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Zinc-morin complex improves pancreatic β-cell function in the HFD-STZ induced experimental type 2 diabetes

V. Sendrayaperumal and S. Subramanian*

Department of Biochemistry, University of Madras, Guindy Campus, Chennai, India

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ABSTRACT

Hyperglycemia induced oxidative stress plays a pivotal role in the initiation and progression of diabetes and its secondary complications. An increase in oxidative damage due to excessive generation of free radicals is associated with etiology of insulin resistance. The sensitivity of pancreatic β-cells to oxidative stress has been attributed to their low content of antioxidants compared with other tissues. Recently, we have synthesized, characterized a new zinc-morin complex and evaluated its antidiabetic potentialin HFD fed-low dose STZ induced type 2 diabetic rats. The present study was aimed to study the role of zinc-morin complex on hyperglycemia mediated oxidative stress in diabetic rats. Oral administration zinc-morin complex at a concentration of 5mg/kg body weight to diabetic rats for a period of 30 days resulted in significant improvement in pancreatic β-cellfunction, which in turn may be due to the increased level of metallothionein, a reservoir of zinc. Further, treatment with zinc-morin complex significantly improved the levels of enzymaticand non-enzymatic antioxidants and reduced the levels of lipid peroxides, nitric oxide, IL-1β and nuclear NF-κB p65 unit in pancreatic tissues of diabetic rats. Ultrastructural observationsfurther evidenced the pancreaticβ-cell protective nature of zinc-morin complex against oxidative damage.

Keywords*: Oxidative stress, insulin resistance, zinc-morin complex.hyperglycemia, antioxidants, metallothionein.*

INTRODUCTION

Oxidative stress is a biological entity accountable for several pathological conditions including diabetes mellitus. The consequences of hyperglycemia induced oxidative environment are the development of insulin resistance, β-cell dysfunction, impaired glucose tolerance, and mitochondrial dysfunction, which leads to diabetes mellitus [1]. Type 2 diabetes mellitus is the most common form of diabetes, which is primarily associated with insulin resistance. As a result, there is an increased formation of anti glycation end products (AGEs) and lipid peroxidation products that exacerbate intracellular oxidative stress, resulting in the loss of molecular integrity, disruption in cellular signaling and homeostasis, followed by inflammation and tissue injury. Pancreatic β-cells are equipped with complex mechanisms to defend ROS toxicity. However, the reduced antioxidant capacity potentially makes the pancreatic β-cells sensitive to ROS mediated signal transduction and cellular response.The maintenance of β-cell function and their protection against oxidative stress mediated tissue damage might delay the onset of diabetes as well as the progression of its complications [2, 3].

The structure–activity relationships and the therapeutic aspects of metals in binding to cellular targets and their action indicate an immense scope for designing novel compounds for the treatment of free radical mediated diseases such as diabetes and cancer. One among such metals of biological importance is zinc, which belongs to the

transition group of the periodic table and is an essential trace element in biological systems. In fact, Insulinis stored as a hexamer containing two Zinc ions in the β-cells of the pancreas.Zinc content in the pancreatic cell is among the highest of the metals [4]. Therefore, zinc appears to be an important metal for insulin-secreting cells, and it may serve as a mediator of insulin storage and secretion [5].

The major contribution of zinc as a pharmacological entity has prompted researchers to identify new zinc-containing compounds for the treatment of diabetes [6, 7]. However, the toxicity of zinc at higher concentrations is a major challenge in exploiting zinc compounds for therapeuticapplications [8].

Zn is involved in a multitude of processes within the pancreas, includinginsulin stoage, secretion, signaling, glucagon secretion and digestive enzyme activity. As a result of this extensive physiological contribution, dysregulation of Zn metabolism within the pancreas impairs a multitude of key processes, including glycemic control [9]. Since the discovery of insulin mimetic actions of zinc, a number of zinc complexes have been synthesized and their antidiabetic properties were studied both *in vitro* as well as *in vivo*. However, most of the zinc complexes so far investigated for their possible antidiabetic and insulin mimic activity were poorly absorbed in their inorganic forms and required high doses, which have been associated with undesirable side effects. In order to circumvent the toxicity, various zinc complexes have been formulated and tested for their antidiabetic activity[10-12]. Recently, we have synthesized, characterized a zinc-morin complex and evaluated the antidiabetic efficacy in HFD fed-low dose STZ induced type 2 diabetic rats [13]. In the present study, an attempt has beenmade to explore the antioxidant role of zinc-morin complex in ameliorating pancreatic β cell dysfunction in type 2 diabetic rats.

