

Protection of pancreatic β -cell by the potential antioxidant bis-o-hydroxycinnamoyl methane, analogue of natural curcuminoid in experimental diabetes.

Anusuya Srinivasan, Venugopal P. Menon

Department of Biochemistry, Annamalai University, Annamalai Nagar, India

Viswanathan Periaswamy

Department of Pathology, Raja Muthiah Medical College, Annamalai University, Annamalai Nagar, India

K.N. Rajasekaran

Department of Chemistry, University of Kerala, Trivandrum, India

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Abstract PURPOSE. To evaluate the antioxidant defense by bis-o-hydroxycinnamoylmethane, analogue of the naturally occurring curcuminoid bis-demethoxycurcumin in streptozotocin induced diabetes in male Wistar rats and its possible protection of pancreatic β -cell against gradual loss under diabetic condition. **METHODS.** Male wistar rats were divided into five groups. Group1 served as control rats. Group2 was control rats treated intragastrically with bis-o-hydroxycinnamoyl methane at a dose of 15mg/kg body weight for 45 days. Group3, 4 and 5 rats were injected with 40mg /kg body weight of streptozotocin to induce diabetes. Group4 rats were treated with the drug similar to group2 and group5 rats treated with the reference drug glibenclamide intragastrically for a similar period. After 45 days, the levels of plasma glucose, glycated hemoglobin, enzymic antioxidants (SOD, CAT) and non-enzymic antioxidants Vit C, Vit E was determined. Histopathological sections of the pancreas were examined. **RESULTS.** The levels of plasma glucose and glycated hemoglobin which were elevated in group3 diabetic rats were reduced after treatment with the drug. The antioxidant levels showed an increase in the case of treated diabetic rats as compared to group3 diabetic rats. The islets were shrunken in group3 diabetic rats in comparison to normal rats. In the treated diabetic rats there was expansion of islets. **CONCLUSIONS.** The experimental drug bis-o-hydroxycinnamoylmethane enhances the antioxidant defense against reactive oxygen species produced under hyperglycemic conditions and thus protects the pan-

creatic β -cell against loss and exhibits antidiabetic property.

INTRODUCTION

Reactive oxygen species play an important role in the pathogenesis and development of complications of diabetes. Reactive oxygen species are produced mainly by the glycation reaction under diabetic conditions (1). Glycation reaction in diabetes occurs in various tissues including β -cells (2, 3). The activity of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase which is low in islet cells when compared to other tissues becomes further worsened under diabetic conditions (4). Earlier reports have shown apoptosis of β -cells (5) and reduced insulin gene transcription (6) by glycation mediated reactive oxygen species. The antioxidant treatment which suppresses apoptosis of β -cells was shown to preserve β -cell function in diabetic mice (7).

The active principle isolated from the rhizomes of the *Curcuma Longa* Linn consists of the components namely curcumin, demethoxycurcumin and bis-demethoxycurcumin collectively known as curcuminoids. All the three constituents are powerful antioxidants and reported to be more active than α -tocopherol (8). The antioxidant activity of bis-demethoxycurcumin (BDMC), which is bis-p-hydroxycinnamoylmethane, was reported to be maximum at a lower concentration than the other two curcuminoids (9, 10).

The synthetic analogs of curcuminoids have also been reported to have antioxidant, antitumor and antimu-

Corresponding Author: Venugopal P.Menon, Center for Micronutrient Research, Annamalai University, Annamalai Nagar, Tamilnadu-608002, India. cmrana@sify.com

tagenic activities (11). The antioxidant activity of naturally occurring curcuminoids and their synthetic analogs were attributed to their potency by inducing enzymes like superoxide dismutase (SOD), catalase (CAT) and also this potential was reported to be more than 30 fold when hydroxyl group is introduced in ortho position (12). Hence in our present study we investigated the protective role of bis-o-hydroxycinnamoylmethane (BDMCA), which is an analogue of bisdemethoxycurcumin BDMC against the oxidative stress in experimental diabetes and its possible protection of pancreatic β -cells. Chemical structures of BDMC and its analog BDMCA are shown in figure 1 and 2 respectively.

