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Original article

Protective effect of kaempferol, a flavonoid compound, on oxidative mitochondrial damage in streptozotocin-induced diabetic rats

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Summary. The present study was designed to investigate the effect of kaempferol on oxidative mitochondrial damage in liver of streptozotocin (STZ)-induced diabetic rats. Diabetes was induced into adult male albino rats of the Wistar strain, by intraperitoneal administration of STZ (40 mg/kg body weight (BW)). Kaempferol (100 mg/kg BW) or glibenclamide (600 µg/kg BW) was administered orally once daily for 45 days. Diabetic rats showed a significant elevation of mitochondrial thiobarbituric acid reactive substances (TBARS) levels in liver as compared to control rats. The level of enzymatic (superoxide dismutase (SOD), glutathione peroxidase (GPx)) and non-enzymatic (reduced glutathione (GSH)) antioxidants were decreased significantly in liver mitochondria of STZ-induced diabetic rats as compared to control rats. Administration of kaempferol or glibenclamide resulted in significant decrease in TBARS and significant increase in SOD, GPx and GSH when compared to diabetic control rats. The activities of mitochondrial enzymes such as isocitrate dehydrogenase (ICDH), alpha-ketoglutarate dehydrogenase (α-KGDH), succinate dehydrogenase (SDH), and malate dehydrogenase (MDH) decreased significantly in STZ-induced diabetic rats. In addition, the activities of mitochondrial respiratory chain enzymes such as NADH dehydrogenase and Cytochrome c-oxidase also decreased significantly in STZ-induced diabetic rats as compared to control rats. Administration of kaempferol or glibenclamide resulted in significant reversal of these enzymes' activities to near normal when compared to diabetic control rats. Thus, obtained results indicate that administration of kaempferol attenuates the mitochondrial damage in STZ-induced diabetic rats.

Key words: albino Wistar rats, streptozotocin, diabetes, mitochondrial damage, kaempferol

Introduction

Diabetes mellitus (DM) is characterized by hyperglycemia due to defective insulin action, insulin secretion or both. The World Health Organization reports that the number of diabetics is expected to increase to 366 million or more by 2030 from 171 million in 2000 (1). Hyperglycaemia is associated with the generation of reactive oxygen species (ROS) and consequent oxidative damage in the liver, kidneys, heart, and pancreas (2). Implication of oxidative stress in the pathogenesis of diabetes is suggested not only by oxygen free radical generation due to non-enzymatic protein glycosylation, auto-oxidation of glucose, impaired glutathione metabolism, alteration in antioxidant enzymes and decreased level ascorbic acid (3, 4). Increased oxidative stress plays an important pathogenic role in the development and progression of diabetes and its complications (5).

Mitochondria are dynamic organelles that not only produce ATP for cellular function, but also participate in a number of intracellular processes such as cell division, the initiation of mitochondrial signaling pathways, modulation of cytosolic metabolic pathways and ultimately determination of cell life or death. In addition, mitochondria are a continuous source of su-

peroxide anions ($O_2^{\cdot-}$) and their ROS products during cell injury (6-8). Hyperglycemia-induced ROS generation within mitochondria plays a major role in the development of diabetic complications (9). Mitochondria are one of the most important cell organelles in diabetes research because of its crucial role as a regulator of energy balance (10). Various NAD/NADP-linked enzymes are intricately involved in the maintenance of the reduced redox state in mitochondria in order to provide the reducing power to generate adenosine triphosphate (ATP) via oxidative phosphorylation (11). DM is associated with mitochondrial dysfunction that may result in increased ROS generation and impaired bioenergetics (12).

Medicinal plant drug discovery continues to provide new and important leads against various pharmacological targets including diabetes (13). Bioflavonoids are currently considered as promising natural substances to develop a modern therapy for diabetes (14). Kaempferol (Fig. 1), a flavonoid, naturally occurs in a variety of fruits, vegetables, wine, and tea. It can be isolated from tea, broccoli, witch-hazel, propslis, grapefruit, and other plants (15). The medicinal properties of kaempferol include antioxidant, anti-inflammatory and anticancer activity (16-18). Several studies have shown that intake of foods containing kaempferol is associated with reductions in mortality, the incidence of myocardial infarction, and the incidence of cerebrovascular disease, as well as with a slightly reduced risk of coronary heart disease (19-21). Previously, in an *in vitro* study, it was shown that kaempferol ameliorates hyperglycemia by improving insulin-stimulated glucose uptake in adipocytes (22). Kaempferol also performs a beneficial role in diabetes by preventing oxidative damage in pancreatic β cells (23). Our previous *in vivo* study found that the administration of kaempferol is having good antihyperglycemic and hypolipidemic activities on STZ-induced diabetic rats (14).

