Assessment of the ameliorative effect of p-coumaric acid and gallic acid on oxidative stress and haematological abnormalities in experimental type 2 diabetes

Adel A*, Eman SA, Sanaa MA, Mohamed BA and Ahmed IY
Molecular Physiology Division, Faculty of Science, Beni-Suef University, Beni Suef, Egypt

Abstract

Background: Diabetes mellitus (DM) is a worldwide health problem. The current study was designed to assess the protective effects of phenolic acids (gallic acid and p-coumaric acid) on oxidative stress and hematological alterations in an experimental rat model of type 2 diabetes mellitus.

Methods: Type 2 diabetes was induced by a single intraperitoneal (i.p.) injection of streptozotocin (65 mg/kg b.wt.), after 15 min of i.p. injection of nicotinamide (120 mg/kg b.wt.). Rats were randomly allocated into four groups as follows: group I (control), group II (diabetic), group III (diabetic rats administered gallic acid, 20 mg/kg body weight, daily for six weeks) and group IV (diabetic rats administered p-coumaric acid, 40 mg/kg body weight, daily for six weeks).

Results: In diabetic rats, it was found that the levels of malondialdehyde and nitric oxide significantly increased, while the activities of superoxide dismutase, catalase, glutathione peroxidase and glutathione-S-transferase, as well as reduced glutathione content, were markedly reduced as compared to those of the control ones. Diabetic rats also showed alterations in the red blood cells count, and its related indices indicating an anemic condition and in the total and differential leukocyte count. All these abnormalities were significantly alleviated following the administration of gallic acid and p-coumaric acid. In conclusion, treatment of diabetic rats with gallic acid and p-coumaric acid markedly diminished the oxidative stress and alleviated the hematological abnormalities that may be attributed to their strong antioxidant activities.

Conclusion: Therefore, both tested phenolic acids can be acted as powerful agents against the development of the oxidative stress status as well as the hematological alterations.

Introduction

Diabetes mellitus (DM) is a serious worldwide medical and social problem. DM is characterized by persistent hyperglycemia with impaired metabolism of glucose, lipids, and protein resulting from the malfunction in insulin secretion and/or insulin action [1, 2]. The 21st century has seen a rise in diabetes and its complications around the world. The incidence of diabetes has increased by 50% over the past 10 years and is accompanied by an increasing burden of morbidity and mortality that is attributable to diabetic complications [3].

Over the past decade, there has been increased interest in oxidative stress and its role in the development of complications of diabetes mellitus [4]. With diabetes hyperglycemia directly leads to reactive oxygen species (ROS) production, increased cellular stress, and glucotoxicity. In an upstream effect of hyperglycemia, production of increased amounts of ROS is also known to have detrimental effects on cellular function [5]. The formation of advanced glycation end products (AGEs), a group of modified proteins and/or lipids with damaging potential, is one contributing factor. On the one hand, it has been reported that AGEs increase reactive oxygen species formation and impair antioxidant systems. Free radicals and oxidative stress induced complications from diabetes include coronary artery disease, neuropathy, nephropathy, and retinopathy and stroke [6,7].

Recently, there has been renewed interest in hematological parameters as predictors of endothelial dysfunction and inflammation, a relationship between HbA1c and hematological indices, and to evaluate the relationship between these parameters and microvascular complications of diabetes [8].

Gallic acid (GA, 3,4,5-trihydroxybenzoic acid) is a polyphenol from plants and a natural product of tannins hydrolysis found abundantly in grapes, different berries, and other fruits as well as in wine [9]. GA has many biological activities such as antihyperglycemic, antihyperlipidemic, antioxidant, anti-inflammatory and hepato-renal protective effects [10-13]. It received much attention because of its potent ability to scavenge ROS, such as superoxide anions, hydrogen peroxide, hydroxyl radicals, and hypochlorous acid [14]. This polyphenol is even more effective than ascorbic acid to prevent lipid peroxidation [15].

p-Coumaric acid (PCA, 4-hydroxyphenyl-2-propenoic acid) is a phenolic acid widely distributed in plants and form a part of the human diet. PCA is present in a plenty of foods, such as grapes, white and red wine, tomato, spinach, coffee, carrot and garlic [16]. PCA has

*Correspondence to: Adel Abd-Elmoneim A, Physiology Division, Faculty of Science, Beni-Suef University, Salah Salem St., 62511, Beni-Suef, Egypt, E-mail: adel_men2020@yahoo.com

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attracted substantial attention due to its several pharmacological and biological actions, such as antidiabetic and anti-inflammatory activities \[11,17,18\]. However, few publications assess the effect of these phenolic compounds against diabetic complications. Thus, the present study was performed to evaluate the protective effects of both phenolic acids (gallic acid and \(p\)-coumaric acid) against oxidative stress, inflammation and hematological alterations in diabetic rats.