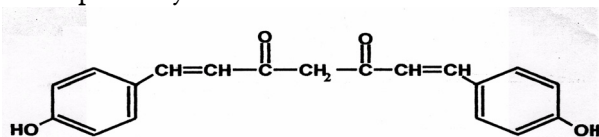


Figure 1: Structure of Bisdemethoxy curcumin (BDMC)

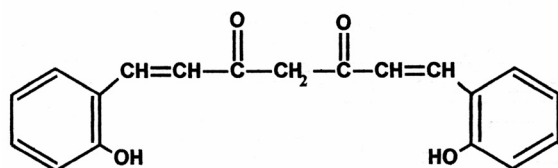


Figure 2: Structure of Bisdemethoxy curcumin analog (BDMCA)

MATERIALS AND METHODS

Animals

Male albino rats of wistar strain were procured from the central animal house, Rajah Muthiah Medical College, Annamalai University and maintained under standard condition with food and water ad libitum. The experimental protocol for the study has the approval of the animal ethics committee of our institution.

Chemicals and Reagents

Streptozotocin was purchased from Sigma Chemical St. Louis Mo USA. Glucose kit was purchased from Span Diagnostics India Ltd, Nitrobluetetrazolium, phenazinemetosulphate, hydrogen peroxide, thiobarbituric acid, acetyl acetone and salicylaldehyde were purchased from Himedia Laboratories, Mumbai, India. All other reagents were of analytical grade.

Drug

The experimental drug bis-o-hydroxycinnamoylmethane was prepared by refluxing a mixture of acetyl acetone, salicylaldehyde and boric acid in DMF adding few drops of catalyst namely 1:1 glacial acetic acid and diethanolamine for 5 to 6 hours. The mixture then poured to 10 % acetic acid was stirred for 2 to 3 hrs. The crude yellow mass was filtered and purified using silica gel column chromatography and the purity checked by TLC and the spectral data verified with the earlier reports (13). The drug was administered orally by intragastric intubation by dissolving a known weight of the drug in water and DMSO in the ratio 100:1(v/v) to get 15mg/kg body weight. The volume 2ml of the drug administered was kept a constant in all groups of rats.

Experimental Induction of diabetes

Diabetes was induced in 9 to 10 weeks old male Wistar rats (150 to 160g body weight) by injection of streptozotocin 40mg kg⁻¹ body weight, dissolved in 0.1M trisodium citrate buffer pH=4.5 intraperitoneally and the control rats were injected with citrate buffer alone. Development of diabetes was first determined by the observations of three classical symptoms polyuria, polydypsia and polyphagia. It was once again verified after two weeks of STZ injections by measuring the plasma glucose levels. Rats with fasting plasma glucose levels less than 10mM/L were excluded from the study.

Experimental Design

Group 1: Control rats given 1ml of vehicle (1% DMSO) alone for 45 days.

Group 2: Rats given 15 mg/kg body weight of BDMCA for 45 days.

Group 3: STZ diabetic rats treated as in group1.

Group 4: STZ diabetic rats treated with the drug as in group2.

Group 5: STZ diabetic treated with the reference drug glibenclamide for a similar period.

The experiment was terminated at the end of 45 days and the animals were fasted overnight.

Blood Sampling

Blood was collected into heparinized tubes by retro-orbital plexus puncture and plasma was separated by centrifugation at 2000g for 10 min.

Preparation of hemolysate

After the separation of plasma, the buffy coat was removed and packed cells (RBCs) were washed thrice with cold physiological saline. RBC lysate was prepared by lysing a known volume of RBC with cold phosphate buffer pH 7.4. The hemolysate was separated by centrifuging at 3000g for 10 min at 2°C and used to measure the activities of enzymic antioxidants superoxide dismutase (SOD) and catalase (CAT).

Histological sections of the Pancreas

The animals from each group were anaesthetized with ether and intra cardiac perfusion of normal saline followed by 10% formalin was done. Pancreas was dissected out, blotted and all the groups were processed for routine paraffin embedding. Sections were cut and stained with haematoxylin and eosin.

