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# Curcuminoids modulates oxidative damage and mitochondrial dysfunction in diabetic rat brain

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**Abstract**

Diabetes exacerbates neuronal injury induced by hyperglycemia mediated oxidative damage and mitochondrial dysfunction. The aim of the present study is to investigate the effects of curcuminoids, polyphenols of *Curcuma longa* (L.) on oxidative stress and mitochondrial impairment in the brain of streptozotocin (STZ)-induced diabetic rats. A marked increase in lipid peroxidation and nitrite levels with simultaneous decrease in endogenous antioxidant marker enzymes was observed in the diabetic rat brain, which was restored to normal levels on curcuminoids treatment. Down-regulation of mitochondrial complex I and IV activity caused by STZ induction was also up-regulated on oral administration of curcuminoids. Moreover, curcuminoids administration profoundly elevated the ATP level, which was earlier reduced in the diabetic brain. These results suggest that curcuminoids exhibit a protective effect by accelerating antioxidant defense mechanisms and attenuating mitochondrial dysfunction in the brain of diabetic rats. Curcuminoids thus may be used as a promising therapeutic agent in preventing and/or delaying the progression of diabetic complications in the brain.

**Keywords:** *Diabetes mellitus, oxidative damage, mitochondria, antioxidant, curcuminoids*

## Introduction

Diabetes mellitus (DM) is an heterogeneous disease characterized by chronic hyperglycemia and requires long-term management. It is often associated with severe complications such as cardiovascular disease, diabetic foot, kidney failure, retinopathy and neuropathy. Cumulative research evidences support that both acute and chronic hyperglycemia produce negative impacts on central as well as peripheral nervous systems leading to end-organ damage [1,2]. One unifying mechanism lying behind this neuronal injury is excessive free radical generation from the auto-oxidation of elevated intracellular glucose levels [3]. Further, the increase in reactive species in diabetes is due to failure or overload of detoxification pathways and dysfunction of the mitochondrial respiratory chain [2,4]. Recent studies indicate that oxidative stress targets brain mitochondrial complexes and accounts for direct neuronal damage [5]. Thus, antioxidant therapy may serve as a promising treatment approach for inhibiting the progression of diabetes-associated complications. Antioxidants such as melatonin and vitamin E have already been reported to prevent diabetes-induced cognitive dysfunction [6]. However, attention was drawn towards the plant-based medicine which can be incorporated

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in the long-term diabetes treatment with minimum side effects.

*Curcuma longa* (L.) is one of the medicinal plants extensively used in the Indian system of medicine for the management of various diseases [7]. Curcuminoids, the active polyphenols of *C.longa* rhizomes, contain curcumin, demethoxycurcumin and bis-demethoxycurcumin, which were shown to have a wide spectrum of pharmacological actions [8]. In diabetes, curcumin was shown to perform a multitude of activities including reduction in glycemic level, elevation in antioxidant status of pancreatic $\beta$-cells and attenuation of the mechanisms involved in diabetic encephalopathy [9–11]. However, the effects of *C. longa* and its derivatives have not been studied for its actions on diabetic brain mitochondrial complexes. Thus, the present study aims to investigate the protective effects of curcuminoids on oxidative damage and mitochondrial dysfunction in the brain of streptozotocin-induced diabetic rats.

## Materials and methods

### *Chemicals*

All chemicals used were of analytical grade and purchased from Sigma-Aldrich (USA) and Merck (Germany). Streptozotocin (STZ) was purchased from Sigma-Aldrich.

### *Plant material and extraction*

Curcuminoids were extracted with hexane and ethanol solvents from rhizomes of *C. longa* and precipitated with petroleum ether. HPLC (Waters, USA) with PDA detector measured yields containing ~78.1% curcumin, 16.5% demethoxycurcumin and 5.4% bis-demethoxycurcumin.