MATERIALS AND METHODS

Chemicals

Zinc acetate, Morin and Streptozotocin were purchased from Sigma Aldrich, Ultra-sensitive ELISA kit for rat insulin (Linco Research, Inc., St. Charles, MO), and all other chemicals used in this study were of analytical grade and obtained from standard commercial suppliers.

Synthesis of zinc-morin complex

The zinc-morin complex was synthesized as reported by us earlier [13]. Briefly,anethanolic solution containing zinc acetate dehydrate (0.2195g, 1mM) was gradually added to ethanolic solution of morin (0.6044g, 2mM). The pH of the medium was adjusted to 7.5 with Tris-HCl buffer and the reaction mixture was constantly stirred, refluxed for 8 hours at 80^{o}C over an oil bath [14]. The resulting precipitate was filtered, washed with absolute ethanol, dried in vacuum and a pale yellow colour solid (yield of 96%) was obtained. The solid product was characterized and used without further purification.

Experimental Animals

Male Albino Wistar rats weighing around 160-180 g were procured from the Tamilnadu Veterinary and Animal Sciences University, Chennai and were housed under standard husbandry conditions (12 h light and dark cycle, relative humidity 55%). The rats were fed with balanced diet (Hindustan Lever Ltd, Bangalore, India) and water ad libitum. The rat diet (pellet) composed of 5% fat, 21% protein, 55% nitrogen-free extract and 4% fiber (w/w) with adequate mineral and vitamin levels for the animals. The experiments were designed and performed in accordance with the current ethical norms approved by the Ministry of Social Justices and Empowerment, Government of India and Institutional Animal Ethics Committee Guidelines for the investigation of experimental pain in conscious animals[IAEC NO:03/10/12].

Experimental design

The rats were divided into two dietary regimens by feeding either normal or high fat diet (HFD) for the initial period of two weeks. The composition of HFD is Powdered NPD – 365g/kg, Lard – 310 g/kg, Caseine – 250g/kg, cholesterol – 10g/kg, vitamin and mineral mix – 60g/kg, DL-methionine – 3g/kg, Yeast powder – 1g/kg, NaCl – 1g/kg[15]. After two weeks of dietary manipulation, the groups of rats fed with HFD were injected intraperitoneallywith a low dose of STZ (35 mg/kg b.w) dissolved in 0.1M cold citrate buffer, pH 4.5). After one week of STZ injection, rats with non-fasting blood glucose levels ≥ 300 mg/dL were randomly selected for the experiment. The rats were allowed to continue to feed on their respective diets until the end of the experiments.

Group 1: Normal control rats.
Group 2: Normal rats treated with zinc-morin complex(5 mg/kg body weight/rat/day) for 30 days.
Group 3: HFD-STZ induced diabetic rats.
Group 4: HFD-STZ induced diabetic rats treated with zinc-morin complex(5 mg/kg body weight/rat/day) for 30 days.

Preparation of pancreatic tissue homogenate
Pancreatic tissues from control and experimental groups of rats were excised, rinsed with ice-cold saline and homogenized in Tris HCI buffer (0.1M. pH 7.4) using Teflon homogenizer and centrifuged at 12,000 g for 30 min at 4°C. The supernatant was pooled and used for the estimations. The protein content in the tissue homogenate was estimated by the method of Lowry et al. [16].

Determination of fasting blood glucose, plasma insulin, HbA1c, HOMA-IR
Fasting blood glucose level was determined by glucose oxidase peroxidase diagnostic enzyme kit (Span Diagnostic Chemicals, Surat, India) and plasma insulin was assayed using rat ELISA kit (Millipore, St. Charles, USA) according to the manufacturer's instructions. Both the analyses were performed according to the manufacturer's instructions. The HbA1c level was estimated in the control and experimental groups of rats [17]. Insulin resistance/sensitivity was assessed by HOMA-IRformula: (fasting glucose (mg/dL) × fasting insulin (μU/L)/405).

