatic damage and oxidative stress using streptozotocin-induced type 1 diabetic rats models.
MATERIAL AND METHODS

Animals Preparation and Induction of Diabetes:
A total of forty male Sprague Dawley rats (250-300 gm) were obtained from the laboratory animal resource unit of Universiti Kebangsaan Malaysia (UKM). Animals were allowed to adapt to laboratory condition for 7 days prior to the experiments and were kept in polypropylene cages at an ambient room temperature with wood shavings and 12 h light/dark cycle. Animals were fed with normal rat's chow and water ad libitum without restriction. Animals handling procedure were performed according to the rules and regulations by the UKM Animal Ethics Committee (UKMAEC).

Diabetes was induced with streptozotocin (Sigma, St, Louis, MO, USA) which was freshly prepared in normal saline by single intravenous injection (through the tail vein) at the dose of 45 mg/kg. Three days after the injection of STZ, blood was obtained via tail vein and diabetes was confirmed by measuring blood glucose level using the glucometer Accu-Check Go (Bayer Germany). The rats with blood glucose level >16 mol/L were considered as diabetic and were further divided into two groups which were diabetic alone (diabetes) (n=10) and diabetic supplemented with TRF (diabetes+TRF) (n=10). Meanwhile, the normal rats were divided into two groups which were non-diabetic (NDM n=10) and non-diabetic supplemented with TRF (NDM+TRF) (n=10). Rats were administered daily with 200 mg/kg of TRF (Malaysian Sime Darby Berhad) through oral gavage for four weeks duration starting from day three of diabetic induction. Meanwhile the normal control and normal +TRF were left untreated.

At the end of the study period the rats were fasted overnight, anesthetized with diethyl ether and blood was withdrawn from orbital sinus which was then collected into tubes containing sodium fluoride for fasting blood glucose analysis. Finally, animals were sacrificed by drug overdose and the pancreas was immediately excised for biochemical and histological analysis.

Homogenate Preparation:
The pancreas was weighed and homogenized in 0.1 M pH 7.4 phosphate buffer solutions (PBS) using homogenizer (Ultra Turrax T25). The homogenates were centrifuged at 3,000 rpm for 10 min at 4°C and the supernatants were stored in at -40°C until further biochemical analysis

Biochemical Analysis:
Fasting blood glucose levels were determined using enzymatic glucose-oxidase kits (Trace Scientific, Melbourne, Australia. Catalogue no. TR 15104). MDA level of pancreas homogenate was determined according to the assessment of thiobarbituric reactive species (TBARS) level as described by Stock and Dormandy (1971). In this calorimetric technique assay, the reaction between MDA with thiobarbituric acid (TBA) that produced MDA-TBA2 adducts was measured spectrophotometrically at 532 nm. Level of protein carbonyl was determined based on Levine, (1990) method. Briefly, the reaction between the carbonyl group proteins with 2,4-dinitriphenylhydrazine (DNPH) formed hydrozone which were measured spectrophotometrically at 370 nm. Reduced glutathione (GSH) was determined according to previous protocols described by Ellman, (1959). Quantification of GSH was achieved through reaction between 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and GSH that formed a yellow-colored complex 5-thio-2-nitrobenzoic acid (TNB) that was measured spectrophotometrically at 412 nm. Determination of SOD activity was performed by using Beyer and Fridovich, (1987) method. In brief, superoxide anion reduced the nitroblue tetrazolium (NBT) to form diformazan and one unit of SOD was considered as amount of enzyme that causes 50% inhibition of NBT reduction. Meanwhile, measurement of total protein was done using Bradford’s method, (1976).

Pancreas Histology:
After animal was sacrificed, pancreas was isolated and sectioned into small pieces. The sectioned pancreas tissue were fixed in 10% formalin solution embedded in paraffin and cut into a tissue section of 3–5 µm thickness. Tissue was fixed overnight on slides and subsequently stained with beta cells specific stain known as Gomori’s aldehyde fuchsins, (1950). Slides were then observed under light microscope for histopathological analysis.