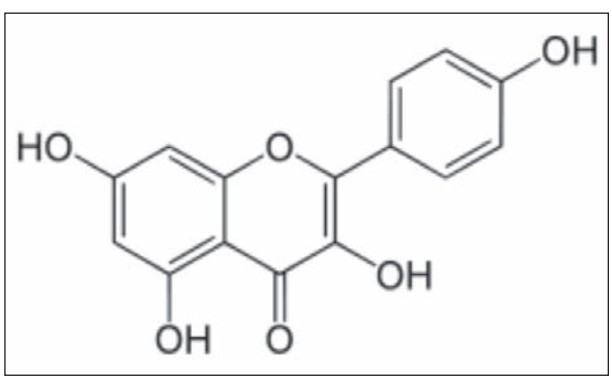

Figure 1. Chemical structure of kaempferol

So far no study has been conducted on the effect of kaempferol on oxidative mitochondrial damage in STZ-induced diabetic rats. Hence, in the present study we have sought to examine the effects of kaempferol on oxidative mitochondrial damage in liver of STZ-induced diabetic rats.

Materials and Methods

Drugs and chemicals

STZ and kaempferol were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade.

Experimental animals

Male albino rats of Wistar strain of body weight (BW) ranging from 180 to 200 g were procured from Central Animal House, King Saud University, and they were maintained in an air-conditioned room (25 ± 1°C) with a 12-h light/12-h dark cycle. The animals were fed *ad libitum* with normal laboratory pellet diet used in the study and procedures involving animals and their care were accordance with the Policy of Research Centre, King Saud University.

Experimental induction of diabetes

The animals were made diabetic by a single intraperitoneal injection of streptozotocin (STZ, 40 mg/kg BW, between 8:00 AM to 9:00 AM) in a freshly prepared citrate buffer (0.1 M, pH 4.5) after an overnight fast. STZ injected animals were given 20% glucose solution for 24 h to prevent initial drug-induced hypoglycaemic mortality. Diabetes was confirmed by measuring the fasting plasma glucose concentration 96 h after induction. Albino rats with a plasma glucose level above 220 mg/dL were considered diabetic and used in this experiment.

Experimental design

The animals were randomly divided into five groups consisting of six animals each. Kaempferol (100 mg/kg BW) or glibenclamide (600 µg/kg BW) was dissolved in 5% DMSO and administered by intubation (p.o.) once a day, between 9 a.m. and 10 a.m., for 45 days. In our previous study, we have chosen 50, 100 and 200 mg/kg doses of kaempferol for tested the glucose lowering action (14). Of the three doses of kaempferol (50, 100 and 200 mg/kg BW), 100 mg gave the maximum improvement in plasma glucose and insulin (14). Hence, the active dose of 100 mg was used in this study.

Group I: Control rats (5% DMSO alone)

Group II: Control rats + kaempferol (100 mg/kg BW)

Group III: Diabetic control

Group IV: Diabetic rats + kaempferol (100 mg/kg BW)

Group V: Diabetic rats + glibenclamide (600 µg/kg BW)

After 45 days administration of kaempferol and glibenclamide, the rats were fasted for 12 h, anesthetized by ketamine (24 mg/kg BW via intramuscular injection) and sacrificed by decapitation. The liver was dissected out immediately and stored for mitochondrial isolation.

Biochemical assays

The mitochondrial fraction of the liver tissue was isolated by the standard method of Johnson and Lardy (24). The concentration of thiobarbituric acid reactive substances (TBARS) in the liver mitochondria fraction was estimated by the method of Niehaus and Samuelsson (25). The activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in the liver mitochondrial fraction were assayed by the method of Kakkar et al. (26) and Rotruck et al. (27) respectively. Reduced glutathione (GSH) was estimated by the method of Ellman (28).

The activity of isocitrate dehydrogenase (ICDH), α-ketoglutarate dehydrogenase (α-KGDH), succinate dehydrogenase (SDH) and malate dehydrogenase (MDH) were assayed in the mitochondria fraction of the liver by the method of Bell and Baron (29), Reed and Mukherjee (30), Slater and Borner (31) and Mehler et al. (32) respectively. NADH-dehydrogenase was assayed by the method of Minakami et al. (33) and cytochrome-c-oxidase was assayed by the method of Pearl et al. (34).

