Antioxidant protection of Malaysian tualang honey in pancreas of normal and streptozotocin-induced diabetic rats

Action protectrice anti-oxydante du miel malésien Tualang sur le pancréas de rats normaux ou diabétiques induits par la streptozotocine

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Résumé

La glucotoxicité contribue à la dysfonction β cellulaire par le biais du stress oxydatif. Notre étude précédente avait démontré que le miel Tualang améliorait le stress oxydatif rénal et induisait un effet hypoglycémiant chez des rats présentant un diabète induit par la streptozotocine (STZ). La présente étude avait pour but d’évaluer si l’effet hypoglycémiant du miel Tualang pouvait en partie être lié à son action protectrice anti-oxydante sur le pancréas. Un diabète a été induit par une seule dose de STZ (60 mg/kg ; voie intrapéritonéale). Les rats diabétiques étaient randomisés en deux groupes dont l’un recevait de l’eau distillée 0,5 ml/j et l’autre du miel Tualang (1 g/kg par jour). En parallèle, deux groupes de rats non diabétiques recevaient de l’eau distillée (0,5 ml/j) ou du miel Tualang (1 g/kg par jour). Les animaux étaient traités oralement pendant 28 jours. À la fin de la période de traitement, les rats traités par le miel avaient une glycémie significativement plus basse (p < 0,05) lorsqu’ils étaient comparés aux rats témoins diabétiques [8,8 (5,8) mmol/L versus 17,9 (2,6) mmol/L ; médiane (interquartile)]. Le pancréas des rats diabétiques témoins contenait des niveaux significativement plus élevés de malondialdéhyde (MDA) ainsi qu’une ascension de l’activité superoxyde dismutase (SOD) et glutathion péroxidase (GPx). L’activité Catalase (CAT) était significativement réduite tandis que la glutathion-S-transférase (GST) et la glutathion réductase (GR) étaient inchangées dans le pancréas des rats diabétiques. Le miel Tualang réduisait significativement les niveaux élevés de MDA (p < 0,05). Le traitement par le miel restaurait également des activités SOD et CAT. Ces résultats suggèrent que l’effet hypoglycémiant du miel Tualang peut être attribué à ses effets anti-oxydants sur le pancréas.

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Mots clés : Stress oxydatif ; Diabète sucré ; Glucotoxicité ; Streptozotocine ; Miel Tualang ; Pancréas

Abstract

Glucotoxicity contributes to β-cell dysfunction through oxidative stress. Our previous study demonstrated that tualang honey ameliorated renal oxidative stress and produced hypoglycemic effect in streptozotocin (STZ)-induced diabetic rats. This present study investigated the hypothesis that hypoglycemic effect of tualang honey might partly be due to protection of pancreas against oxidative stress. Diabetes was induced by a single dose of STZ (60 mg/kg; ip). Diabetic rats were randomly divided into two groups and administered distilled water (0.5 ml/d) and tualang honey (1.0 g/kg/d). Similarly, two groups of non-diabetic rats received distilled water (0.5 ml/d) and tualang honey (1.0 g/kg/d). The animals were treated orally for 28 days. At the end of the treatment period, the honey-treated diabetic rats had significantly (p < 0.05) reduced blood glucose levels [8.8 (5.8) mmol/L; median (interquartile range)] compared with the diabetic control rats [17.9 (2.6) mmol/L]. The pancreas of diabetic control rats showed significantly increased levels of malondialdehyde (MDA) and up-regulation of superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities. Catalase

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1. Introduction

Reactive oxygen species (ROS) is implicated in the etiology of diabetes induced by chemical agents such as alloxan and streptozotocin (STZ) in experimental animals [1]. Besides the pathogenesis of diabetes, ROS is associated with diabetic status and this condition has been proposed as one of the pathogenic mechanisms of diabetic complications [2]. Chronic hyperglycemia impairs β-cell function and insulin sensitivity, a phenomenon known as glucotoxicity [3]. Glutotoxicity is believed to contribute to β-cell dysfunction through oxidative stress, a consequence of increased mitochondrial generation of ROS that follows excessive glucose metabolism [4]. The pancreatic β-cells are highly susceptible to oxidative stress because they have very low expressions and activities of anti-oxidative enzymes [5]. The role of oxidative stress on pancreatic β-cells is further reinforced by studies which showed that alloxan generates ROS in the pancreas and that antioxidant drug, n-acetylcysteine (NAC) inhibits NF-κB activation and reduces hyperglycemia [6]. Studies have shown that over-expression of antioxidant enzymes protects against increased levels of free radicals in β-cells and its micro milieu [7,8].