Estimation of zinc in pancreas
The pancreas was removed, quickly weighed and acid digested with a triple acid mixture comprising of nitric acid, sulphuric acid and perchloric acid in the ratio of 11:6:3 respectively for the complete removal of organic contents. The digested sample was made up to 100 ml using deionized water and this sample is used for the assay of zinc through atomic absorption spectroscopy (GBC-Avanta, Australia) using hollow cathode lamps [18].

Determination of metallothionein
The pancreatic tissue samples were saturated by addition of $CdCl_2$. The excess Cd^{2+} and all the Cd-binding ligands other thanmetallothioneinin the samples were selectively removed by addition of red blood cell hemolyzates and a subsequent heating step. Since it is known that 1 mole of metallothionein binds with 6 or 7 g-atoms of Cd^{2+}, the actual concentration of metallothioneinin pancreas is calculated after estimation of Cd^{2+} in the heated supernatant[19].

Assay of antioxidant status
The activities of enzymatic antioxidants such as SOD [20], Catalase [21], GlutothionePeroxidase (GPx) [22], glutathione-S-transferase (GST)[23] were assayed in the pancreatic tissues. The levels of lipid peroxides [24], hydroperoxides[25] were determined in pancreatic tissue homogenate of control and experimental groups of rats.

Assay of pancreatic NF-κB p65 unit
The nuclear levels of NF-κB free p65 may correlate positively with the activation of NF-κB pathway. The NF-κB/p65 ActivELlSA kit (Imgenex. San Dieio 6) was used to quantify NF-κB free p65 in the nuclear fraction of pancreatic tissue. The analysis was done according to the manufacturer's instructions.

Assay of proinflammatory cytokines
The levels of proinflammatory cytokine IL-1β in pancreas of control and experimental groups of rats were determined byspecific ELISA kits according to the manufacturer's instructions (Biosource. CAQ5).

Determination of nitric oxide (NO)
NO level in pancreas was assayed indirectly by measuring the nitrite concentration by colorimetric method based on the Griess reaction [26].

Transmission Electron Microscopy
A portion of pancreas (about 1mm3) from control and experimental groups of rats were fixed in 3% glutaraldehyde in sodium phosphate buffer (0.2 M, pH 7.4) for 3 h at 4°C. Tissue samples were washed with the same buffer, post-fixed in 1% osmium tetroxide and sodium phosphate buffer (0.2 M, pH 7.4) for 1 h at 4°C. The samples were again washed with the same buffer for 3 h at 4°C, dehydrated with graded series of ethanol and embedded in Araldite. Thin sections were cut with LKBUM4 ultramicrotome using a diamond knife, mounted on a copper grid and stained

with 2% uranyl acetate and Reynolds lead citrate [27]. The grids were examined under a Philips EM201C transmission electron microscope.

Statistical analysis

The values are expressed as mean values of six rats in each group ± standard deviation (S.D). Data analysis was done with SPSS 16 student software. Hypothesis testing method included one way analysis of variance (ANOVA) followed by post hoc testing performed with least significance difference (LSD). Values of 0.001; 0.01; $P<0.05$ was considered to indicate statistical significance.

RESULTS

The levels of fasting blood glucose, glycosylated hemoglobin, plasma insulin and urine sugar in all the groups of rats are shown in Table 1. Diabetic rats showed a significant decrease in plasma insulin level but in case of diabetic rats treated with zinc-morin, there was a significant increase in plasma insulin level. The increased levels of fasting blood glucose and glycosylated hemoglobin in diabetic rats were significantly reduced in zinc-morin complex treated diabetic rats. However, there was no significant alteration in the levels of fasting blood glucose, glycosylated hemoglobin and plasma insulin in control rats treated with the complex.

Fig.1shows HOMA IR of control and experimental group of rats. HOMA IR was higher in diabetic rats indicating the state of insulin resistance. Upon treatment with zinc-morin complex, HOMA IRwas significantly reduced. However, there was no significant difference in the HOMA IR value of control rats and control rats treated with zinc-morin complex.

