m-Coumaric acid attenuates non-catalytic protein glycosylation in the retinas of diabetic rats

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In this study, we investigated the inhibitory effects of m-coumaric acid on the glycosylation of proteins in the retinas of diabetic rats. Male rats were divided into two main groups, Group I (normal control) and Group II (diabetic); Group II was further divided into four subgroups: Group IIa (diabetic control), Group IIb (diabetic rats were given m-coumaric acid orally [150 mg/kg, body weight (bw)/day]), Group IIc (diabetic rats were given HCA m-coumaric acid orally [300 mg/kg bw/day]), and Group IId (diabetic rats were given insulin [10 units/kg bw/day]) as a positive control). The treatment lasted for six weeks, and the data obtained suggested that m-coumaric acid reduced glucose and glycated hemoglobin levels, which further decreased the formation of glucose-derived advanced glycation end products. Hence, it protected the tissues from the detrimental effects of hyperglycemia and enhanced antioxidant activity. In conclusion, m-coumaric acid could be a potential candidate to prevent the onset and progression of retinopathy in diabetic patients. © Pesticide Science Society of Japan

Keywords: diabetic rats, AGEs, HCA, m-coumaric acid, hyperglycemia, retinopathy.

Introduction

Protein glycosylation is a complex cascade of sequential steps that begins with the non-catalytic binding of sugar, such as glucose, fructose or their derivatives to the amino group of a protein. The molecular rearrangement of functional proteins occurs before the production of glycated proteins (GPs) followed by the crosslinking of these proteins.1)

These modified proteins are not recycled in different organs, such as the collagen of connective tissue, nephrons, retinas, or blood vessels, leading to the subsequent dysfunction of these organs. In diabetic patients, the rate of advanced glycation end products (AGEs) was increased, resulting in a deleterious effect on vital tissues such as retinas, neurons, nephrons, and the heart.2) The most common symptom diagnosed in diabetic patients is the formation of glycated hemoglobin (HbA1c) due to the bonding between hemoglobin and glucose often referred to as glycation.3) The detection of HbA1c in blood samples has been used by doctors to diagnose type 2 diabetes. According to a report from the World Health Organization (WHO), a HbA1c level of 6.5% has been recommended as a cutoff point for diagnosing diabetes.4) Additionally, aldose reductase plays a significant role in the conversion of glucose-6-phosphate to sorbitol, which acts as a mediator in the development of retinopathy.5) Phenolic compounds are widely known for their potent antioxidant and anticancer properties. Prolonged intake of foods rich in polyphenols, such as pomegranates, can prevent some diseases due to their high phenolic content, which acts as a natural defense against environmental stress worldwide. Numerous phenolic compounds have been shown to prevent non-enzymatic reactions of proteins with monosaccharides in vivo.6)

Hydroxycinnamic acid (HCA) is a sesquiterpene coumarin that is regarded as one of the most important phenolic com-
pounds isolated from various fruits and plants. The effect of HCA on renal cell function is of particular importance, as nephropathy develops in 5–10% of patients with both types of diabetes. Moreover, previous studies have demonstrated that HCA has an anti-inflammatory effect on the kidneys of diabetic rats by suppressing NF-β activation. Several HCA derivatives, such as cinnamic acid, p-coumaric acid, ferulic acid, caffeic acid, chlorogenic acid, and rosmarinic acid are efficient antiglycation agents. Since HCA is easily absorbed in both the stomach and intestines as compared to other phenolic compounds, it may be an active antiglycation agent. The action of phenolic compounds on protein glycation is attributed to their antioxidant potential in preventing the oxidation and subsequent formation of AGEs, which is often referred to as antioxidation. This study aimed to investigate the impact of m-coumaric acid on the glycosylation of proteins in the retinas of diabetic rats to forestall the development of complications such as retinopathy.