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using SPSS software package 9.05. Results were expressed as mean ± standard deviation (S.D) from six rats in each group. P values < 0.05 were considered as significant.

Results and Discussion

Diabetes is a complex metabolic disorder with characteristic modulation of glucose metabolism leading to excessive ROS production and generation of various diabetic complications such as nephropathy, neuropathy, cardiopathy and even hepatopathy. The liver is intimately involved in the pathogenesis of diabetes, where hepatic insulin resistance is regarded as a key contributing element to high fasting blood glucose (35) and ketone body formation, and thus to the development of diabetic complications. In insulin dependent diabetes mellitus (IDDM) various agents like interleukin-1 beta, interferon gamma, tumor necrosis factor alpha, alloxan and streptozotocin could operate by forming free radicals that could attack the mitochondrial genome (36). The structural damage of tissues or complications in diabetes mellitus may be due to oxidative stress. Oxidative stress may play an important role in the development of mitochondrial damage associated diabetic complications (37). The superoxide anion radical is generated by liver mitochondria under both physiological and pathological conditions. Table 1 depicts the levels of TBARS, activities of enzymic antioxidants (SOD and GPx) and the levels of non-enzymic antioxidant (GSH) in the liver mitochondria of normal and STZ-induced diabetic rats. In our study mitochondrial levels of TBARS in liver significantly increased in STZ-induced diabetic rats. It indicates that increased level of mitochondrial TBARS in diabetic rats cause lipid peroxidation, which

Table 1. Effect of kaempferol on TBARS and the activities of enzymatic antioxidants and level of GSH in mitochondrial fraction of the liver of STZ-diabetic rats

Groups	TBARS (mmol/mg protein)	SOD (Ua/mg protein)	GPx (Ub/mg protein)	GSH (μg/mg protein)
Control	1.76 ± 0.10[a]	2.85 ± 0.14[a]	7.92 ± 0.68[a]	13.56 ± 1.10[a]
Control + kaempferol (100 mg/kg BW)	1.81 ± 0.14[a]	2.83 ± 0.21[a]	8.05 ± 0.76[a]	13.48 ± 1.02[a]
Diabetic control	3.02 ± 0.28[b]	1.55 ± 0.10[b]	4.78 ± 0.29[b]	8.01 ± 0.64[b]
Diabetic + kaempferol (100 mg/kg BW)	1.62 ± 0.12[c]	2.43 ± 0.22[c]	6.45 ± 0.55[c]	11.93 ± 0.98[c]
Diabetic + glibenclamide (600 μg/kg BW)	1.68 ± 0.15[c,d]	2.80 ± 0.19[a]	6.87 ± 0.44[c]	12.07 ± 1.17[c]

Values are means ± S.D for six rats in each group.
Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT).
U^a- Enzyme concentration required for 50% inhibition of NBT reduction/min.
U^b- μmol of reduced glutathione consumed/min.

may alter the structure integrity of mitochondrial membranes resulting in mitochondrial dysfunctions. When kaempferol was administered in diabetic rats the level of TBARS significantly reverted to that of near normal rats. Kaempferol has been shown to possess antioxidant and anti-inflammatory effects (16, 17). Thus antioxidative action of kaempferol may have an excellent ability to scavenge hydroxyl and peroxyl radicals.

Increased production of oxygen free radicals in diabetes is suggested by protein glycosylation and auto-oxidation of glucose and decreased availability of enzymatic and non-enzymatic antioxidants (38). SOD catalyzes the conversion of superoxide anion to hydrogen peroxide (H_2O_2). H_2O_2 is cleared from the system by the activity of catalase and glutathione peroxidase. In present study the activities of SOD and GPx were decreased significantly in the liver mitochondria of diabetic rats, which is probably due to an increased generation of accumulation of ROS and severe oxidative stress by STZ. GSH is a major defence mechanism against oxidative stress within mitochondria. DM has been found to profoundly alter mitochondria, including the selective depletion of mitochondrial GSH (39). In our study the level of GSH significantly decreased in liver mitochondria of STZ-induced diabetic rats. It has been suggested that depletion of mitochondrial GSH resulted in depletion of other antioxidants and enhanced the susceptibility of cells to further damage. This condition may aggravate the overproduction of mitochondrial ROS. Administration of kaempferol and glibenclamide to diabetic rats significantly reversed these enzymatic and non-enzymatic antioxidants towards normalcy. Some antioxidants are suggested to have beneficial effects in the treatment of oxidative stress-associated diseases including diabetes (40, 41). Kaempferol has good antioxidant and anti-inflammatory effects (16, 17). The beneficial effects of kaempferol may also be attributed to improved antioxidant activity in tissues, which potentially reduces the membrane lipid peroxides (42).