Honey is a supersaturated sugar solution of which fructose and glucose are the predominant constituents [9]. In addition to carbohydrates, honey contains protein including enzymes, amino acids, vitamins and minerals, antioxidants such as catalase, peroxidase, alkaloids, polyphenols and flavonoids [10–14]. Generally, honey consists of variable compositions. These differences depend on floral sources, geographical origin, total compositional variations have been reported to influence the antioxidant properties and other therapeutic effects of honey in both in vitro and in vivo studies [15,16]. These compositional variations have been reported to influence the antioxidant properties and other therapeutic effects of honey in both in vitro and in vivo studies [15,16]. Tualang honey is produced by Apis dorsata, the bees which build their hives on tualang tree (Koompasia excelsa). In our previous study, we have reported that tualang honey reduced hyperglycemia and ameliorated oxidative stress in kidney of STZ-induced diabetic rats [19]. Based on our results, we have hypothesized that hypoglycemic effect of tualang honey might have been mediated partly through ameliorating oxidative stress in the pancreas. In the literature, interest on the role and use of natural antioxidants for prevention of oxidative stress and free radical damage in diabetes has recently increased. So far, there are no available data about the effect of honey on pancreas in STZ-induced diabetic animals. Therefore, this study was carried out to investigate the effect of chronic hyperglycemia on lipid peroxidation and free radical scavenging enzymes in pancreas of normal and diabetic rats supplemented and not supplemented with tualang honey (AgroMas®, Malaysia).

2. Materials and methods

2.1. Chemicals

STZ, tris(hydroxymethyl)aminomethane-HCl (Tris-HCl), thiobarbituric acid (TBA), reduced glutathione (GSH), oxidized glutathione (GSSG) and glutathione reductase (GR) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Superoxide dismutase (SOD) and Glutathione peroxidase (GPx) assay kits were purchased from Cayman (MI, USA). Bio-Rad protein assay kit was purchased from Bio-Rad (USA). All other chemicals used were of analytical grade.

2.2. Composition and preparation of tualang honey

Tualang honey (AgroMas®, Malaysia) was supplied by Federal Agricultural Marketing Authority (FAMA), Kedah, Malaysia. The composition of tualang honey is presented as follows: total reducing sugar (67.5%) [fructose (29.6%), glucose (30.0%), maltose (7.9%); fructose/glucose ratio (0.99)], sucrose (0.6%) and water (20.0%). It was diluted with 0.5 mL of distilled water and prepared freshly each time it was administered.

2.3. Experimental animals

Twenty-four male Sprague-Dawley rats weighing 250–300 g, housed in a well ventilated animal room at ambient temperature (25 ± 2 °C) with 12-h light and dark cycles, were used in this study. The animals were bred in Laboratory Animal Research Unit of Universiti Sains Malaysia, Health Campus, Kelantan, Malaysia. An ethical approval was obtained from the Animal Ethics Committee of Universiti Sains Malaysia, Malaysia. The care and handling of our animals followed the Institutional Guidelines for the Care and Use of Animals for Scientific Purposes from Helsinki Declaration. Rats had free access to standard chow and drinking water ad libitum, unless otherwise stated.

2.4. Induction of diabetes and treatment

After an overnight fast, diabetes was induced by a single dose of STZ (60 mg/kg body weight) administered intraperitoneally in citrate buffer (0.1 mol/L, pH 4.5). Control rats received citrate buffer alone without STZ. Two days after STZ injection, fasting blood samples were collected from the tail vein and used for the estimation of blood glucose concentrations using an Accu-Chek Glucometer (Roche, Germany). Animals with blood glucose concentrations equal to 12 mmol/L or greater with symptoms of diabetes mellitus such as polyuria, polydipsia, polyphagia and...
weight loss were considered diabetic and included in the study. The animals were randomly divided into four groups consisting of six animals and treated as follows:

- Group 1: normal rats given distilled water (0.5 mL);
- Group 2: normal rats administered tualang honey (1.0 g/kg body weight);
- Group 3: diabetic rats administered distilled water (0.5 mL);
- Group 4: diabetic rats treated with tualang honey (1.0 g/kg body weight).