The level of zinc in the pancreatic tissues of control and experimental group of rats were shown in Fig.2. An increase in thelevel of zinc was observed in control rats treated with zinc-morin complex. There was a significant reduction in the level of zinc in diabetic rats whereas diabetic rats treated with zinc-morin complexsignificantly improved the zinc levelswhen compared with diabetic rats.

The levels of metallothionein in control and experimental groups of rats are shown in Fig. 3. The decreased levels of metallothionein in diabetic rats were significantly increased upon oral administration withzinc-morin complex. Thesignificant difference in metallothionein level was observed in control rats treated with the zinc-morin complex compared to normal rats.

Table 2 depicts the activities of enzymatic antioxidants such as SOD, Catalase, GPx, and GST in pancreatic tissues of control and experimental groups of rats. The activities were significantly diminished in the pancreatic tissues of diabetic group of rats. Oral treatment of zinc-morin complex significantly improved the activities of these enzymic antioxidants to near normalcy in pancreatic tissues of diabetic rats.

Table 3 exemplifies the levels of lipid peroxides and hydro peroxides in pancreatic tissue of control and experimental groups of rats. The significant increase noted in the levels of lipid peroxides and hydro peroxides in pancreatic tissue of diabetic group of rats, declined significantly to near normalcy by the treatment of zinc-morin complex. Whereas, zinc-morin complex treated control rats did not show any significant difference in the levels of lipid peroxides and hydro peroxides when compared to control rats.

The levels of inflammatory markers in pancreatic tissues of control and experimental groups of rats were shown in table 4. The pancreatic tissues of diabetic group of rats showed high levels of nuclear NF-κB p65 unit compared to control rats. Upon zinc-morin complex treatment to diabetic rats there was a significant reduction in the level of nuclear NF-κB p65 unit when compared to diabetic rats. The concentration of nitric oxide in the form of nitrite in pancreatic tissues of control and experimental groups of rats were examined. Diabetic rats showed significantly increased level of nitrite in pancreatic tissues compared to control rats whereas, the level of nitrite was significantly reduced in diabetic rats treatment with zinc-morin complex. The levels of IL-1βin pancreatic tissues of control and experimental groups of rats were analyzed. There was a significant increase in the levels of IL-1βin tissues of diabetic group of rats compared to control rats. However, oral administration of zinc-morin complex to diabetic rats significantly reduced the level of IL-1βin tissues compared to diabetic rats.

The ultrastructural changes in the pancreatic cells of control and experimental groups of rats are shown in Fig. 4A–D. Fig. 4A represents the electron micrograph of pancreaticcell of control group of rats showing normal cellular organelles such as mitochondria, endoplasmic reticulum, Golgi complex and large number of secretory granules containing insulin distributed in the cytoplasm. The electron micrograph of pancreatic cell of diabetic group of rats (Fig. 4C) revealed the degeneration of cell with loss of nuclear envelope and vacuolization with ballooning appearance of mitochondria as well as dilation of the rough endoplasmic reticulum. Amarked decrease in the number of secretory granules with insulin was observed in the cells of the diabetic group of rats. The electron micrograph (Fig. 4D) apparently shows the pancreatic b-cell protective nature of zinc-morin complex in diabetic group of rats by means of moderate increase in secretory granules with insulin, organized nuclear structure, less swelling of mitochondria and no vacuolarization of cytoplasmic region of b-cells. The cells of normal rats treated with zinc-morin complex (Fig. 4B) showed similar pattern of ultra structure of cells of normal control rats.