### *Induction of diabetes*

Diabetes mellitus was induced in overnight-fasted rats by a single intraperitoneal injection of STZ dissolved in citrate buffer (0.1 M, pH 4.5) in a dose of 60 mg/kg body weight [12]. Hyperglycemia was confirmed by elevated glucose levels in plasma, determined at 72 h and then on day 7 after STZ injection by using GOD- POD (glucose oxidase-peroxidase) kit. The animals with blood glucose concentration of more than 200 mg/dl were included in the study.

### *Experimental design*

Male Wistar rats of average weight, 150–200 g, and age 4–5 months were used in the study. Rats were caged under controlled temperature, 20–24°C, relative humidity, 45–55% and 12 h light/dark cycle. They were fed standard laboratory diet and water given *ad libitum*. All procedures were approved by the University's ethics committee. Treatment was conducted; a single dose daily for 1 month beginning from the 7$^{th}$ day of induction. Rats were divided into four groups with eight rats per group.

- Group I: normal control rats.
- Group II: diabetic control rats.
- Group III: diabetic rats treated with vitamin E as standard antioxidant (100 mg/kg bw orally).
- Group IV: diabetic rats treated with curcuminoids (120 mg/kg bw orally).

After completion of the treatment schedule, the rats were deeply anaesthetized with ether and then sacrificed by cervical dislocation. Their brain was quickly collected and cerebellum and brain stem was removed. The remaining cortex was maintained at $-80$°C until use. Half of the cortex was used for mitochondrial complex analysis while the other half was used for biochemical assays.

### *Determination of oxidative stress markers*

Brain tissue samples were homogenized in ice-cold 0.1 M Tris-Cl buffer (pH 7.4). Lipid peroxidation was determined as the amount of thiobarbituric acid reactive substances (TBARS) content as described by Okhawa et al. [13]. Glutathione (GSH) level was determined according to the method of Ellman [14]. Superoxide dismutase (SOD) activity was analysed by the method of Misra and Fridovich [15], while catalase and glutathione peroxidase (Gpx) activity were estimated as described elsewhere [16,17].

Total nitrite concentration was used as an indicator of nitric oxide (NO) synthesis. Briefly, nitrates in the samples were reduced to nitrites by incubating samples in a reaction mixture containing 1 IU/ml nitrate reductase, 500 μM NADPH and 50 μM FAD for 20 min; 100 IU/ml lactate dehydrogenase and 100 mm sodium pyruvate were further added, making the final reaction volume 500 μl that was subsequently incubated for 10 min [18]. Total nitrites were assayed by adding 500 μl Griess reagent (4% sulphanilamide and 0.2% naphthylendiamide in 10% phosphoric acid) to each sample [19]. Protein content was estimated by the Lowry et al. [20] method.

### *Isolation of brain mitochondria*

Brain mitochondria were isolated by the method of Lai and Clark [21] with little modifications. Briefly, brain was homogenized in the isolation medium consisting of 10 mm Tris (pH 7.5), EDTA (1 mM) and sucrose (0.32 mM). The homogenate was centrifuged at 1500 *g* for 5 min. The supernatant was removed and re-centrifuged at 15 000 *g* for 10 min. The pellet was resuspended in 5 ml of isolation medium and layered on a discontinuous gradient of 7 and 13% Ficoll and centrifuged at 90 000 *g* for

30 min. The pellet consisting of isolated brain mitochondria was resuspended in the isolation medium and used for complex analysis.

*Assay of complex I*

The reaction buffer consisted of potassium phosphate buffer (35 mM, pH 7.2), magnesium chloride (5 mM), EDTA (1 mM), sodium cyanide (2.65 mM), bovine serum albumin (0.1%, w/v) and antimycin A (1 μg/ml). Mitochondrial protein (60–80 mg) and ubiquinone (50 μM final concentration) were added and the reaction was initiated by the addition of NADH (5 mm final concentration). The rate of decrease in absorbance was monitored at 340 nm for 15 min. A similar assay was carried out in the presence of rotenone (100 μM) to measure rotenone-insensitive enzyme activity. The rotenone-sensitive complex I activity was calculated by subtracting the activity measured in the presence of rotenone from the total activity [22].