The animals were treated by oral gavage once daily for 4 weeks. Fasting blood glucose and body weight were measured weekly. After 4 weeks of treatment, the animals were fasted overnight and sacrificed by decapitation. Pancreata were rapidly dissected and washed in ice-cold normal saline (0.9% NaCl), clots washed off, then frozen in liquid nitrogen and stored at −80°C till use.

2.5. Preparation of homogenates

Ten percent (w/v) homogenation of pancreatic tissues were made in Tris-HCl (0.1 M, pH 7.4) using an ice-chilled glass homogenizing vessel in a homogenizer fitted with Teflon pestle (Glas-Col, USA) at 900 rpm. The suspended mixture was centrifuged at 1000 × g for 10 min at 4°C in a refrigerated centrifuge. The resulting supernatant was used for the assay of total protein, activities of antioxidant enzymes and malondialdehyde (MDA) concentrations.

2.6. Sample assays

2.6.1. Lipid peroxidation assay

Lipid peroxidation was determined as MDA according to the method of Ohkawa et al. [20]. Briefly, 100 μL of pancreatic homogenates or MDA standards were pipetted into test tubes containing 1.5 mL of 20% (w/v) glacial acetic acid (pH 3.5), 200 μL of 8.1% (w/v) sodium dodecyl sulphate (SDS), 1.5 mL of 0.8% (w/v) thiobarbituric acid (TBA) and 700 μL of distilled water. The test tubes were incubated at 95°C for 60 min with a marble on top of each test tube. After incubation, the test tubes were cooled and then centrifuged at 3000 × g for 10 min. The amount of MDA formed was measured spectrophotometrically at 532 nm. 1,1,3,3-Tetraethoxypropane (TEP), a form of MDA, was used as standard in this assay. The concentration of MDA was expressed as nmol of MDA per mg protein.

2.6.2. Superoxide dismutase (SOD) assay

Superoxide dismutase (SOD) activity was measured using Cayman assay kit according to the manufacturer’s instructions. This assay kit utilizes a tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD was defined as the amount of enzyme needed to exhibit 50% dismutation of superoxide radical. The SOD assay measures all the three types of SOD (Cu/Zn, Mn, and FeSOD).

2.6.3. Glutathione peroxidase (GPx) assay

Glutathione peroxidase (GPx) activity was measured using Cayman assay kits according to the manufacturer’s instructions. This kit measures GPx activity indirectly by a coupled reaction with glutathione reductase (GR). Oxidized glutathione (GSSG), produced upon reduction of hydroperoxide by GPx, is recycled to its reduced state by GR and NADPH. The oxidation of NADPH is accompanied by a decrease in absorbance at 340 nm. One unit of GPx was defined as the amount of enzyme that catalyzes the oxidation of 1 nmol of NADPH per minute at 25°C.

2.6.4. Catalase (CAT) assay

CAT activity was measured according to the method of Gott [21]. Briefly, this assay involves the incubation of sample test tube containing 0.5 mL of hydrogen peroxide and 0.1 mL of pancreatic homogenate. After incubation at 37°C for 60 s, the enzymatic reaction was stopped by addition of 0.5 mL of ammonium molybdate solution. The yellow complex of ammonium molybdate and hydrogen peroxide was then measured spectrophotometrically at 405 nm. One unit of CAT was defined as the amount of enzyme that catalyzes the decomposition of 1 μmol of hydrogen peroxide per minute.

2.6.5. Glutathione reductase (GR) assay

Glutathione reductase (GR) activity was assayed according to the method of Goldberg and Spooner [22]. Briefly, 1 mL of 2.728 mM GSSG solution and 40 μL of pancreatic homogenate were incubated for 5 min at 37°C. After incubation, the reaction was initiated by addition of 200 μL of 1.054 mM NADPH solution. The decrease in absorbance was measured at 340 nm using spectrophotometer and recorded every 30 s over a period of 5 min. One unit of GR was defined as the amount of enzyme that catalyzes the oxidation of 1 nmol of NADPH per minute.