Statistical Analysis:
Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) version 15.0. Normality distribution of the data was verified by Shapiro-Wilk normality test. One way ANOVA was used for between group comparison which followed by Tukey post-hoc test to compare the difference between groups. All results were expressed as means ± standard error means (SEM) and significant value was considered at p<0.05.
Results:

Body Weight and Glucose Level:

After four weeks of the study, diabetic and diabetic+TRF groups showed significantly lower (p<0.05) body weights compared to both the non-diabetic groups. The body weight in diabetic+TRF group showed significantly higher (p<0.05) compared to the diabetic group without TRF supplementation (Table 1). Fasting blood glucose levels in the diabetic and diabetic+TRF groups were significantly higher (p<0.05) compared to the both non-diabetic groups. However, supplementation of TRF was able to reduce glucose level in the diabetic rats as demonstrated by significantly lower (p<0.05) level of fasting blood glucose in the diabetic+TRF group compared to the diabetic group alone (Table 1).

Effect of TRF on the Pancreatic Oxidative Stress and Antioxidants Levels:

As demonstrated in Figure 1, a significantly higher level (p<0.05) of MDA was observed in pancreas homogenate of diabetes group alone as compared to other groups. Moreover, supplementation of TRF for 28 consecutive days caused a significant reduction (p<0.05) of MDA level in pancreas homogenate of diabetes+TRF group than in diabetes group alone and interestingly, the MDA level was reduced to almost a similar level as those observed in the NDM group. Similarly, the protein carbonyl of diabetes group alone also showed significantly higher values compared to both the non-diabetes groups and TRF supplementation significantly reduced the levels of protein carbonyl in diabetes+TRF group.

Meanwhile SOD activity was significantly low (p<0.05) in diabetes group alone compared to the other experimental groups. TRF supplementation significantly enhanced (p<0.05) SOD activities in diabetes+TRF group than those of diabetes group alone (Figure 3). Meanwhile, GSH was found to be significantly decreased (p<0.05) in the diabetes groups alone compared to the non-diabetes groups (Figure 4). However, TRF supplementation was able to increase GSH level in diabetic conditions as evidenced with significantly higher (p<0.05) level of these antioxidants in diabetes+TRF rats than in diabetic rats alone. Furthermore, the improved level was near to the value recorded for both the non-diabetes groups, suggesting the ability of TRF at 200 mg/kg to restore the GSH level in diabetic condition to normal state.

Histological Analysis:

Figure 5A to 5D showed the histology of pancreas from each of experimental group. Pancreas of NDM (Figure 7A) and NDM+TRF (Figure 7B) rats exhibited normal histology features as demonstrated with high intensity of purple-violet colour which marked the presence of beta cells in the islet of Langerhans. On the other hand, histological finding of pancreas from diabetic group alone revealed a complete destruction of the islets of Langerhans with the presence of beta cells was hardly observable (Figure 7C). However, TRF supplementation able to minimize the pancreatic damage as evidenced with the presence of beta cells in islets of Langerhans in diabetes+TRF rats compared to the diabetes group without TRF supplementation.

Table 1: The body weight and fasting blood glucose following four weeks TRF supplementation. Values were expressed as means ± SEM. Data were analyzed by ANOVA followed by Tukey post-hoc test for groups’ comparison.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weight (gram)</th>
<th>Blood Glucose (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDM</td>
<td>336.28 ± 4.68</td>
<td>8.01 ± 0.28</td>
</tr>
<tr>
<td>NDM + TRF</td>
<td>293.72 ± 10.23</td>
<td>6.35 ± 0.56</td>
</tr>
<tr>
<td>Diabetes</td>
<td>241.76 ± 7.07ab</td>
<td>29.66 ± 0.87ab</td>
</tr>
<tr>
<td>Diabetes + TRF</td>
<td>274.22 ± 11.09abc</td>
<td>24.98 ± 4.16abc</td>
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</table>

* p<0.05 compared to the NDM, * p<0.05 compared to the NDM+TRF, 
* p<0.05 compared to the diabetes group.