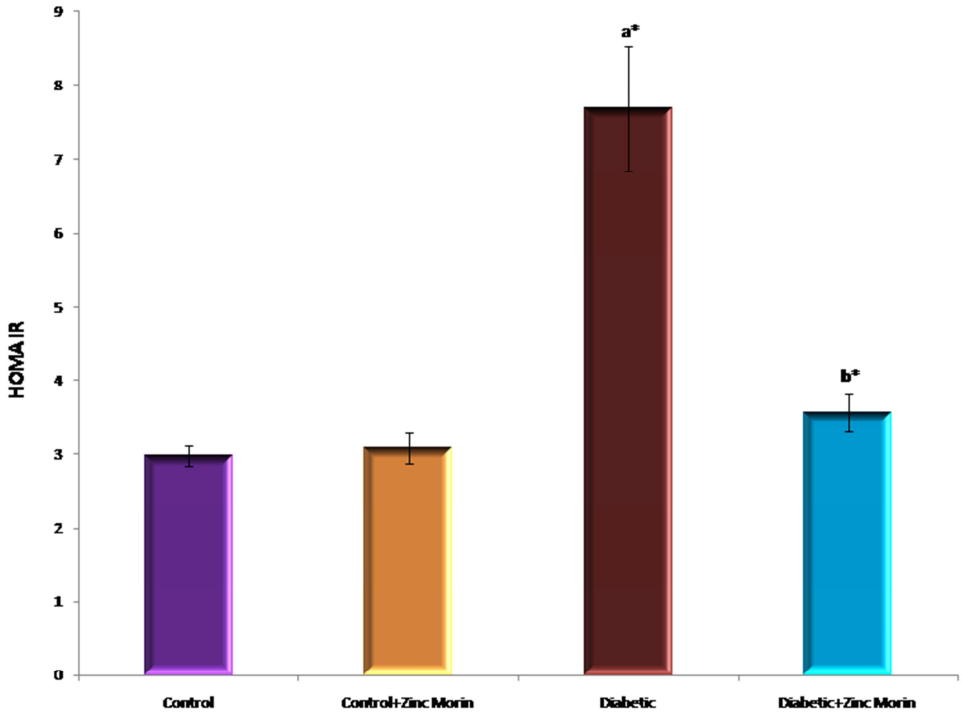

Figure 1

Fig 1: Effect of zinc-morinon the levels of HOMA-IR in plasma of control and experimentalgroups of rats.Values are expressed as mean ± SD for groups of six rats in each.Statisticalsignificance was determined by one way ANOVA followedby post hoc test LSD. *p < 0.001; #p <0.01; @p < 0.05.[a] compared with control; [b] compared with diabetic rats