Materials and Methods

1. Animal model
Male albino rats 150±20 g (n=75) were obtained from the animal house at King Fahad Medical Research Center, King Abdulaziz University (KAU), Jeddah, Saudi Arabia and housed in clean, sterile cages. Animals received rat chow and tap water ad libitum. All experiments were performed according to the standards of Animal Ethics Committee at KAU. A control group (Group I) of 15 rats received a single injection of 0.25 mol/L citrurate buffer. The other 60 rats were injected with a single dose of streptozocin (STZ) (120 mg/kg bw) i.p. Blood sugar levels were checked every week to confirm the development of diabetes mellitus (DM). If the blood glucose level was more than 250 mg/dL, it was considered diabetic, and if it was less than the stated value, it was excluded. Diabetic rats were divided into four groups: Group IIA, diabetic but untreated; Group IIB, diabetic rats administered with intragastric m-coumaric acid (150 mg/kg bw/day); Group IIC, diabetic rats administered with intragastric m-coumaric acid (300 mg/kg bw/day); and Group IID, diabetic rats maintained on insulin subcutaneously (10 units/kg bw/day) as a positive control. The doses of m-coumaric acid were given according to the guidelines as mentioned. The treatment lasted for six weeks; before sacrifice, animals were fasted overnight (12 hr), and then 10% thiopental was given as anesthesia. Blood and retinas were collected directly from animals. The serum was isolated and analyzed for malondialdehyde (MDA), glycated hemoglobin, fructosamine and total antioxidant activity. Retinas was rinsed in phosphate buffer saline to remove excess blood and stored at −80°C until further analysis.

2. Measurement of glycated hemoglobin
The glycated hemoglobin (HbA1c) level was measured using the HemoCue (HbA1c 501) System (HemoCue, Inc., USA). Initially, the cartridge was inserted into the cartridge compartment; then the reagent pack was prepared and applied to the specimen: 20 μL of whole blood was dropped on sterilized slides and drawn in by capillary action to the tip of the reagent pack. Finally, the reagent pack was inserted into the cartridge, and the result was observed after 5 min.

3. Measurement of malondialdehyde, fructosamine, and total antioxidant activity
The collected blood was centrifuged for 10 min at 2000 rpm, and the serum was collected and MDA levels were determined using a commercially available Lipid Peroxidation (MDA) Assay Kit (Abcam, UK). Subsequently, the serum fructosamine concentration was also assayed by using fructosamine colorimetric assay serum plasma 15–1000 μmol/L Sentinel Diagnostics. Finally, the serum total antioxidant activity was analyzed by using a Total Antioxidant Capacity Assay Kit (Abcam, UK). Briefly, in the antioxidant assay, Cu²⁺ was reduced to Cu⁺ by serum antioxidants. The resulting Cu⁺ specifically formed a colored complex with the dye reagent. The color intensity at 570 nm is proportional to the total antioxidant activity in the serum.

4. Determination of aldose reductase
Serum aldose reductase (AR) activity was determined spectrophotometrically by monitoring the decrease in nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) absorption at 340 nm at 37°C using DL-glyceraldehyde as a substrate. The AR activity represents the difference between the rate of NADPH oxidation with and without the substrate (μmol/mg protein).

5. Biochemical analysis of retinal tissue
Retinal tissue (0.1 g) was homogenized in 2 mL of phosphate buffer (pH 7.3) containing a protease inhibitor. Samples were centrifuged at 12,000 rpm for 10 min at 5°C. The filtrate was collected to determine MDA, reduced glutathione (GSH), catalase and superoxide dismutase (SOD) levels by using a commercial kit (BIO-RAD, England) and the tumor necrosis factor (TNF-α), interleukin-1 (IL-1), and AGE levels using ELISA kits. Protein levels were determined using a Folin reagent with a standard curve.

6. Statistical analysis
All experiments were performed in duplicate, and the results were expressed as the mean±S.D. A one-way analysis of variance (ANOVA) was performed using GraphPad Prism 6 (Graph Pad Software, USA) to compare the differences. The individual comparisons were carried out by employing Tukey’s test. Significant differences are indicated as * p<0.05, ** p<0.01 and *** p<0.001.