Fig. 1: Comparison of MDA levels between groups. Values were expressed as means ± SEM. a p<0.05 compared to the NDM group, b p<0.05 compared to the NDM+TRF group, c p<0.05 compared to the diabetes group. Data were analyzed by ANOVA followed by Tukey post-hoc test for groups’ comparison.
Fig. 2: Comparison of protein carbonyl levels between groups. Values were expressed as means ± SEM. a
p<0.05 compared to the NDM group, b p<0.05 compared to the NDM+TRF group, c p<0.05 compared
to the diabetes group. Data were analyzed by ANOVA followed by Tukey post-hoc test for groups’
comparison.

Fig. 3: Comparison of SOD activity among experimental groups. Values were expressed as means ± SEM. a
p<0.05 compared to the NDM group, b p<0.05 as compared to the diabetes group. Data were analyzed by
ANOVA followed by Tukey post-hoc test for groups’ comparison.

Fig. 4: Comparison of glutathione levels among experimental groups. Values were expressed as means ± SEM. a
p<0.05 compared to the NDM group, b p<0.05 compared to the NDM+TRF group, c p<0.05 compared
to the diabetes group. Data were analyzed by ANOVA followed by Tukey post-hoc test for groups’
comparison.
Discussion:

Damaged pancreatic islet cells are clinically associated with the development of diabetes. Coskun, et al., (2005) reported that pancreas of diabetic rats displayed degeneration, necrosis and destruction of beta cells in the islets of Langerhans. STZ is a commonly used agent in experimental diabetes due to selective destruction on beta cells of the islet of Langerhans. Depletion of intracellular nicotinamide dinucleotide (NAD), DNA strand breaks and methylation in islet cells has been proposed as the mechanism by which STZ destroys β-cells of the pancreas (Coskun, et al., 2005). In addition, STZ administration also produced free radicals which directly causing oxidative damage to the beta cells of the pancreas. In this study, STZ-induced diabetes was successfully achieved as evidenced by significantly high level of blood glucose in diabetic rats. The histological finding of the pancreas also demonstrated that a remarkable tissue destruction with a very low intensity of beta cells in islets of Langerhans that could be observed.

Supplementation of TRF at 200 mg/kg for 28 consecutive days was able to improve blood glucose levels. However, the improvement was not enough to restore blood glucose level to the normal state. TRF is a potent antioxidant that has both tocotrienol and tocopherol and it is able to reduce oxidative stress in many pathological conditions (Budin, et al., 2009). We speculate that the reduction of blood glucose levels in this study may be due to its antioxidant activities. Furthermore, histologically, Gomori aldehyde fuchsin staining showed that TRF supplementation was able to partially restore the histology of the pancreas. Therefore in this study, the TRF mainly acted as an antioxidant rather than antidiabetic because of its antioxidative actions on beta cells of the pancreas. Hence, the blood glucose levels did not reach its normal levels.

Our finding also in accordance with Coskun, et al., (2005) and Jin, et al., (2008) whose had demonstrated that antioxidant supplementation can improve glycemic status. Budin, et al., (2007) also proved that alpha lipoic acid able to reduce blood glucose and pancreatic damage in of type 1 induced diabetic rats. Furthermore, tocotrienol supplementation significantly increased the insulin levels and reduced the blood glucose in diabetic induced rats in dose dependent manner (Kuhad, et al., 2009). In addition, vitamin E supplementation increased the production of insulin by protecting the destruction of beta cells (Luostarinen, et al., 1995). According to Asayama, et al., (1994) the destruction of pancreas in diabetic rats could be prevented with vitamin E because it’s directly acts on the islets of Langerhans.
Previous study showed an increase of free radicals, lipid peroxidation and protein oxidation were occurred in diabetes (Gallou, et al., 1993). Our study also demonstrate that MDA and protein carbonyl were significantly higher in the pancreas of diabetic group alone and these results are in line with study conducted by Ramachandran, et al., (2004) which also found that the oxidative stress condition occurred in the pancreas of diabetic rats. The increase in oxidative stress is associated with the destruction of cellular component that continuously occurs during the pathogenesis of DM which may play an important role in tissue injury and the progression of diabetes complications.