BIOCHEMICAL INVESTIGATIONS

Plasma glucose

It was measured by enzymic method using kit supplied by Span Diagnostics, India Ltd (14). To 10 µl of plasma 1 ml of glucose reagent was added and incubated at 37°C for 15 min. Absorbance was read at 510 nm against reagent blank and a standard was also processed similarly.

Glycated hemoglobin

It was measured by the method of Sudhakar Nayak and Pattabiraman (15). 200 µl of the lysate was mixed with 1.8 ml of 0.3 M oxalic acid and the mixture was hydrolyzed for 4 hours. Cooled and added 1.0 ml of 40% TCA. After centrifugation for 20 minutes 1.5 ml of the supernatant was treated with 0.05 ml of 80% phenol and 3 ml of concentrated sulfuric acid. Tubes were incubated at 37°C for 30 minutes and the color developed was read at 480 nm. Standard fructose in the range of 10-40 µg was processed similarly.

Non protein thiol

It was measured by the method of Ellman (16). 0.5 ml of plasma was pipetted out and precipitated with 2.0 ml of 5% TCA. After the centrifugation 1.0 ml of the supernatant was taken and 0.5 ml of Ellman's reagent and 3.0 ml of phosphate buffer were added. The yellow color developed was read at 412 nm. A series of standard was treated in a similar manner along with a blank containing 3.5 ml of buffer. The values were expressed as mg/dl.

Vitamin C

It was measured using DNPH by the method of Roe and Kuther (17). Mixed 0.5 ml of plasma with 1.5 ml of 6% TCA. Allowed to stand for 5 minutes and centrifuged. To the supernatant

0.3 g of acid washed norit was added, shaken vigorously and filtered. 0.5 ml DNPH was added to 2 ml of the filtrate, stoppered and placed in a water bath for 3 hours at 37°C. The tubes were removed from water bath and placed in ice-cold water and added 2.5 ml of 85% H₂SO₄ drop by drop. The tubes were mixed well and allowed to stand for 30 minutes at room temperature. A set of standards containing 20-100 µg of ascorbic acid were taken and processed similarly, along with a blank containing 2.0 ml of 4% TCA. The yellow color developed was read colorimetrically at 540 nm.

Superoxide Dismutase

The activity of SOD was assayed by the method of Kakkar et al (18). To 1.2 ml of sodium pyrophosphate buffer, 0.1 ml of phenazine methosulphate, 0.3 ml of nitroblue tetrazolium, 0.2 ml of NADH, 1.1 ml of water and 0.1 ml of hemolysate was added and incubated exactly for 90 seconds at 30°C. The reaction was started by the addition of NADH and stopped by the addition of 1 ml of glacial acetic acid. Control was carried out simultaneously. The reaction mixture was stirred vigorously and shaken with 4 ml of n-butanol. The mixture was allowed to stand for 10 minutes, centrifuged and the chromogen extracted in butanol layer was read at 560 nm against butanol blank. One unit of the enzyme activity is defined as the enzyme reaction which gave 50% inhibition of NBT reduction in one minute under the assay conditions and expressed as specific activity in units / mg Hb.

Catalase

It was assayed by the method of Sinha (19). 0.1 ml of hemolysate and 1.5ml of phosphate buffer were added. To this, 0.4ml of hydrogen peroxide was added and the reaction was arrested after 30 and 60 second by the addition of 2.0 ml dichromate acetic acid reagent. A control was also carried out simultaneously. All the tubes were heated in a boiling water bath for exactly 10 minutes, cooled and absorbance read at 620nm. Standards in the range of 2-10 μ moles were taken and processed as the test. The activity of catalase was expressed as μ moles of hydrogen peroxide consumed/min/mg Hb.

Statistical Analysis

All data are presented as the mean \pm SD from 6 rats in each group. One-way analysis of variance (ANOVA) followed by least significant difference point test was used to identify significantly different groups. $P < 0.05$ indicates significant differences between group mean.

RESULTS

Table-1 shows the levels of plasma glucose and glycated hemoglobin in normal and experimental rats. The levels of glucose and glycated hemoglobin were elevated significantly in the group3 diabetic control rats when compared with normal rats in group1 and 2. After treatment with BDMCA the levels of glucose and glycated hemoglobin were significantly lowered.