Results

The body weight of the rats at the beginning of the study was similar in all groups. At the end of the experiment, diabetic animals showed a significant weight loss (p<0.001). The injection of rats with STZ resulted in a significant increase in blood glucose levels in the diabetic group as compared with the control group (p=0.01). Treatment with 150 or 300 mg m-coumaric
acid/kg bw or insulin resulted in a significant decrease in blood glucose compared with the untreated diabetic animals (p<0.05, Table 1). Because of diabetes, HbA1c was significantly increased (p<0.05) in the untreated diabetic group. Treatment of animals with 150 or 300 mg m-coumaric acid/kg bw or insulin improved two parameters in a dose-dependent manner (Table 1). The hypoglycemic action exerted by m-coumaric acid was lower than that by insulin (p<0.05).

The antioxidant activities of GSH, catalase, and SOD were significantly reduced in the retinas of diabetic animals because of the STZ injection. Supplementation with various concentrations of m-coumaric acid or insulin resulted in a significant elevation of the GSH (p<0.05 for each) level and the activities of catalase and SOD (p<0.001, <0.01 and <0.05 respectively) in a dose-dependent manner (Table 2). The degree of lipid peroxidation in the retinas was significantly elevated because of diabetes, while the administration of m-coumaric acid resulted in a dose-dependent decrease in MDA levels (Table 2). Serum aldolase reductase (Fig. 1A) was significantly reduced (p<0.01) in diabetic rats as compared to the control. Unexpectedly, the total antioxidant activity was increased (p<0.001) in diabetic rats as compared to the control. This may be due to the triggered hyperactive antioxidant defense system following the initial dose. m-coumaric acid supplementation (150 or 300 mg/kg bw) or insulin elevated these activities in a dose-dependent manner.

It was also established that both TNFα and IL-1 play a significant role in the pathogenesis of diabetic retinopathy. Upon the administration of an STZ injection, the retinal levels of TNFα and IL-1 became significantly elevated (Fig. 1C) (p<0.001) for each group indicating a considerable rise in the levels of inflammation compared to normal control rats. The administration of m-coumaric acid (150 or 300 mg/kg bw) or insulin resulted in a significant dose-dependent reduction of these levels (p<0.01). However, in the untreated diabetic group, enhanced formation of AGEs was observed as compared to the healthy control group. m-coumaric acid supplementation (150 or 300 mg/kg bw) or

### Table 1. Body weights and levels of glucose and HbA1c levels in all groups

| PARTICULARS         | Group I       | Group IIa      | Group IIb      | Group IIc      | Group IIId     |
|---------------------|---------------|----------------|----------------|----------------|---------------|
| Initial body weight (g) | 120.13±9.5   | 125.44±7.1     | 129.19±6.8     | 122±7.2        | 131.33±6.5    |
| Final body weight (g)  | 199.5±8.2    | 150.8±7.2<sup>b</sup> | 167.3±6.3<sup>c</sup> | 165.5±8.9<sup>b,c</sup> | 210±12.3<sup>c</sup> |
| Glucose (mg/dL)      | 92.78±0.45   | 275.35±1.45<sup>b</sup> | 145.92±1.4<sup>c</sup> | 137.71±1.23<sup>c</sup> | 102.21±0.57<sup>c</sup> |
| HbA1c (%)            | 5.54±0.41    | 8.42±0.34<sup>b</sup> | 6.8±0.54<sup>d</sup> | 6.8±0.38<sup>c</sup> | 5.8±0.52<sup>d</sup> |

<sup>a</sup>Data are represented as mean±S.D., <sup>b</sup>comparison with control group and <sup>c</sup>comparison with untreated diabetes group.