Mitochondria are important subcellular organelles involved in energy production and are susceptible to oxidative stress. The mitochondrial enzymes (ICDH, α-KGDH, SDH and MDH) catalyze the oxidation of several substrates through the tricarboxylic acid (TCA) cycle, yielding reduced equivalents, which are channelled through the respiratory chain for the synthesis of ATP by oxidative phosphorylation. STZ-elicited ROS leads to oxidative insult, which may be the reason for increased susceptibility of mitochondrial proteins to oxidative damage. Therefore, it is highly probable that STZ-associated elevations in ROS and lipid peroxides might have the effect of inactivating mitochondrial proteins, which would diminish mitochondrial function and ultimately lead to some of the toxic effects that have been observed in the mitochondrion of tissues of diabetic rats. Figure 2 and 3 represents the activities of TCA cycle enzymes and respiratory chain enzymes in the liver mitochondria of normal and STZ-induced diabetic rats. In the present study the activities of mitochondrial enzymes such as ICDH, α-KGDH, SDH, and MDH were decreased significantly in STZ-induced diabetic rats. A decreased activity of

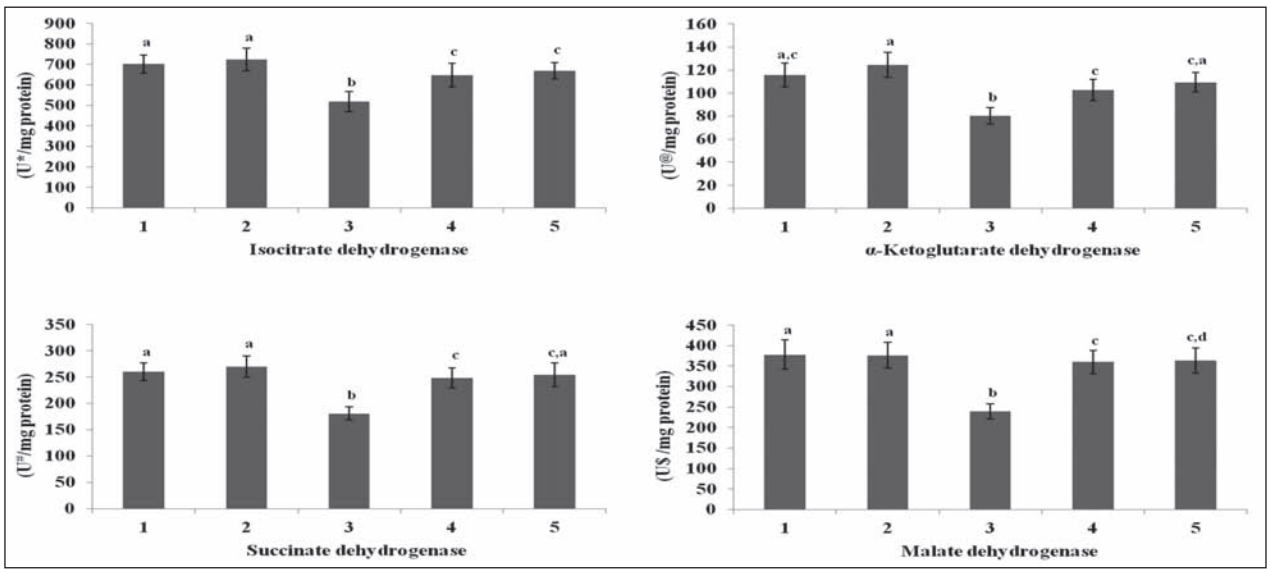

Figure 2. Effect of kaempferol on the activities of mitochondrial respiratory chain enzymes in the liver of STZ-diabetic rats.
Group 1: Control
Group 2: Control + kaempferol (100 mg/kg BW)
Group 3: Diabetic control
Group 4: Diabetic control + kaempferol (100 mg/kg BW)
Group 4: Diabetic control + glibenclamide (600 mg/kg BW)
Values are means ± S.D. for six rats in each groups.
Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT).
U* – nmol of α-ketoglutarate formed/h; U@ – nmol of ferrocyanide formed/h; U# – nmol of succinate oxidized/min; U$ – nmol of NADH oxidized/min.