*Assay of complex II*

The reaction mixture consisted of mitochondrial protein (40–60 μg), phenol indo-2,6-dichlorophenol (DCIP, 70 μM), phenazine methosulphate, potassium phosphate buffer (50 mM, pH 7.4), EDTA (0.1 mM) and bovine serum albumin (0.1%, w:v). The reaction was initiated by the addition of sodium succinate (50 μl, final concentration 20 μm) and the rate of decrease in absorbance at 600 nm was monitored [23].

*Assay of complex II–III*

The reaction mixture consisted of mitochondrial protein (50–100 μg) and sodium succinate (100 mM), 100 mM potassium phosphate buffer (pH 7.4), 0.3 mm EDTA and 1 mm sodium cyanide. The reaction was initiated by the addition of cytochrome *c* (50 μM) and the rate of increase in absorbance at 550 nm was measured in the presence and absence of antimycin A (2.5 μg). Activity of complex II–III was calculated by subtracting the activity measured in the presence of antimycin from the total activity measured in the absence of antimycin [24].

*Assay of complex IV*

Activity of cytochrome *c* oxidase was measured using the method of Gibson and Hilf [25]. The reaction mixture consisted of mitochondrial protein (40–60 μg) in potassium phosphate buffer (0.5 M, pH 7.5). The reaction was initiated by addition of reduced cytochrome *c* (1.2 mM). Cytochrome *c* was reduced by using sodium dithionite and the rate of change in absorbance was monitored at 550 nm. The net enzyme activity was calculated by using the molar extinction co-efficient of cytochrome *c* (21 $M^{-1}$cm).

*Analysis of ATP content*

Mitochondrial suspension was assayed for ATP by separation in a reverse-phase high performance liquid chromatography. An isocratic elution with 100 mM phosphate buffer ($KH_2PO_4$) pH 6.5 and 1.0% methanol was performed with a flow rate of 1 ml/min. The required time for each analysis was 8 min at wavelength of 254 nm. The chromatography apparatus was from Waters with UV detector with column symmetry, C18 (5 μm, $3.9 \times 150$ mm).

*Statistical analysis*

All results are presented as mean $\pm$ SD. The inter-group variation was measured by one way analysis of variance (ANOVA) and the level of significance was considered at $p < 0.05$

## Results

*Biochemical parameters*

The STZ-induced rats had shown significant increase of $343.42 \pm 11.03$% in blood glucose level in comparison to control on the 7$^{th}$ day of induction, which remains unchanged during the study. Oral administration of curcuminoids significantly decreases the glucose level by $60.29 \pm 12.5$% in comparison to the STZ-induced group. However, vitamin E was found to show an insignificant effect on glucose level (Figure 1). The body weight of STZ-induced rat was also lower than the control group (control: $201 \pm 1.01$, STZ: $138 \pm 1.26$, data not shown).

*Antioxidant profile*

Table I represents the activity of oxidative stress marker enzymes in the different experimental groups. There was significant elevation in the TBARS content, with a concomitant reduction in the total glutathione content in the diabetic rat brain. Treatment of curcuminoids and vitamin E reverses the TBARS content back to normal level and increases the endogenous GSH content. The activities of SOD, catalase and GPx enzymes were also found to be declined in the brain of STZ-induced rats which was restored to normal level on curcuminoids and vitamin E treatment.

*Total NO content*

Figure 2 illustrates the level of total nitrite content in the experimental group. STZ induction causes a marked elevation in the nitrite level. Curcuminoids and vit E treatment considerably reduced the NO content, thus inhibiting the NO generation.