Materials and methods

Experimental animals

Adult male albino rats (\textit{Rattus norvegicus}) weighing about 130 ± 10 g were used in the present study. They were obtained from National Research Center (NRC), Dokki, Giza, Egypt. They were kept under observation for two weeks before the onset of the experiment to exclude any inter-current infection. The chosen animals were housed in plastic good aerated cages at the normal atmospheric temperature (25 ± 5°C), humidity (55 ± 5%) and normal 12 hours light/dark cycle. During the entire period of study, the rats were provided with water and normal basal diet with known composition \textit{ad libitum}. The animal procedures were conducted according to the principles and guidelines of the Canadian Council on Animal Care \[19\].

Type 2 diabetes was induced in overnight fasted rats by single intraperitoneal (i.p.) injection of streptozotocin (STZ) (65 mg/kg b.wt.), freshly dissolved in cold citrate buffer (pH 4.5), after 15 min of i.p. injection of nicotinamide (NA) (120 mg/kg b.wt.) prepared in normal physiological saline \[20\]. After measuring plasma glucose concentration, seven days after injection, rats with a 2-hour plasma glucose level ranging from 200-300 mg/dl were considered mildly diabetic and included in the experiment.

Experimental design

Rats were randomly allocated into four groups as follows:

- **Group I**: served as control and were orally administered an equivalent volume of vehicle.
- **Group II**: served as diabetic and were orally administered an equivalent volume of vehicle.
- **Group III**: acted as diabetic rats were orally treated with gallic acid (20 mg/kg b.wt.) for six weeks \[10\].
- **Group IV**: acted as diabetic rats were orally treated with \(p\)-coumaric acid (40 mg/kg b.wt.) for six weeks \[17\].

All treatments were dissolved in 0.5% carboxymethylcellulose (CMC) and given daily by gastric intubation. By the end of the sixth week, rats were sacrificed under diethyl ether anesthesia. Two blood samples were collected from each rat. The first was collected into a tube containing ethylene-diamine-tetra-acetic acid (EDTA) and immediately preserved in the refrigerator for subsequent analysis of blood glycosylated hemoglobin (HbA1c) and complete blood count (CBC). The second was allowed to coagulate at room temperature for further serum separation. The clear, sera were quickly removed, divided into three portions for each individual animal, and kept at -20°C for subsequent analysis. Part of liver homogenized at 4°C with ten times (w/v) in 0.9% saline. The homogenate was centrifuged at 3000 r.p.m. for five minutes to remove cellular debris, supernatant was kept at -20°C, and used for biochemical analysis.

Lab examinations

Hepatic levels of lipid peroxidation (LPO), nitric oxide (NO), and reduced glutathione (GSH) were estimated according to the methods of Preuss et al., Miranda et al. and Beutler et al. respectively \[21-23\]. Furthermore, the activities of hepatic superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione-S-transferase (GST) were detected according to the methods ofMarklund and Marklund, Cohen et al., Matkovics et al. and Mannervik and Gutenberg \[24-27\]. Serum tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) was assayed by sandwich ELISA using reagent kit purchased from R&D Systems, Inc. (USA) according to the manufacturer’s protocol.

Red blood cells count (RBCs in 10\(^6\)/μl) and white blood cells (WBCs in 10\(^3\)/μl) were estimated according to the visual method of Dacie and Lewis \[28\]. Hematocrit (HCT %) was and the blood hemoglobin concentration (Hb in g/dl) in samples were assayed according to the method of Alexander and Griffiths \[29\]. Mean corpuscular volume (MCV in fl), mean corpusular hemoglobin (MCH in pg) and mean corpuscular hemoglobin concentration (MCHC in %) were calculated as outlined in Dacie and Lewis \[28\]. Platelets count (10\(^3\)/μl) was detected as mentioned by Trowbridge et al. \[30\]. Differential leukocytes count [neutrophils (%), Lymphocytes (%), Monocytes (%), Eosinophils (%) and Basophils (%)] were estimated using the method of Osim et al. \[31\]. Blood HbA1c percentage was assessed according to the method of Bisse and Abraham using reagent kits purchased from Biosystems S.A. (Spain) \[32\].

