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Induction of Heme Oxygenase-1 Improves Glucose Tolerance and Enhances Insulin Sensitivity in Obese Diabetic Rats

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Obesity is a worldwide problem. It is considered to be a major risk factor for many systemic diseases like type 2 diabetes mellitus (T2D), cardiovascular, renal and orthopedic diseases. Recent evidence has underscored the emerging role of the heme oxygenase system in many diseases. The present study was designed to investigate the effects of induction of the HO system by hemin on glucose tolerance and insulin sensitivity in obese diabetic albino rats as an experimental model of T2D.

Methodology: 18 male albino rats were divided into three groups: 1- Control group: fed standard pellet chow diet and injected with the vehicle. 2- T2D group: animals were fed high fat diet (HFD) and received single i.p. injection of STZ (35 mg/kg) for induction of diabetes. 3- T2D+Hemin treated group: animals were fed HFD Then, animals received single i.p. injection of STZ (35 mg/kg) for induction of T2D. Hemin (15 mg/kg i.p.) was given twice weekly for 4 weeks after induction of diabetes. Pancreatic tissues and serum were collected and evaluated by Histopathological and biochemical assay for histopatological lesions and biochemical parameters (blood glucose level,

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insulin level and sensitivity, hemoxygenase activity, lipid peroxides, TNF-α and total anti-oxidant capacity).

Results: Pancreatic tissues of diabetic group show severe inflammation with loss of pancreatic architecture, interstitial edema, and marked vacuolization. Hemin therapy preserves pancreatic architecture and reduced the inflammatory lesions and greatly reduced vacuolization in diabetic rats. Hemin treatment decreased the elevated glucose level in diabetic rats from 37.75±2.37 to 22.23±1.69 (mmol/L), lipid peroxides decreased from 22.23±1.69 to1.85±0.24 (µmol/L), TNF- α decreased from 58.95±5.29 to 35.17±3.52 (pg/ml). On the hand hemin treatment increased the total antioxidant activity in diabetic rats from 22.38±2.75 to 70.33±5.29 (µm/mg) and increased insulin level from 3.16±0.48 in diabetic group to 9.21±0.8 (µU/L), hemin treatment also enhanced insulin sensitivity.

Conclusion: The results of the present study demonstrated that treatment with hemin induced protection against obesity induced type 2 diabetes most probably by its antioxidant effect and its effect on insulin production and insulin sensitivity. These findings are likely to motivate further research and indicate new approaches for treatment of diabetes.

Keywords: High fat diet; obesity; type 2 diabetes; hemin; hemoxygenase; insulin sensitivity.

1. INTRODUCTION

Diabetes mellitus is one of the chronic diseases that have a progressively increasing incidence rate all over the world [1]. The pathogenesis of diabetes on the body results from disturbed carbohydrate, protein and fat metabolism secondary to decreased insulin secretion or insulin action on different tissues. Two types of diabetes are known based on the cause and age of onset, Type 1 diabetes (T1D) (juvenile onset) and type 2 diabetes (T2D) (maturity onset). T2D had a wider prevalence and accounts for nearly 90% of diagnosed cases of diabetes mellitus [2,3,4] in the developed countries [5].

Obesity is one of the major health problems. The prevalence of obesity is increasing at an alarming rate in many parts of the world. Obesity increases the risk for several clinical conditions, including cardiovascular diseases, renal diseases and insulin resistant T2D [6].

A common characteristic for both type 1 and type 2 diabetes is the persistently elevated plasma glucose level; although they have different etiologies. T1D is said to be due to relative or absolute lack of insulin production while Increased insulin resistance is reported by many reports to be the underlying cause of T2D; although other reports indicate also that insulin resistance is not the only cause for T2D and that decreased number of pancreatic beta cell with islet dysfunction can contribute also to the etiology of T2D [7,8].

Hyperglycemia is considered as one of the stressors which increase the formation of oxidative metabolic end products and promotes intense inflammatory reactions in different tissues including pancreatic tissues [9,10]. Hyperglycemia can lead to oxidative damage of cells of pancreatic acini with decreased number of acinar cells with resultant decreased production of insulin [11]. So exploring new approaches that can decrease oxidative stress and suppress the inflammatory processes can help maintaining normal cell structure and function of pancreatic islet cells that would preserve insulin production which would be of great beneficial effect in the course of treatment of T2D.