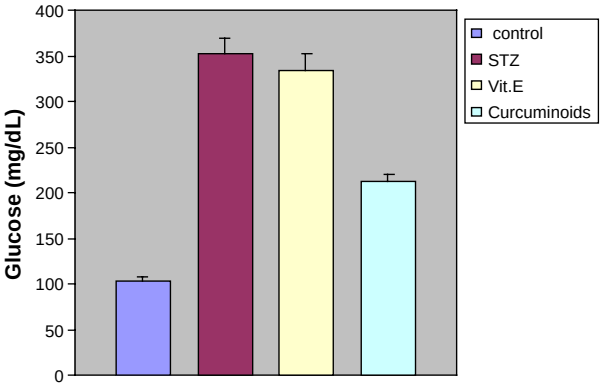


Figure 1. Blood glucose level (mg/dL) in STZ-induced rats followed by vitamin E or curcuminoids treatment. Values are given as mean ± SD for eight rats in each group. Values are statistically significant at $p < 0.05$ for control vs STZ and STZ vs curcuminoids-treated group.

*Mitochondrial complex activity*

Impairments of electron transfer in specific mitochondrial complexes were observed in the brain of STZ-induced rats (Table II). The activity of Complex I and IV was down-regulated by 46.42 ± 0.23 and 34.44 ± 0.09%, respectively, in STZ-induced rat brain, whereas curcuminoids or vitamin E treatment significantly up-regulated the complex I activity by 230.59 ± 0.3, 193.82 ± 0.27% and complex IV activity by 221.72 ± 0.11, 231.03 ± 0.15%, respectively, in comparison to the STZ untreated group. On the other hand, activity of complex II and II–III remains insignificantly altered in the diabetic rat brain. Indeed, a higher *succinate cytochrome c reductase* activity (175.6 ± 0.13% vs control) was noticed in the diabetic rat brain. Oral administration of curcuminoids and vitamin E did not significantly affect complex II and II–III activity.

*ATP content*

ATP levels in brain mitochondria of STZ-induced rats were decreased compared to the normal control, as indicated in Figure 3. Administration of curcuminoids and vitamin E profoundly increased the depleted ATP levels.

## Discussion

Chronic hyperglycemia induces oxidative damage, which leads to direct neuronal injury. Oxidative damage occurs due to the increased formation of free radicals, reduction in the endogenous antioxidant defense system or both. Therefore, antioxidant therapy that target oxidative stress would help in preventing and/or delaying the progression of diabetes and associated neuronal injury. Curcuminoids primarily exhibited potent free radical scavenging activity due to the presence of phenolic –OH groups, thus inhibiting direct neuronal injury [26].

In the present study, STZ-induced rats were used as an experimental model for diabetes, since they provides a relevant example of endogenous chronic oxidative stress due to the resulting hyperglycemia [27]. Curcuminoids significantly reduces the elevated blood glucose level, whereas, vitamin E shows an insignificant effect. Earlier studies reported the hypoglycemic activity of turmeric and curcumin due to its antioxidant property as well as effect on glucose metabolism [9,28].

The elevated intracellular glucose concentration in the brain undergoes auto-oxidation, in the presence of free metal ions, subsequently causing an imbalance in the oxidant/antioxidant system [29]. Elevated oxidative stress due to this imbalance leads to neuronal injury through oxidized proteins, damaged DNA and augmented levels of lipid peroxidation, as indicated by increased TBARS content on STZ induction in the present study [30,31]. Curcuminoids and vitamin E treatment significantly reduces this enhanced lipid peroxidation and the marked activity is consistent with the previously published reports of curcumin and vitamin E [32,33].

Reduction in the endogenous GSH content occurs in parallel with increased lipid peroxidation in diabetes, as also observed in this study [34]. Curcuminoids and vitamin E protected this STZ-induced depletion of GSH. Curcuminoids appear to increase the GSH content by reducing the influx of glucose through the polyol pathway which is responsible for the depletion of NADPH, required for glutathione reductase activity [35].