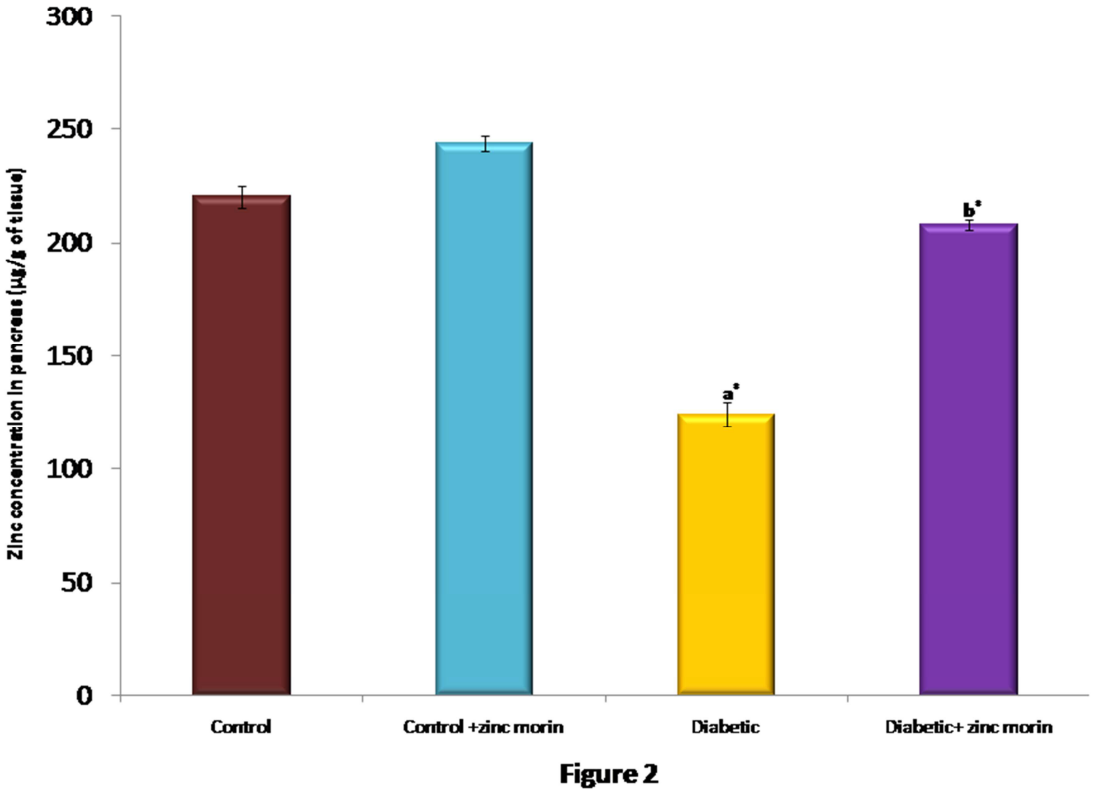

Fig 2: Effect of zinc-morinon the levels of Zincin pancreas of control and experimentalgroups of rats.Values are expressed as mean ± SD for groups of six rats in each.Statisticalsignificance was determined by one way ANOVA followedby post hoc test LSD. *p < 0.001; #p < 0.01; @p < 0.05.[a] compared with control; [b] compared with diabetic rats

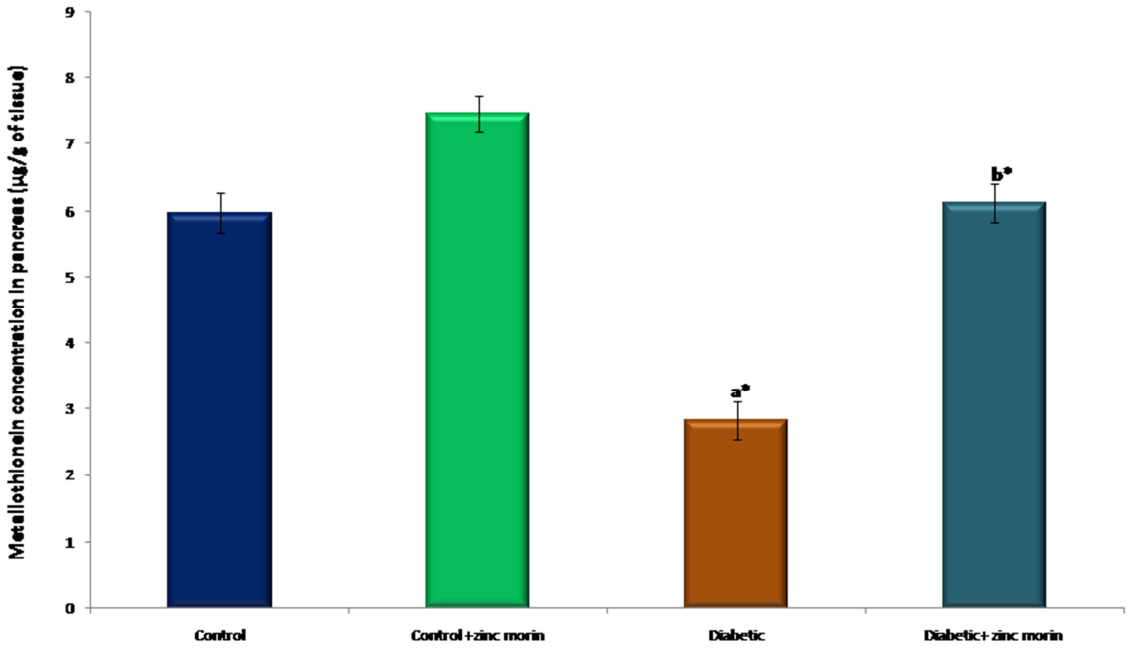

Figure 3

Fig 3: Effect of zinc-morinon the levels of Metallothionein in pancreas of control and experimentalgroups of rats.Values are expressed as mean ± SD for groups of six rats in each.Statisticalsignificance was determined by one way ANOVA followedby post hoc test LSD. *p < 0.001; #p < 0.01; @p < 0.05.[a] compared with control; [b] compared with diabetic rats