Table 1: Levels of plasma glucose and glycated hemoglobin in experimental diabetic rats.

| Group | Glucose mM / L | Glycated hemoglobin % glycated |
|-----------------------------|---------------------------------|---------------------------------|
| 1. Normal | 3.905 \pm 0.353 | 6.687 \pm 0.701 |
| 2. Normal + Drug | 3.440 \pm 0.200 | 6.697 \pm 0.667 |
| 3. Diabetic | 17.090 \pm 1.455 ¹ | 20.702 \pm 1.438 ¹ |
| 4. Diabetic + drug | 5.645 \pm 0.558 ² | 8.709 \pm 0.724 ² |
| 5. Diabetic + glibenclamide | 6.152 \pm 0.490 ² | 8.724 \pm 1.107 ² |

Values are mean \pm SD from 6 rats in each group

1 - diabetic control animals (group 3) compared with normal control (group 1).

2 - diabetic treated animals (Group 4 and 5) compared with diabetic control (group 2).

* The values are significant at pQ 0.05 level.

Table-2 shows the levels of plasma non protein thiols and ascorbic acid. The levels were significantly lowered in diabetic rats of group3 when compared with those in normal control rats of group1 and 2. Administration of BDMCA and glibenclamide to STZ diabetic rats in

group 4 and 5 increased significantly the levels of non protein thiols and ascorbic acid as compared to that of diabetic rats in group 3.

Table 2: Levels of non-protein thiols and ascorbic acid in the plasma of experimental diabetic rats.

| Group | GSH mg / 100 ml | Ascorbic acid mg / 100 ml |
|----------------------------|---------------------------------|--------------------------------|
| 1 Normal | 26.250 \pm 2.622 | 2.668 \pm 0.129 |
| 2 Normal + Drug | 26.250 \pm 2.622 | 2.610 \pm 0.130 |
| 3 Diabetic | 15.833 \pm 2.582 ¹ | 1.542 \pm 0.292 ² |
| 4 Diabetic + drug | 22.500 \pm 2.236 ² | 2.542 \pm 0.189 ² |
| 5 Diabetic + glibenclamide | 22.917 \pm 2.323 ² | 2.655 \pm 0.123 ² |

Values are mean \pm SD from 6 rats in each group

1 - diabetic control animals (group 3) compared with normal control (group 1).

2 - diabetic treated animals (Group 4 and 5) compared with diabetic control (group 2).

* The values are significant at pQ 0.05 level.

Table-3 depicts the activities of superoxide dismutase (SOD) and catalase in the RBC lysate of normal and experimental rats. Diabetic rats in group3 showed a significant decrease in the activity of these enzymes when compared with normal rats in group1 and 2. Administration of BDMCA and glibenclamide to diabetic rats in group 4 and 5 increased the activity as compared with that of diabetic rats in group3 for both enzymes.

Table 3: Levels of superoxide dismutase and catalase in the RBC lysate of experimental diabetic rats.

| Group | SOD ^u | CAT ^u |
|----------------------------|--------------------------------|--------------------------------|
| 1 Normal | 3.173 \pm 0.147 | 2.810 \pm 0.094 |
| 2 Normal + Drug | 3.257 \pm 0.128 | 2.883 \pm 0.123 |
| 3 Diabetic | 1.628 \pm 0.108 ¹ | 1.730 \pm 0.132 ¹ |
| 4 Diabetic + drug | 3.005 \pm 0.089 ² | 2.823 \pm 0.159 ² |
| 5 Diabetic + glibenclamide | 2.935 \pm 0.107 ² | 2.630 \pm 0.078 ² |

Values are mean \pm SD from 6 rats in each group

1- diabetic control animals (group 3) compared with normal control (group 1.)

2- diabetic treated animals (Group 4 and 5) compared with diabetic control (group 3)

• The values are significant at pQ 0.05 levels.

• u - μ moles of H₂O₂ utilized / min / mg Hb

• u - Enzyme required for 50 % inhibition of NBT reduction / min / mg / Hb

Figure 3 to 7 depicts the islet cells of the pancreas of rat in different groups. Fig 3 shows normal islet cells. The islets were shrunken in group3 diabetic rat when compared with group1 control rat. Treatment of diabetic rat with the drug BDMCA in group4 and glibenclamide in group5 showed moderate expansion of islets.