A prominent imbalance between reactive oxygen species (ROS) production and endogenous

Table I. Effect of curcuminoids or vitamin E on lipid peroxidation and endogenous antioxidant marker enzymes in diabetic rat brain.

| Group | TBARS (nm/mg protein) | GSH (μg/mg protein) | SOD (IU/mg protein) | Catalase (μM $H_2O_2$ oxidized/min/mg protein) | GPx (μM glutathione oxidized/min/mg protein) |
|---|---|---|---|---|---|
| Normal control | 0.300 ± 0.04 | 4.13 ± 1.77 | 49.72 ± 13.84 | 16.67 ± 4.43 | 3.14 ± 1.42 |
| STZ control | 0.895 ± 0.27 | 1.12 ± 0.31 | 14.88 ± 1.58 | 5.29 ± 1.60 | 0.53 ± 0.15 |
| STZ + vitamin E | 0.623 ± 0.104 | 3.69 ± 0.29 | 58.61 ± 13.38 | 10.09 ± 2.75 | 2.09 ± 0.42 |
| STZ + curcuminoids | 0.455 ± 0.05 | 2.71 ± 0.37 | 48.31 ± 12.49 | 16.68 ± 3.68 | 2.97 ± 0.52 |

Values are given as mean ± SD for eight rats in each group. Values are statistically significant at $p < 0.05$ for control vs STZ, STZ vs vitamin E and STZ vs curcuminoids-treated group.

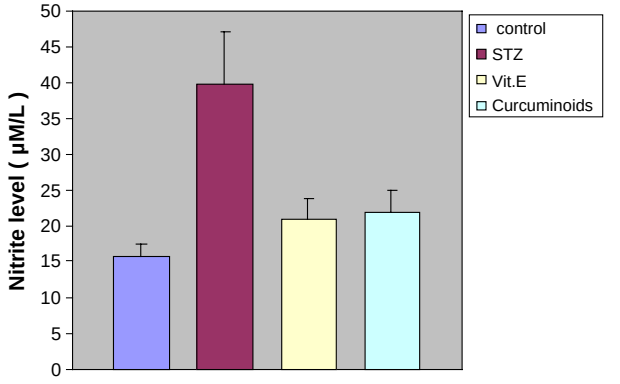


Figure 2. Effect of vitamin E or curcuminoids on total nitrite level in the brain of diabetic rats. Data are expressed as mean ± SD; $n = 8$ in each experimental group. Values are statistically significant at $p < 0.05$ for control vs STZ, STZ vs vitamin E and STZ vs curcuminoids-treated group.

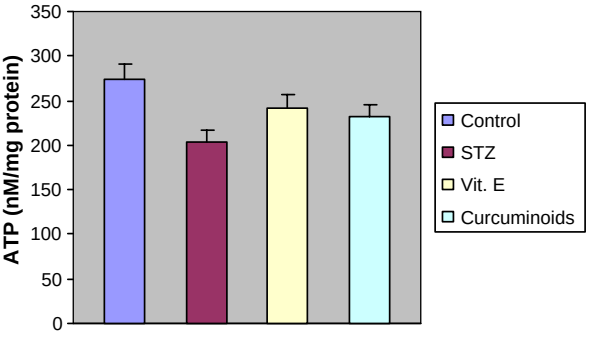


Figure 3. Alterations in ATP level on STZ induction followed by vitamin E or curcuminoids on ATP content in brain mitochondria. ATP content was measured by HPLC. Values are given as mean ± SD for eight rats in each group. Values are statistically significant at $p < 0.05$ for control vs STZ, STZ vs vitamin E and STZ vs curcuminoids-treated group.

antioxidant defense mechanism has been confirmed by reduced activity of catalase, SOD and GPx, on STZ induction in the present study [36]. Decreased GPx and catalase activity indicates elevated $H_2O_2$ production, while reduced SOD activity reflect enhanced protein glycation, as one of the mechanism in diabetes [37]. Treatment with curcuminoids and vitamin E significantly potentiates above enzyme activities and the results are in agreement with the previous reports [38]. Thus, our findings suggest that curcuminoids and vitamin E exhibits potent antioxidant activity by scavenging free radicals and restoring the imbalance between oxidants/antioxidant homeostasis developed during diabetes.