### Table 2. Serum and retina malondialdehyde (MDA) levels and reduced glutathione (GSH), catalase, and superoxide dismutase (SOD) activity (p* value, all groups vs. control; p** value, treated vs. untreated) in retinas of different groups (mean±S.D.)

| Groups         | Serum MDA µmol/L | Retina |                        |                        |                        |
|----------------|------------------|--------|------------------------|------------------------|------------------------|
|                | MDA µmol/mg protein | GSH µg/mg protein | Catalase U/mg protein | SOD U/mg protein |
| Group I (n=15) | Range 24–15.6     | 98–196 | 104–546                | 992–2349               | 956–2623               |
|                | Mean±S.D. 75±12   | 66.7±28 | 284±52                | 1249±140               | 1545±191               |
| Group IIa (n=12) | Range 40–89       | 190–261 | 68.7–221.5             | 109.5–642              | 453–1342               |
|                | Mean±S.E. 48±5.9   | 188±37  | 123.6±19.3             | 358±65                 | 760±78                 |
|                | p* value <0.01     | N.S.   | <0.01                  | <0.001                 | <0.001                 |
| Group IIb (n=13) | Range 33–56       | 149–213 | 121–496                | 706–2002               | 876–1987               |
|                | Mean±S.E. 31±12.2  | 142±32  | 259±50                | 843±134               | 1021±1                 |
|                | p* value <0.01     | <0.001 | <0.001                 | <0.001                 | <0.001                 |
| Group IIc (n=13) | Range 16–73.4     | 136–261 | 69.6–213.9             | 456–1632               | 962–1316               |
|                | Mean±S.E. 33±2.7   | 133±25  | 128±16.4              | 987±56                 | 836±53                 |
|                | p** value <0.001   | <0.001 | <0.05                 | <0.001                 | <0.001                 |
| Group IIId (n=14) | Range 22–73.4     | 156–261 | 69.6–213.9             | 546–1756               | 624–2116               |
|                | Mean±S.E. 38±6.7   | 121±25  | 128±16.4              | 1112±86                | 1233±153               |
|                | p** value <0.001   | N.S.   | <0.05                 | <0.001                 | <0.001                 |
insulin resulted in a significant reduction of AGEs in a dose-dependent manner as indicated (Fig. 1C).

**Discussion**

Cinnamic acid is a natural flavonoid found in many plants and fruits. The flavonoid HCA is an intermediate metabolite synthesized from tyrosine. HCA exerts specific anabolic effects on bone in *in vitro* conditions. A recent study has reported that HCA stimulated the osteoblastic bone formation and inhibited osteoclastic bone resorption *in vitro*.13) HCA also inhibits nuclear factor kappa B (NF-κB) signaling, which is activated by TNF-α or the receptor activator of the NF-κB ligand, indicating that a molecular mechanism could be present through which HCA exerts an anabolic effect on the bone.

Moreover, previous studies have shown the role of HCA derivatives as antifungal agents that can be used for plant protection.14) Since HCA is present in cereals and many other plants, it is beneficial for human consumption in addition to being toxic to pathogens. Recent investigations have revealed that cinnamic acid derivatives such as ferulic acid (3-methoxy-4-hydroxycinnamic acid) and isoferulic acid (3-hydroxy-4-methoxycinnamic acid) are the most bioactive components present in the rhizomes of *Cimicifuga heracleifolia*. This medicinal plant is an anti-inflammatory drug source frequently used in Japanese traditional medicine and has an additional use as an AGEs inhibitor.15–17) All of these reported studies are in good agreement with our findings. *m*-Coumaric acid, which was used in this study, is a hydroxycinnamic acid that is a hydroxy derivative of cinnamic acid. Dorantes *et al.*, reported the inhibition of growth of some foodborne pathogenic bacteria by *m*-coumaric acid that was extracted from *Capsicum annum*.18)

The present study also demonstrated that interference with the overproduction of ROS by *m*-coumaric acid (150 or 300 mg/kg bw) in the diabetic rats was indicated by the reduction of glucose and HbA1c levels as compared to the untreated group. Moreover, *m*-coumaric acid normalized the parameters of oxidative stress in diabetic retinas and prevented the activation of major pathways involved in hyperglycemia-induced vascular damage. *m*-Coumaric acid also repressed downstream effectors of vascular response to injury. Additionally, the reduction of free radical overproduction suggests an indirect AGE-inhibiting effect of *m*-coumaric acid. The results obtained are very close to those for insulin, which was used as a positive control. Interestingly, these results are also in accordance with those of a previous study19) which demonstrated that phenolic compounds reduce diabetic complications with the inhibition of free radical production.