Statistical analysis

The experimental results were expressed as mean ± standard error (SE) and subjected to One-Way Analysis of Variance (ANOVA), using a computer software package (SPSS version 20, IBM Corp., 2011) and followed by Duncan’s Multiple Range Test (DMRT) to determine the significant differences between groups, at \(P < 0.05\).

Results

HbA1c concentration exhibited a significant elevation in the diabetic rats and ameliorated after treatment with gallic acid or \(p\)-coumaric acid (Figure 1). Data regarding the effect of gallic acid and \(p\)-coumaric acid on oxidative stress markers in the liver of diabetic rats were shown in (Table 1). The level of malondialdehyde (MDA), a marker of lipid peroxidation, revealed a significant increase in diabetic rats as compared to the control rats. Treatment with either gallic acid or \(p\)-coumaric exhibited a significant amelioration in MDA concentration.

![Figure 1. Glycosylated hemoglobin, HbA1c, (%) of control, diabetic and diabetic rat treated with gallic acid and \(p\)-coumaric acid](image-url)
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NO is another marker of oxidative stress, expressed as nitrite, showed the same behavioral pattern as MDA.

Data describing the effect of treatments on the non-enzymatic antioxidant parameters, total thiols and reduced glutathione (GSH), in the liver of diabetic rats were presented in (Table 2). Both treatments showed a significant increase of total thiols and GSH concentrations which were decreased in the non-treated diabetic rats.

The activities of antioxidant enzymes, catalase, superoxide dismutase, glutathione peroxidase, and glutathione-S-transferase exhibited a significant decrease in the diabetic rats as compared to the control ones. The daily oral administration of gallic acid or p-coumaric acid produced a marked alleviation of these alterations. The effect of gallic acid on the above-mentioned antioxidant enzymes seemed to be more potent than p-coumaric acid (Table 2).

The level of TNF-α in all experimental groups was shown in figure 2. Values of TNF-α obtained from diabetic rats revealed a significant increase as compared to control rats. Treatment of diabetic rats with gallic acid or p-coumaric acid showed a profound decrease in the TNF-α concentration (Figure 2).

Tables 3&4 described the effect of treatments on RBCs count and its indices. Results obtained from diabetic rats showed a significant decrease in RBCs count, Hb content, HCT, MCV, MCH, and MCHC when compared to corresponding control rats (Tables 3 and 4). Consequently, diabetic rats suffer from hypochromic anemia with anisocytosis. Orally treated diabetic rats returned the levels of these parameters nearly to the normal values. The platelets count of diabetic rats exhibited a significant decrease as compared to normal ones. Upon treatment, platelets count was improved and returned near to normal values.

As showed in table 5, total WBCs count revealed a significant increase in diabetic rats as compared to control rats. Moreover, differential WBCs count of the diabetic group showed significantly increased in neutrophils, eosinophils, and monocytes as compared to control group. All these recorded changes were improved after treatments with the two tested agents. In contrast, there was no significant increase in both basophils and lymphocytes (Table 5).

**Discussion**

Oxidative stress is defined as an imbalance between cellular antioxidant capacity and reactive oxygen species (ROS) production [33]. Oxidative stress plays a major role in the pathogenesis of diabetes and its complications [34]. Hyperglycemia causes tissue damage through multiple mechanisms including increased flux of glucose and other

| Group           | Parameter          | LPO (nmol MDA/100mg tissue) | NO (nmol nitrite/100mg tissue) |
|-----------------|--------------------|------------------------------|-------------------------------|
| Control         | RBCs (106/μl)      | 22.14 ± 0.12a                | 8.95 ± 0.20b                  |
| Diabetic        |                    | 30.74 ± 0.36b                | 15.44 ± 0.69b                 |
| Diabetic + GA   |                    | 24.16 ± 0.11b                | 10.02 ± 0.14a                 |
| Diabetic + PCA  |                    | 26.82 ± 0.51b                | 12.25 ± 0.51b                 |