In addition to ROS, NO was also implicated as the major source of end-organ damage in diabetes by altering mitochondrial respiratory chain complexes [39,40]. However, NO is involved in the wide spectrum of physiological processes, it is evident that its action as a signalling or neurotoxic molecule depends on its concentration. Excessive NO generation is the consequence of elevated nitric oxide synthase (NOS) activity in mitochondria of diabetic rat brain. In this study, a sharp elevation in the nitrite level was observed in the STZ-induced rats which was significantly reduced after vitamin E and curcuminoids treatment. NO rapidly diffuses into the neurons and reacts with superoxide anion to form peroxynitrite ($ONOO^-$) which affects energy conservation mechanisms and oxidative post-translation modification of proteins, subsequently leading to neuronal cell death [41]. The elevated $ONOO^-$ concentration acts as a powerful oxidant and causes damage to mitochondrial respiratory chain complexes. In the present study, selective down-regulation of mitochondrial complexes I and IV activity was noticed, whereas insignificant changes were observed in complexes II and II–III activities. Indeed, an elevated level of succinate dehydrogenase activity was found as reported earlier [42]. Selective damage to complex I activity by $ONOO^-$ appears to be the result of protein modifications in the form of S-nitrosation [43,44], whereas inhibition of Complex IV activity may be due to irreversible binding of $ONOO^-$ in competition with molecular oxygen [45,46]. The study findings show that both curcuminoids and vitamin E markedly up-regulated the mitochondrial complex I and IV activity due to potent $ONOO^-$ radical scavenging property [47]. In addition, curcumin has shown the potential to inhibit expression of inducible NOS which may also contribute to the elevation of mitochondrial complexes activity [48].

Depletion of ATP content was observed in the diabetic rat brain as a consequence of impaired mitochondrial respiratory chain activity. Curcuminoids or vitamin E treatment profoundly restored the

Table II. Status of mitochondrial complexes activity after administration of vitamin E or curcuminoids in STZ-induced diabetic rat brain.

| Group | Complex I (µM NADH oxidized/min/mg protein) | Complex II (µM DCIP reduced/min/mg protein) | Complex II–III (µM cyt-c reduced/min/mg protein) | Complex IV (µM cyt-c oxidized/min/mg protein) |
|---|---|---|---|---|
| Normal control | 1.465 ± 0.385 | 0.127 ± 0.156 | 1.23 ± 0.663 | 0.842 ± 0.095 |
| STZ control | 0.68 ± 0.08 | 0.223 ± 0.108 | 0.16 ± 0.065 | 0.29 ± 0.084 |
| STZ + vitamin E | 1.318 ± 0.46 | 0.201 ± 0.08 | 0.218 ± 0.025 | 0.67 ± 0.221 |
| STZ + curcuminoids | 1.568 ± 0.51 | 0.172 ± 0.039 | 0.492 ± 0.295 | 0.643 ± 0.137 |

Values are given as mean ± SD for eight rats in each group. Values are statistically significant at $p < 0.05$ only for Complex I and IV while insignificant changes are observed in Complex II and II–III when compared within control vs STZ, STZ vs vitamin E and STZ vs curcuminoids-treated group.

ATP content. Hence, the findings of the current study suggest the protective effect of curcuminoids and vitamin E on reactive nitrogen species mediated mitochondrial dysfunction in the brain of diabetic rats.

In conclusion, the data in our study suggest that curcuminoids exhibit a protective effect against oxidative damage and mitochondrial dysfunction in the diabetic rat brain. Since curcuminoids have shown multiple targeted actions, it is assumed that the protective effect was also due to its synergistic activity. It is now apparent that the future approach to treat diabetes and its associated complications must consider either the use of individual drugs or drug combinations having multi-pharmacological activities. Curcuminoids may be ideal because they contain a variety of compounds with different known pharmacological actions. However, further research is needed, for the better understanding of the mechanism of action of curcuminoids by which it modulates ROS/RNS levels and alters protein modification involved in mitochondrial dysfunction.



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