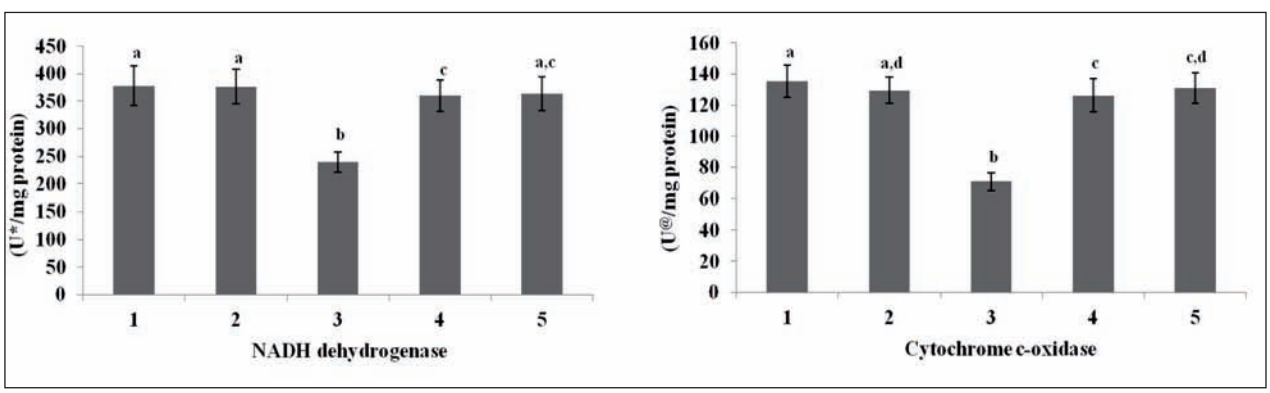

Figure 3. Effect of kaempferol on the activities of mitochondrial tricarboxylic acid cycle enzymes in the liver of STZ-diabetic rats.
Group 1: Control
Group 2: Control + kaempferol (100 mg/kg BW)
Group 3: Diabetic control
Group 4: Diabetic control + kaempferol (100 mg/kg BW)
Group 4: Diabetic control + glibenclamide (600 mg/kg BW)
Values are means ± S.D for six rats in each group.
Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT).
U* – nmol of NADH oxidized/min; U@ – change in OD x 10^{-2}/min

mitochondrial TCA cycle enzymes has observed in the STZ-diabetic rats (43). Inhibition of these enzymes by ROS may affect the mitochondrial substrate oxidation, resulting in reduced oxidation of substrates, reduced rate of transfer of reducing equivalents to molecular oxygen and depletion of cellular energy (44). Oral administration of kaempferol and glibenclamide to diabetic rats the activities of TCA cycle enzymes significantly reversed to near normal rats. Thus results indicate that kaempferol may improve the mitochondrial antioxidant defence system, and minimizing the mitochondrial damage associated with diabetes complications.

Cytochrome c oxidase and NADH dehydrogenase are present in the inner mitochondrial membrane and are involved in the synthesis of high-energy compound ATP. NADH-dehydrogenase constitute complex I of the electron transport chain, which passes electron from NADH to coenzyme Q. Cytochrome c-oxidase donates electrons directly to molecular oxygen and constitutes complex IV. In the present study, the activity of these enzymes significantly decreased in STZ-induced diabetic rats, which might be due to depletion of reducing equivalents like NADH and NADPH, which are utilized for the formation of reduced glutathione to counter oxidative damage of mitochondrial components. Administration of kaempferol and glibenclamide significantly increased these enzymes activities in STZ-induced diabetic rats due to its free radical scavenging activity.

In conclusion, the results suggest that kaempferol could maintain liver mitochondrial function in STZ-induced diabetic rats. The possible mechanisms for the observed preventive effects of kaempferol could be due to scavenging of free radicals and improving antioxidant status. This study may be beneficial to prevent mitochondrial damage associated with diabetes complications. Further detailed investigation is necessary to understand kaempferol's mechanism of action and establish its therapeutic potential in the treatment of diabetes and diabetic complications.

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