Following 28 days of TRF supplementation, the level of MDA and protein carbonyl in diabetic+TRF rats was significantly lower than diabetic rats alone and the level was almost similar to those recorded in non-diabetes groups. This result indicated the role of TRF in reducing lipid peroxidation which is beneficial in overcoming diabetes-related complications. According to Musalmah, et al., (2001), supplementation of TRF at 200 mg/kg able to improved wound healing in type 1 induced diabetic rats. TRF is a vitamin E with a mixture of tocotrienol and tocopherol (Weber, et al., 1997). Vitamin E is a fat soluble antioxidant with low molar concentration and acts as antioxidant breaking chain on cell membrane. It assists in protecting PUFA on cell membranes from oxidative stress by deactivate free radicals at early stage of radical productions (Horwitt, et al., 1986) and administration of vitamin E can reduce the level of protein carbonyl in tissue homogenate of diabetic rats Ardestani, et al., (2008). Moreover, vitamin E also acts in preventing the formation of carboxymethyl lysine resulting from protein glycation of albumin (Schleicher, et al., 1997).

In this study, we observed that the activity of SOD in pancreas of diabetic rats was significantly lower than other groups. This result was similar to earlier findings by Jang, et al., (2000) and Aksoy, et al. (2005) who demonstrated that activity of SOD is decreased in pancreas tissue of diabetic rats. Decrease in SOD activity occurs when the enzyme was used to neutralize reactive superoxide anions to a more stable compound of hydrogen peroxide (Maritim, et al., 2003). STZ administration involved the production of free radicals such as hydroxyl radicals and nitric oxide (Kaneto, et al., 1995). Previous researcher found that the islets cells of pancreas contain low levels of antioxidant defense enzymes such as SOD and catalase compared to the other tissue and this may account for the susceptibility of cells to free radicals induce oxidative damage (Kakkar, et al., 1998). The current study revealed that 28 days supplementation of TRF to diabetic rats improved the SOD status and the improvement effect was remarkable whereby the SOD activities in diabetic rats supplemented with TRF is higher than those presented in normal groups. TRF supplementation possibly helps the increase the antioxidant defense mechanism in the pancreas and therefore maintains the antioxidant status. Our results were in line with previous study (Seifi, et al., 2010) who demonstrated that supplementation of natural product which contained antioxidant properties such as Artemisia campestris able to minimize lipid and protein oxidation and improve the antioxidant status of the pancreas in streptozotocin diabetic rats. Furthermore Budin, et al., (2007) also showed that SOD activity in pancreas was increased following antioxidant supplementation which is alpha-lipoic acid.

Lack of antioxidant defense mechanism also contributed towards increase in cellular susceptibility against oxidative injury (Dominguez, et al., 1998). GSH is the frontier antioxidant defense mechanism that is present in large amount in cells and it also possesses free radical scavenging property (Kamalakkannan and Prince, 2006; Coats and Ahola, 1979). In this study, GSH level was found to be significantly decreased in diabetic group. The study conducted by Abdel-Wahab, et al., (2000) also showed decreased levels of GSH in pancreas of diabetic rats. The decline of GSH in tissue is potentially due to either of lack of NADPH or excessive consumption of GSH for neutralization of peroxide radicals (Gumieniczek, 2005). Supplementation of TRF to diabetic rats was able to restore the GSH level in diabetic conditions to nearly normal state and this is possibly due to the reduction of oxidative stress as shown in this study. The findings are in line with Rajasekaran, et al., (2005) who demonstrated that GSH is increased after the diabetic rats supplemented with antioxidants.

**Conclusion:**

Following four weeks of study, oral supplementation of 200 mg/kg of TRF was found to have beneficial effect in reducing hyperglycemic status in diabetes. TRF also reduced the oxidative stress and simultaneously increased the antioxidant level in pancreas of diabetic rats. Supplementation of TRF at the dose of 200 mg/kg for 28 days reduces oxidative stress and has protective effects on diabetic-induced pancreatic damage in streptozotocin-induced diabetic rats.

**REFERENCES**