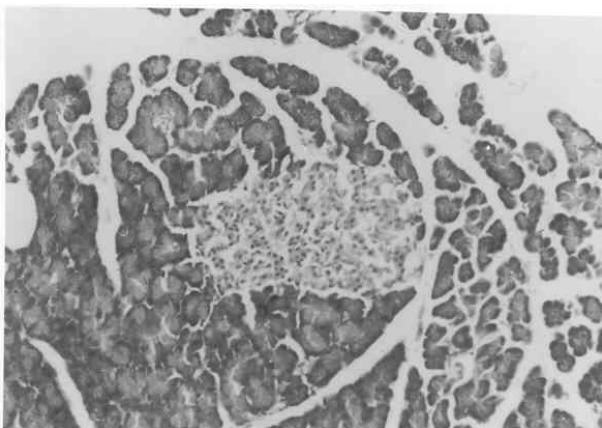


Figure 3: Typical photomicrograph of the pancreas of control rat H and E x 10.

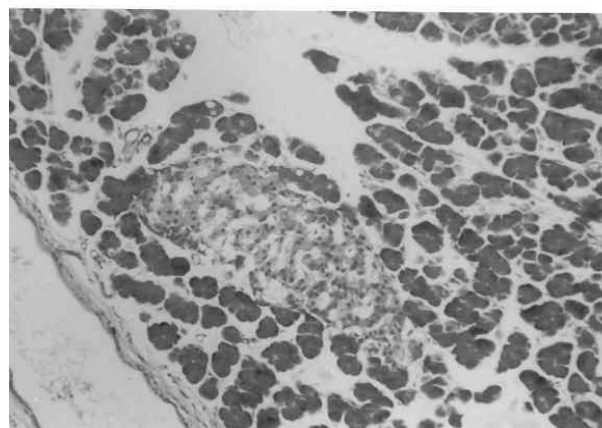


Figure 6: Typical photomicrograph of the pancreas of STZ induced diabetic rats treated with 15mg / kg b.wt of BDMC-A. H and E x 10 shows moderate expansion of islets.

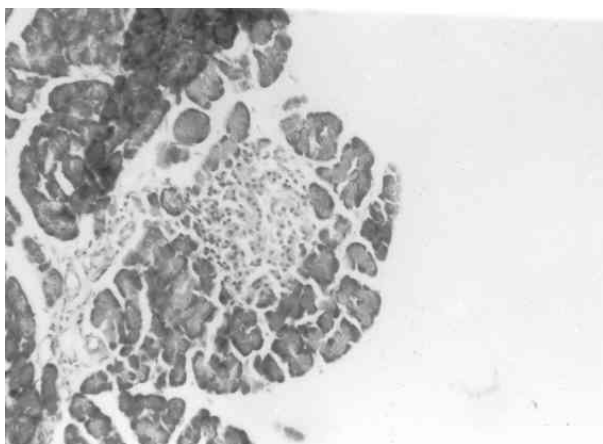


Figure 4: Typical photomicrograph of the pancreas of 15mg / kg b.wt of BDMCA treated control rat H and E x 10 shows normal islets.

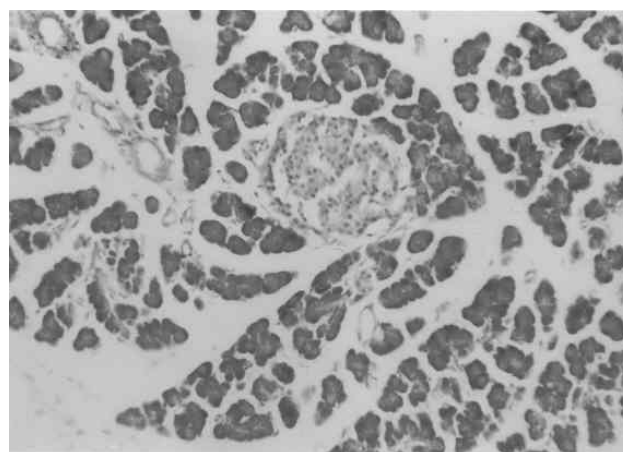


Figure 7: Typical photomicrograph of the pancreas of STZ induced diabetic rats treated with 600 µg/kg b.wt of Glibenclamide. H and E x 10 shows moderate expansion of islets.