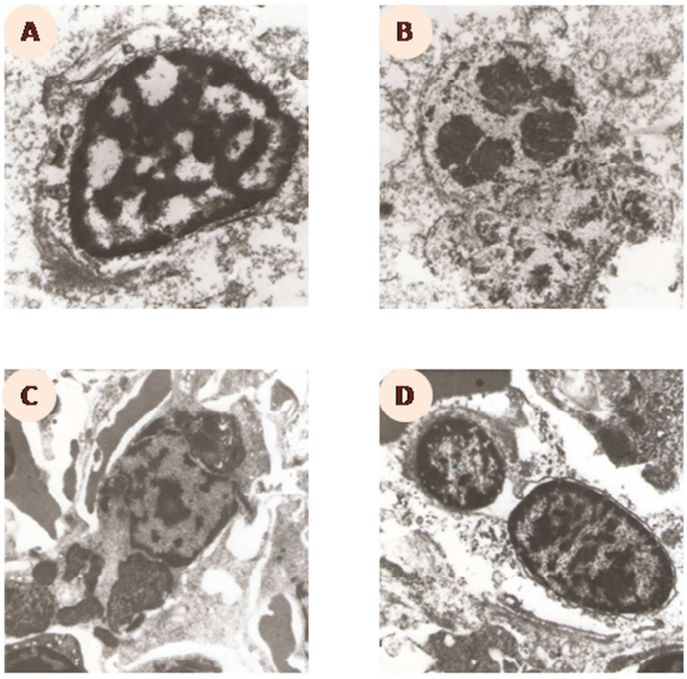

Figure 4

Fig 4 A-D: Transmission electron micrographs of (A) Control; (B) Drug Control (C) Diabetic Control (D) Diabetic + zinc-morin complex at 15,000x magnification

DISCUSSION

The HFD rat model with low dose of STZ has been known to induce insulin resistance and mild impairment of insulin secretion, which is similar to the clinical features of type 2 diabetes [28]. An interrelationship between diabetes and essential trace elements have been reported[29-31]. Reduction in the levels of some minerals has been associated with development of diabetes. Because of its enhancing or potentiating effect on insulin's activity, zinc is one of the mostly attributed minerals to diabetes [32]. The acceptance of zinc compounds as promising therapeutic antidiabetic agents has been slowed due to the chronic toxicity associated with zinc accumulation. In order to circumvent the toxic effects of zinc, we have previously studied a combinational approach wherein a zinc-flavonol complex was synthesized, characterized and its toxic as well as insulin sensitizing potential was evaluated in HFD-low dose STZ-induced experimental diabetes in rats [13].

In the present study, HFD-STZ induced diabetic rats showed a significant increase in the levels of blood glucose in diabetic rats indicating that insulin resistance has been established in HFD-STZ induced rats. Hyperglycemia is the essential feature of diabetes mellitus resulting in oxidative stress mediated tissue damage contributing to diabetes and its associated complications [33]. Oral administration of zinc-morincomplex improvesinsulin sensitivity, which is evident from the results offasting blood glucose, plasma insulin and HOMA-IR. Zinc-morin complex administration augments insulin stimulated glucose uptake into the peripheral tissues. The levels of plasma insulin were moderately decreased in HFD-STZ induced diabetic rats. Though the level was not decreased to a greater extent, the insulin level in HFD-STZ diabetic rats could not facilitate glucose uptake due to the insulin resistant state. There was a improved insulin sensitivity and a rise in insulin level in the complex treated diabetic ratssuggesting that zinc-morincomplex exhibit significant insulin sensitization potential as well as improvement in

the glucose homeostasis probably due to improved pancreatic β-cell function which is evident from improved plasma insulin level.

Reduced β-cell mass is a significant factor in diminishing insulin secretion in type 2 diabetes mellitus. The balance of cell proliferation and death through necrosis or apoptosis determines net β-cell mass. The present study showed that there was increased β-cell apoptosis and decreased β-cell proliferation in the HFD-STZ diabetic rats. Oral administration of zinc-morin complex to HFD- STZ induced diabetic rats resulted in the recovery of β-cell area. The zinc-morincomplex increases β-cell area by inducing β-cell proliferation and inhibiting cell apoptosis. Previous studies have reported that chronic hyperglycemia can induce β-cell apoptosis and that lipotoxicity may contribute to the β-cell death [34, 35].