2.6.6. Glutathione-S-transferase (GST) assay

Glutathione-S-transferase (GST) activity was assayed according to the method of Habig et al. [23]. Briefly, 2 mL of 0.3 M potassium phosphate buffer (pH 6.35), 75 μL of 30 mM CDNB solution, 725 μL of distilled water and 0.1 mL of pancreatic homogenate were pipetted into a test tube. The test tube was vortexed and incubated at 37°C for 10 min. After incubation, the reaction was initiated by addition of 100 μL of 30 mM reduced glutathione solution. The decrease in absorbance was measured spectrophotometrically at 340 nm and recorded every 30 s for 4 min. One unit of GST was defined as the amount of enzyme that catalyzes the conjugation of 1 nmol of GSH-CDNB per minute.

2.6.7. Protein assay

Protein concentration was estimated using Bio-Rad protein assay kit based on the method of Bradford [24]. The assay is a dye-binding assay in which a differential color change of a dye, with maximum absorbance at 595 nm, occurs in response to various concentrations of protein.
Table 1 shows the effect of tualang honey on fasting blood glucose and body weight in normal and diabetic rats supplemented and not supplemented with tualang honey. Results are expressed as median (interquartile range) and \( n = 6 \) for all groups.

\( p \leq 0.05 \) compared to Diabetic + \( \text{dH}_2\text{O} \); \( * p \leq 0.01 \) compared to Normal + \( \text{dH}_2\text{O} \).

Table 2 summarizes the activities of antioxidant enzymes and levels of lipid peroxidation in normal and diabetic rats supplemented and not supplemented with tualang honey. Results are expressed as median (interquartile range) and \( n = 6 \) for all groups.

\( * p \leq 0.05 \); \( * * p \leq 0.01 \) compared to Normal + \( \text{dH}_2\text{O} \); \( * * * p \leq 0.05 \); \( * * * * p \leq 0.01 \) compared to Diabetic + \( \text{dH}_2\text{O} \).

### 2.7. Statistical analysis

Data were analyzed using SPSS 12.0.1. Values are expressed as median (interquartile range). Statistical significance of difference was assessed by Kruskal-Wallis H test followed by Mann-Whitney U test. \( p \) value \( < 0.05 \) was considered significant.

### 3. Results

3.1. Effect of tualang honey on fasting blood glucose and change in body weight in normal and diabetic rats supplemented and not supplemented with tualang honey

Table 1 summarizes the levels of blood glucose and changes in body weight in normal control rats supplemented and not supplemented with tualang honey as well as diabetic control rats supplemented and not supplemented with tualang honey. A significant \( (p < 0.01) \) increase in blood glucose level was observed in diabetic control rats compared with normal control rats. As shown in Table 1, the blood glucose levels of the honey-treated diabetic rats were significantly lower than those of the untreated diabetic rats \( 8.8 (5.8) \) and \( 17.0 (4.4) \) mmol/L, respectively; \( p < 0.05 \). Table 1 also shows that, in contrast to normal healthy rats, there was a significant \( (p < 0.01) \) weight loss among the diabetic control rats. The diabetic rats supplemented with tualang honey showed a significant \( (p < 0.05) \) increase in body weight compared with diabetic control rats.

3.2. Effect of tualang honey on antioxidant enzyme activities and levels of malondialdehyde in the pancreata of normal and diabetic rats supplemented and not supplemented with tualang honey

Table 2 summarizes the activities of antioxidant enzymes and levels of malondialdehyde in the pancreata of normal and diabetic rats supplemented and not supplemented with tualang honey. The table shows a significant \( (p < 0.05) \) increase in the activity of pancreatic SOD in diabetic control rats compared with normal control rats. The honey-treated diabetic rats had a significantly \( (p < 0.01) \) down-regulated pancreatic SOD activity compared with diabetic control rats. There was a significant \( (p < 0.01) \) decrease in the CAT activity in diabetic control. Oral administration of tualang honey to diabetic rats significantly \( (p < 0.05) \) elevated pancreatic CAT activity. The pancreatic GPX activity was significantly \( (p < 0.01) \) up-regulated in diabetic control rats and treatment with tualang honey did not down-regulate pancreatic GPX activity. There was no significant change in the activities of pancreatic GST and GR in the diabetic rats. The levels of pancreatic MDA were significantly \( (p < 0.01) \) elevated in diabetic control rats compared with normal rats. Treatment with tualang honey significantly \( (p < 0.05) \) decreased the levels of pancreatic MDA.