Data are expressed as Mean ± SE of six rats from each group. Means not sharing a common superscript symbol(s) differ significantly at P < 0.05

| Group           | Parameter          | Total thiols (nmol/100mg tissue) | GSH (nmol/100mg tissue) | SOD (U/g tissue) | CAT (Kx10⁴U) | GPx (mU/100mg tissue) | GST (U/100mg tissue) |
|-----------------|--------------------|----------------------------------|-------------------------|------------------|--------------|---------------------|---------------------|
| Control         |                    | 493.86 ± 4.38d                  | 270.83 ± 12.66d         | 16.79 ± 0.24a    | 47.67 ± 5.15a  | 98.90 ± 0.86a       | 189.90 ± 1.02a      |
| Diabetic        |                    | 385.29 ± 3.17d                  | 92.08 ± 12.24d          | 10.24 ± 0.21a    | 19.56 ± 1.5a   | 56.93 ± 1.6a        | 145.95 ± 1.40a      |
| Diabetic + GA   |                    | 457.35 ± 4.26d                  | 212.02 ± 4.43d          | 14.85 ± 0.32a    | 38.40 ± 0.39b  | 82.50 ± 1.32a       | 167.52 ± 1.21a      |
| Diabetic + PCA  |                    | 425.73 ± 3.39d                  | 186.48 ± 5.14d          | 13.86 ± 0.40b    | 35.78 ± 0.96e  | 73.33 ± 1.33b       | 159.73 ± 0.74e      |

Data are expressed as Mean ± SE of six rats from each group. Means not sharing a common superscript symbol(s) differ significantly at P < 0.05

| Group           | Parameter          | RBCs (106/μl) | HCT (%) | HB (g/dl) | Platelets (10⁵/μl) |
|-----------------|--------------------|--------------|---------|-----------|-------------------|
| Control         |                    | 6.80 ± 0.21b  | 49.70 ± 0.75c | 15.55 ± 0.31a | 341.00 ± 15.24a |
| Diabetic        |                    | 5.70 ± 0.21a  | 35.60 ± 1.79b | 10.71 ± 0.53b | 214.16 ± 3.98b  |
| Diabetic + GA   |                    | 6.43 ± 0.16a  | 46.60 ± 0.41b | 14.10 ± 0.11b | 311.50 ± 14.76b |
| Diabetic + PCA  |                    | 6.31 ± 0.16b  | 45.50 ± 0.67b | 13.96 ± 0.47b | 281.00 ± 4.32b  |

Data are expressed as Mean ± SE of six rats from each group. Means not sharing a common superscript symbol(s) differ significantly at P < 0.05

| Group           | Parameter          | MCV (fl) | MCH (pg) | MCHC (%) |
|-----------------|--------------------|----------|----------|----------|
| Control         |                    | 73.26 ± 2.63a | 22.95 ± 0.81a | 31.38 ± 0.45a |
| Diabetic        |                    | 63.61 ± 2.62a | 18.71 ± 0.79a | 29.61 ± 0.39a |
| Diabetic + GA   |                    | 70.96 ± 1.04a | 21.95 ± 0.71a | 30.23 ± 0.03a |
| Diabetic + PCA  |                    | 73.13 ± 1.84a | 22.06 ± 0.41a | 30.11 ± 0.81a |

Data are expressed as Mean ± SE of six rats from each group. Means not sharing a common superscript symbol(s) differ significantly at P < 0.05
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Hyperglycemia causes an increase in the oxidative stress that is responsible for the development of hematological complications in diabetic patients [52]. In the present study, the observed decreased levels of RBCs, Hb, HCT, MCV, MCH and MCHC in diabetic rats indicating an anemic condition. These findings are in accordance with the previous finding of Edet et al. [53]. The development of anemia in DM has been explained with increased non-enzymatic glycosylation of RBC membrane proteins [54]. Oxidation of these proteins and persistent hyperglycemia in DM causes an increase in the production of lipid peroxides leading to hemolysis of RBC [55].

Table 5. Total and differential WBCs count of control, diabetic and diabetic rats treated with gallic acid and p-coumaric acid

| Group        | Parameter WBCs (10^9/mm³) | Neutrophils (%) | Lymphocytes (%) | Monocytes (%) | Eosinophils (%) |
|--------------|---------------------------|-----------------|-----------------|---------------|-----------------|
| Control      | 7.33 ± 0.64a              | 37.16 ± 2.30a   | 52.33 ± 1.76a   | 5.50 ± 0.42a  | 4.00 ± 0.89a    |
| Diabetic     | 10.71 ± 0.98a             | 52.33 ± 1.83a   | 47.50 ± 3.89a   | 7.50 ± 0.67a  | 2.00 ± 0.25a    |
| Diabetic + GA| 7.90 ± 0.44a              | 41.16 ± 2.44a   | 50.33 ± 4.31a   | 6.66 ± 0.49a  | 2.66 ± 0.33a    |
| Diabetic + PCA| 8.41 ± 0.30a             | 45.16 ± 1.95a   | 46.50 ± 1.47a   | 6.66 ± 0.61a  | 2.66 ± 0.33a    |