The important role of hemoxygenase (HO) system in many disease mechanisms such as cardiovascular, renal diseases and diabetes had been highlighted by several studies [12,13,14]. The HO enzyme is an intracellular enzyme present in 2 isoforms, the inducible (HO-1) and constitutive (HO-2) isoforms [15,16]. Recent evidences suggested the presence of certain motifs for inflammatory/oxidative transcription factors like NF-kB, AP-1, AP-2, and a motif for glucocorticoid-responsive elements in the HO-1 gene promoter [17,18], suggesting important regulatory role for HO-1 in many cellular activities including cellular defense and glucose metabolism [19,20].

Both HO-1 and HO-2 catalyze a similar biochemical reaction by acting on heme which leads to the generation of equimolar amounts of biliverdin, free iron and carbon monoxide (CO) [21]. CO is known to be an activator of soluble guanylate cyclase with potential antiapoptotic effect and bilirubin act as an antioxidant *in vivo*

and in vitro [22,23,24], while the iron helps the formation of the of the antioxidant, ferritin [25,26].

HO-2 is stimulated by normal physiological processes [27,28]. On the other hand many agents that induce HO-1 activity are associated with oxidative stress and inflammation [29,30] as well as conditions of disturbed homeostasis such as hyperglycemia, hypertension and hyperlipidemias [21,31,32]. This reflects the fact that HO-1 is a rate limiting step regulating many pathophysiological pathways in our body.

Pathophysiological stimuli inside the body that are capable for activating HO-1 result in only moderate increase in HO-1 level that is not enough to stimulate downstream signaling pathway to keep cellular homeostasis. In order to obtain a more powerful induction of HO-1, certain pharmacological agents can be used for this aim, like hemin, copper protoporphyrin, cobalt protoporphyrin or stannous mesoporphyrin [31,33].

Giving all the above mentioned facts it appears that disturbed body homeostasis that may occurs in certain diseases such as hypertension, diabetes and hyperlipidemia can induce HO-1 activation that could be a target for new approaches in the way of treatment of such diseases. Therefore, the present study was designed to investigate the effects of induction of the HO system by hemin on glucose tolerance and insulin sensitivity in obese diabetic albino rats as an experimental model of T2D.

2. MATERIALS AND METHODS

2.1 Animals

Eighteen male albino rats weighing about (100-150 g) were procured from the central animal facility of the Institute. Animals were housed at room temperature with normal light/dark cycles. All rats were provided with commercially available rat normal pellet diet and water *ad libitum*, animals were left to acclimatize for one week prior to the experimental manipulation. Principles of laboratory animal care were followed, and the experimental procedures used in this study were approved by the Animal Care and Use Committee of Faculty of Medicine, Minia University, Egypt.

2.2 Diets Protocol

- ***Standard diet (SD)***

SD consisted of 16% protein, 60% carbohydrates, 3% fat, 5% minerals and ash, and 12% moisture. This was stored at ambient temperature.

- ***High fat diet (HFD)***

HFD was composed of high fat (26.9%), high simple carbohydrate (18.6%), normal protein (15.9%) and low minerals (2.1%). This was stored at 4℃. Feeding rats this high fat diet is a well characterized model that results in hyperglycaemia, hyperinsulinemia, insulin resistance, defective islet compensation, and impaired glucose tolerance [34,35,36].

2.3 Method of Induction of Type 2 DM (T2D)

Animals were fed high fat diet HFD for the initial period of 2 weeks. After 2 weeks of dietary manipulation, animals were given single intra-peritoneal (i.p.) injection of freshly prepared Streptozotocin (STZ) solution (in 0.1 M citrate buffer, pH 4.5), at a dose of 35 mg/kg body weight. The diabetic state was ascertained by monitoring blood glucose levels using a standard Randox glucose kit. Blood glucose levels above 200 mg/dl were considered diabetic and were selected for further histological and biochemical analysis [37].

2.4 Experimental Design

Animals were randomly divided into the following experimental groups (n = 6):

1. **Control group:** Animals were fed standard diet for the whole period of experiment, they acted as normal untreated control and received vehicle only.
2. **T2D group:** Animals were fed HFD for 6 weeks. On the 2nd week, animals received single i.p. injection of STZ (35 mg/kg) for induction of T2D. These animals were sacrificed 4 weeks after STZ administration.
3. **T2D+Hemin treated group:** animals were fed HFD for 2 weeks. Then, animals received single i.p. injection of STZ (35 mg/kg) for induction of T2D. Hemin (15

mg/kg i.p.) [38,39] was given twice weekly for 4 weeks after induction of diabetes.