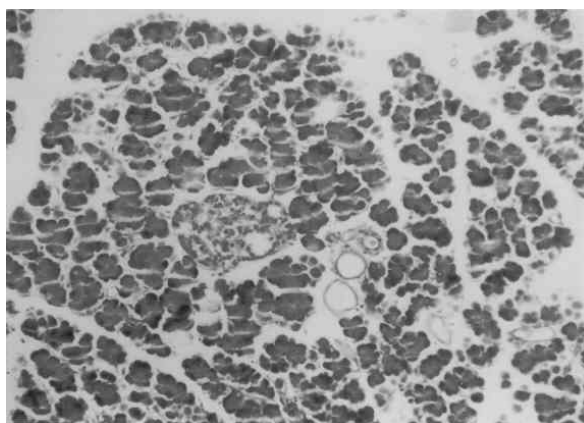


Figure 5: Typical photomicrograph of the pancreas of STZ induced diabetic control rats H and E x 10 shows shrunken islets.

DISCUSSION

The islet β cells are susceptible to damage caused by oxygen free radicals (20) since the antioxidant defense system is weak under diabetic condition (21). The levels of antioxidant defense system are altered in STZ induced diabetic rats which is in good correlation with the present observation (22).

Non protein thiols like glutathione are one of the important primary defenses that counteract the oxidative stress. We observed a lower level of plasma non protein thiols in STZ diabetic rats which is consistent with the earlier report (22, 23). The observed decrease may be due to utilization of non protein thiols by increased oxygen free radicals produced in hyperglyce-

mic conditions associated with diabetes mellitus.

Vitamin C is a well known antioxidant and quenches singlet molecular oxygen in aqueous medium (24). We observed a decrease in level of plasma vitamin C. Decreased level of plasma ascorbic acid have been reported in experimental diabetes(25). The decrease in the level of vitamin C may be due to increased utilization against ROS produced in large amounts in diabetes or decrease in the non protein thiols like GSH as GSH is required for the recycling of vitamin C.

The two important enzymic antioxidants namely superoxide dismutase and catalase in the RBC lysate is found to have decreased activity in STZ diabetic control rats. A similar decrease in the activity of these two enzymes in hemolysate of STZ diabetic rats was reported earlier (26). The increase in the level of oxygen radicals superoxide $O_2^{\bullet-}$ and OH^{\bullet} in experimental diabetes and the protection by SOD and catalase were reported by Uchigata et al (27).

Treatment of diabetic rats with the drug BDMCA for 45 days increased the levels of non-enzymic antioxidants vitamin C and non protein thiols in plasma. The activities of SOD and CAT were also increased in RBC lysate of treated diabetic rats. The antioxidant activity of the drug BDMCA might have been due to the inhibition of glycation of the antioxidant enzymes SOD and CAT. Glucose which forms Schiff's base with proteins has been reported to have high affinity for proteins especially those containing transition metal ions (28). Increased glycated Cu-Zn-SOD has been reported in diabetes. Earlier reports have shown that curcumin inhibits advanced glycation end products in STZ diabetes in rats (29). In the present study also we observed a decrease in the glycated hemoglobin in rats treated with the drug BDMCA. The inhibitory action may be due to the presence of the central 1, 3-diketone system as in curcumin. Enhancement of antioxidants SOD and CAT by BDMCA thus might be explained as due to prevention of glycation. Furthermore the presence of -OH group in the O-position increase its antioxidant potential through intermolecular hydrogen bonding involving the -SH group of non protein thiols and enzymes (30).

Increase in the activity of the enzymes catalase and SOD might have protected β -cells against damage by

reactive oxygen species. Treatment with the antioxidant N-acetyl cysteine in diabetic mice was shown to have suppressed β -cell apoptosis and decrease blood glucose level (7). Hence we conclude that the drug used in the present study offers protection of β -cells against ROS mediated damage by enhancing antioxidants and reduces hyperglycemia in chemically induced diabetes.

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