Data are expressed as Mean ± SE of six rats from each group. Means not sharing a common superscript symbol(s) differ significantly at P < 0.05 (DMRT)

Figure 2. Tumor necrosis factor alpha (TNF-α) of control, diabetic and diabetic rats treated with gallic acid and p-coumaric acid

The results also revealed that daily administration of GA and PCA to diabetic rats significantly increased the activities of antioxidant enzymes (SOD, CAT, GPx, and GST) and the levels of total thiol and GSH in the liver of diabetic rats. This could be due to decreased oxidative stress as evidenced by decreased LPO. In accordance with our results, Patel and Goyal, Ahad et al. and Huang et al. reported that GA reverted back the altered levels of these antioxidants in different tissues (liver, kidney, and heart) and different experimental animal models of diabetes [43-45]. GA significantly increased the activities of enzymatic antioxidants (SOD, CAT, GPx, GR, and GST) in the heart and non-enzymatic antioxidants (GSH, vitamin C, and E) in plasma and the heart in experimentally induced myocardial infarction model in Wistar rats [46]. Furthermore, Amalan and Vijayakumar reported that SOD, CAT, and GPx activities elevated and LPO level decreased indicating the efficacy of PCA in attenuating the oxidative stress in diabetic liver [47].

On the other hand, numerous evidences were correlated with oxidative stress and inflammation with the complications of diabetes as a result of NF-κB activation [48]. The activated NF-κB regulates various pro-inflammatory mediators such as iNOS, and pro-inflammatory cytokines such as interleukins (ILs) and TNF-α [49].

The treatment of diabetic rats with GA and PCA significantly reduced the levels of serum TNF-α, proving the anti-inflammatory effects of both agents. GA also has an inhibitory activity on p38 MAPK activation and downstream TNF-α and IL-6 production [50]. Furthermore, PCA and UA enhanced the inhibitory effects against inflammation via inactivation of both NF-κB and MAPKs signaling pathways. Zhao et al. found that PCA may inhibit the production of inflammatory cytokines induced by lipopolysaccharide (LPS) in RAW264.7 macrophage cells through blocking NF-κB and MAPKs signaling pathways [51].
deformability, reduced erythrocyte survival, and lipid fluidity [56]. Following GA and PCA administration, the alterations of RBCs and its related indices were considerably improved. This finding could be due to radical scavenging and antioxidant activity of both agents resulting in decreased hemolysis. It may also occur via stimulating formation or secretion of erythropoietin, which stimulates stem cells in the bone marrow of rats to produce new RBCs [57].

The present data also revealed obvious alterations in the total and differential leukocyte count of diabetic rats, especially an elevation of WBCs count, neutrophils, and monocytes. These results are in agreement with those of others [58,59]. The mechanism of leukocytosis in diabetes is exactly unknown. However, leukocytosis in diabetes may be achieved through the release of cytokines such as TNF-α, NF-κB and transforming growth factor 1 [60]. Interleukins and TNF-α are released from activated leukocytes and cause endothelial dysfunction [61]. Moreover, Heidland et al. reported that leukocytes can be activated by glycation end products, oxidative stress, angiostatin II resulting from hyperglycemia, and can produce factors like tumor necrosis factor-α and interleukin β1 that involve chronic diabetes complication pathogenesis [62].

Platelets are the fragment of cells that participate in blood clotting, they initiate repair of blood vessels walls and are also considered as an acute phase reactant to infection or inflammation [63]. In line with the study of Zakrzeska et al., the obtained data indicated a reduction in the platelet count of diabetic rats relative to control ones [64]. Hyperglycemia may represent a causal factor for in vivo platelet activation and may be responsible for nonenzymatic glycation of platelet glycoproteins, causing changes in their structure and conformation, as well as alterations of membrane lipid dynamics. Furthermore, hyperglycemia-induced oxidative stress is responsible for enhanced peroxidation of arachidonic acid to form biologically active isoprostanes, which represents an important biochemical link between impaired glycemic control and persistent platelet activation [65]. On the other hand, there was no significant change in platelet counts after treatment with GA or PCA although platelet counts moderately increased.

Conclusion

Gallic acid and p-coumaric acid are powerful antioxidants that revealed many beneficial effects in diabetic rats such as decreasing oxidative stress, improving antioxidant status, diminishing inflammation and relieving hematological abnormalities. Thus, both agents are good candidates for management of diabetes and diabetic complications.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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