Zinc homeostasis is maintained and regulated by zinc transport proteins and zinc binding proteins like Metallothionein (MT) [36]. Considering that MT displays antioxidant capacities and it seems to capable of preventing chemically induced diabetes in animals[37]. This observed effect of zinc-morinwas associated with increased levels of MT. Discussing the mechanism underlying protection against HFD-STZ by zinc-morin complex, we assumed that zinc induced MT provides defense against free radicals generated by HFD-STZ. This assumption is based on ROS which are increased during inflammatory reactions and participate in β cell destruction[38-40]. The observed increase in the levels of zinc and MT in the complex treated rats exemplifies the antioxidant potential of zinc-morin complex.

The function of pancreatic β-cells is limited due to its susceptibility and more sensitivity to the free radical toxicity and limiting competence for inactivation by the endogenous antioxidants [41]. Lipid peroxidation is used as an index of oxidative tissue mediated damage. The degree of tissue damage mediated by free radicals depends on the balance between free radical generation and the endogenous antioxidant defense mechanism[42]. The increase oxidative stress induces the peroxidation of polyunsaturated fatty acids and lead to the formation of TBARS and MDA, as byproducts of LPO. In the present study, the levels of oxidative stress markers such as lipid peroxides and hydroperoxides were increased significantly in pancreas of HFD-STZ group. In addition, the levels of activities of enzymatic antioxidants such as SOD, CAT, GPx, and GST were significantly improved upon treatment with zinc-morin complex. Treatment with the zinc-morin complex significantly declined the levels of these oxidative stress markers with improved antioxidant status indicating the antioxidant potential of the complex.

The ultrastructural observations made on the pancreatic tissues substantiate the claim that zinc complex protects the pancreatic β cells from hyperglycemia mediated oxidative stress. The amelioration of ultrastructural changes in the diabetic rats treated with the complex could be due to the antioxidant potential of the complex thereby protecting the pancreatic tissues. It has been reported that Zinc may counteract the deleterious effects of oxidative stress, which contribute to reduced insulin resistance, and mayalso protect pancreatic β cells from glucolipotoxicity [43].

Chronic inflammation is strongly associated with type 2 diabetes and insulin resistance [44]. Inflammation causes insulin resistance via inhibiting the signaling downstream of insulin receptor. Hyperglycemia mediated oxidative stress leads to the superactivation of stress-sensitive signaling pathways including NF-kB. The deterioration of pancreatic β-cells due to chronic oxidative stress results in the activation of transcription factor, NF-kB and release of the proinflammatory cytokines. Studies have suggested the role for pro-inflammatory cytokines in regulating insulin sensitivity. It has been reported that human with T2DM exhibited higher levels of pro-inflammatory cytokines such as TNF-α, IL-1 β, and IL-6 [45]. IL-1 β is an important cytokine mediator of pancreatic islet injury and is secreted by activated macrophages and neutrophils [46]. IL-1 β causes a decrease in glucose stimulated insulin biosynthesis and secretion and in high doses, apoptosis of the pancreatic islets. IL-1β also leads to down-regulation of GLUT2 and glucokinase mRNA expression, enzymes that transport and phosphorylate glucose in the pancreatic islet, respectively [47]. IL-1 β lead to cell membrane damage, DNA strand breaks, although this event may be mediated by NO [48]. Oral administration of zinc-morin complex to HFD-STZ induced diabetic rats decreases the levels of NF-kB, IL-1 β, and NO indicating the anti-inflammatory activity of zinc-morin complex.

CONCLUSION

In addition to the biochemical alterations, the results of the histological and ultrastructural observations of the pancreatic tissues revealed that zinc-morin complex acts as a potent antidiabetic agent by ameliorating the glucolipotoxicity induced oxidative stress in pancreas of HFD/STZ-induced diabetic rats.

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