### 4. Discussion

Both clinical and experimental models of diabetes mellitus are associated with increased oxidative stress caused by
chronic hyperglycemia [25]. Oxidative stress is postulated as one of the mechanisms by which glucotoxicity produces its deleterious effects [26]. STZ has a selective cytotoxic action on pancreatic β-cells. Although the exact mechanism by which STZ produces its β-cell cytotoxic effect is not fully understood, it is believed to be mediated by release of nitric oxide (NO) and ROS resulting in alkylation of DNA [27]. In the present study, we examined the possible protective effect of tualang honey on pancreatic tissue in STZ-induced diabetic rats. In our previous study, we have demonstrated that tualang honey at a dose of 1.0 g/kg improved body weight, decreased blood glucose levels and ameliorated oxidative stress in kidneys of STZ-induced diabetic rats [19]. We had postulated that the antihyperglycemic effect of tualang honey might partly be due to protection of pancreatic β-cells against STZ-induced diabetic oxidative stress. In the present study, tualang honey treatment at a dose of 1.0 g/kg body weight decreased elevated blood glucose and moderately increased body weight in STZ-induced diabetic rats. This is consistent with our previous findings [19].

ROS attack membrane phospholipids and cause the conversion of unsaturated fatty acids to lipid peroxides. Peroxidation of fatty acids containing three or more double bonds generates MDA. These lipid peroxides are highly toxic products that may bring about inactivation and damage of membrane bound enzymes, proteins and cell membranes either through direct attack by free radicals or through chemical modification by its end products, such as MDA [28]. In our study, pancreatic MDA levels were significantly increased in diabetic control rats. This is consistent with other studies showing that diabetes is associated with elevated levels of lipid peroxides [29]. Tualang honey treatment decreased the elevated MDA. Increased levels of pancreatic MDA in diabetic rats might be a consequence of increased ROS formation and decreased antioxidants caused by STZ as well as hyperglycemia, which in view of augmented effect of oxygen free radicals might have led to accumulation of MDA [4,27,29].

Our data showed that superoxide dismutase (SOD) activity was up-regulated while catalase (CAT) activity was down-regulated in the pancreas of diabetic control rats. Other investigators also reported similar findings [30,31]. Increased activity of SOD could be due to its induction by increased generation of superoxide anions. This is often a defense mechanism to protect the pancreatic β-cells from the detrimental effect of increased superoxide production [7,32]. Although SOD converts superoxide radicals to hydrogen peroxide, experimental evidence show that over-expression of SOD activity may promote toxicity of superoxide-radical-forming compounds [33]. Enhanced SOD activity might lead to an increased turnover of hydrogen peroxide generation. Catalase (CAT) catalyzes the conversion of hydrogen peroxide to water and oxygen. The down-regulation of CAT activity might be a consequence of glycation [34], and inhibition by elevated levels of superoxide [35]. In line with these data, enhanced SOD and reduced CAT activities might result in accumulation of hydrogen peroxide which might undergo Fenton reaction in the presence of transition metal ions such as copper and iron to generate hydroxyl radicals [36]. Hydroxyl radical is a highly reactive species proposed as the main initiator of most free radical induced tissue damage [37]. This could have contributed to the significantly elevated levels of MDA observed in the diabetic control rats. The pancreas of diabetic rats showed increased activity of glutathione peroxidase (GPX). The elevated GPx activity in the diabetic rat pancreas might be a compensatory mechanism to detoxify organic and inorganic peroxides including excess hydrogen peroxide generated by increased SOD activity [38,39]. Our results showed that the activities of glutathione reductase (GR) and glutathione-S-transferase (GST) did not change in diabetic rat pancreas. This may indicate that these enzymes (GR and GST) do not play significant roles in protecting the pancreas against oxidative stress.

In conclusion, our results suggest that pancreatic tissues of diabetic rats are exposed to an increased oxidative stress. Tualang honey supplementation showed protective effects on the pancreas against STZ-induced diabetic oxidative stress. This is evident by the reduced levels of lipid peroxidation marker, MDA. Tualang honey administration also restored pancreatic SOD and catalase activities, while GPx activity remained up-regulated. These protective effects of tualang honey on the diabetic pancreas against oxidative stress might be responsible for its hypoglycemic effect. Our data showed that tualang honey did not produce any effect on blood glucose, antioxidant enzymes and other oxidative stress markers in non-diabetic pancreas. Further studies are required to elucidate the exact mechanism by which tualang honey protects the pancreas against oxidative stress.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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References