Both preclinical and clinical studies have shown the significance of IL-1 in the pathogenesis of proliferative diabetic retinopathy.20) Retinal IL-1 was found to be significantly increased in diabetic rats as compared to normal control rats, *m*-coumaric acid treatment (150 or 300 mg/kg bw) attenuated the expression of IL-1 as compared to the untreated group. Various studies have shown that phenolic compounds such as HCA *m*-coumaric acid, inhibit IL-1-mediated diabetic retinopathy and cataracts.21)

TNF-α contributes to the development of diabetic retinopa-
and significantly higher levels of TNF-α are found in patients with either type 1 or type 2 diabetes as compared to similarly aged healthy control subjects. 23) TNF-α plays a significant role in the apoptotic pathway of retinal endothelial cells during early and late stages of diabetic retinopathy in a rat model. 19) In the present study, TNF-α expression was significantly increased in the diabetic rats as compared to normal ones and the groups treated with m-coumaric acid (150 or 300 mg/kg bw) showed a significant reduction in TNF-α levels in relation to the untreated diabetic group.

The major constituents of plasma that contribute to total antioxidant capacity are albumin, urate, ascorbate, alpha tocopherol, and bilirubin. 24) Therefore, measurement of total plasma antioxidant capacity may give a more precise indication of the relationship between antioxidants and the pathogenesis.

Therefore, the measurement of total plasma antioxidant capacity may give a more precise indication of the relationship between antioxidants and the pathogenesis. In the present study, we demonstrated a significant decrease in the total plasma antioxidant activity in the diabetic group as compared to the control group. After treatment with different dosages of m-coumaric acid, the total antioxidant activity of diabetic rats was increased compared to that of the untreated diabetic group. Our results are in accordance with the results of some previous studies that suggest the possible role of free radicals in the pathogenesis of diabetic mellitus. 25) Our study is also supported by the similar results of Ahmad et al., which showed an imbalance between the production of free radicals and the neutralization by a complex antioxidant system in diabetic rats. 26)

Fructose amine and aldose reductase play a crucial role in the development of diabetic retinopathy through the binding of tissue protein and the elevation of sorbitol. m-Coumaric acid was found to reduce fructosamine and enhance aldose reductase in diabetic rats as compared to the untreated group. This clearly demonstrates that m-coumaric acid is a natural food supplement with a broad spectrum of beneficial biochemical and cellular biological effects due to its ability to reduce the hyperglycemia-induced overproduction of ROS.

Additionally, the carbonyl group of glucose is nucleophilic and attacks free amine groups of protein that can donate an electron and thus protein loses its function. 27) To protect the functional protein from glycation, electrophilic agents could be introduced to prevent protein glycation. Therefore, the mechanism of action of m-coumaric acid may be that the hydroxyl group on the 3d position of the aromatic ring is ready to donate an electron, which traps the glucose. On the other hand, the carboxylic group (−COOH) and the adjacent C=O neutralize the free radicals. Therefore, m-coumaric acid could be further investigated as an active antiglycation drug to protect patients from diabetic complications.

Conclusions

The data obtained in this study suggests that the interaction of m-coumaric acid with glucose could decrease the levels of glucose and could potentially reduce the formation of glucose-derived AGEs; hence protecting the tissues from the detrimental effects of hyperglycemia. However, these reactions are slow, so it is unclear whether they are physiologically relevant, and their resulting impact on overall health of an individual needs to be investigated. In conclusion, it can be suggested that m-coumaric acid may have benefits for the prevention of the onset and progression of retinopathy in diabetic patients.

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