At the end of the experiment, animals were decapitated and plasma was collected for further analysis. The pancreas was also taken for histological analysis.

2.5 Chemicals and Drug Preparations

Streptozotocin (STZ) and hemin were purchased from Sigma chemical company, St Louis, MO, USA. All other chemicals and solvents used in the study were of analytical grade and were obtained from Sigma chemical company unless otherwise mentioned.

Hemin was dissolved in 0.1 M NaOH, titrated to pH 7.4 with 0.1 M HCl, and diluted 1:10 with phosphate buffer as previously reported [38,39]. For the control group, animals received the vehicle used to dissolve hemin. Each injection was 0.5 ml and was given twice weekly for 4 weeks.

2.6 Histological Examination of Pancreas

Pancreatic tissue obtained from duodenal and splenic lobes were fixed in 10% formalin, processed, and paraffin embedded; then sections 5 µm thickness were cut and stained with hematoxylin and eosin for histological analysis. Whole pancreatic sections were examined by a pathologist for acinar cell necrosis, vacuolization, interstitial edema, fibrosis, and mononuclear cell infiltration.

2.7 Biochemical Assay

2.7.1 Determination of HO activity

HO activity was measured as bilirubin production as previously reported [38,39].

2.7.2 Determination of glucose, insulin and insulin resistance by homeostasis model assessment (HOMA) of insulin resistance

Plasma glucose level was determined using enzymatic colorimetric method (Spectrum, Egyptian Company for Biotechnology (S.A.E), Egypt) [40]. It depends on the enzymatic oxidation of glucose in the presence of glucose oxidase forming hydrogen peroxide which further reacts under catalysis of peroxidase with phenol and 4-aminoantipyrine to form a red violet quinoneimine dye the intensity of which can be measured against a reagent blank at 546 nm.

Plasma insulin level was determined by Insulin Enzyme-Linked Immunosorbent assay (ELISA) [41] following the manufacturer's instructions.

Homeostasis model assessment of insulin resistance (HOMA-IR) analysis was used to assess insulin resistance. Values for HOMA-IR were calculated according to the following formula [42];

Fasting plasma glucose (mg/dl) X fasting insulin (µU/ml) / 22.5

2.7.3 Determination of lipid peroxides as markers of oxidative stress

Plasma malondialdehyde (MDA) level, as an indicator of lipid peroxidation, was determined by colorimetric method using a commercially available kit (Biodiagnostic, Egypt) as previously described [43] and based on the reaction of thiobarbituric acid (TBA) with MDA in acidic medium at temperature of 95℃ for 30 min to form TBA reactive product, the absorbance of the resultant pink product can be measured at 534 nm using a spectrophotometer.

2.7.4 Determination of plasma total antioxidant capacity

Plasma total antioxidant capacity was measured by colorimetric method using commercially available kit (Biodiagnostic, Egypt) according to the manufacturer's instructions.

2.7.5 Determination of plasma tumor necrosis factor-α (TNF α)

Plasma concentration of TNFα was measured by using rat TNFα ELISA kit (Biosource, USA) according to the manufacturer's protocol.

2.8 Statistical Analysis

Data were represented as mean ± standard error of the mean (M ± SEM). Statistical analysis was performed using Graph pad Prism 5 software and significance difference between groups was done by one-way ANOVA with a P value of ≤0.05 was considered statistically significant.

3. RESULTS

3.1 Hemin Therapy Reduced Hyperglycaemia

4 weeks of Hemin therapy reduced plasma glucose level (mmol/L) in diabetic rats. Fig. 1 shows that in T2D group there is about 4 fold increases in blood glucose level 37.75±2.37 compared to control group 8.03±0.91. Hemin treatment significantly reduced this hyperglycaemia 22.23±1.69.

3.2 Hemin Therapy Increased Plasma Insulin Level

Fig. 2 shows the effect of Hemin treatment on plasma insulin level (µU/L) of diabetic rats. Hemin therapy significantly increased plasma insulin in diabetic rats from 3.16±0.48 to 9.21±0.8 that approaches normal control value 10.37±0.99.

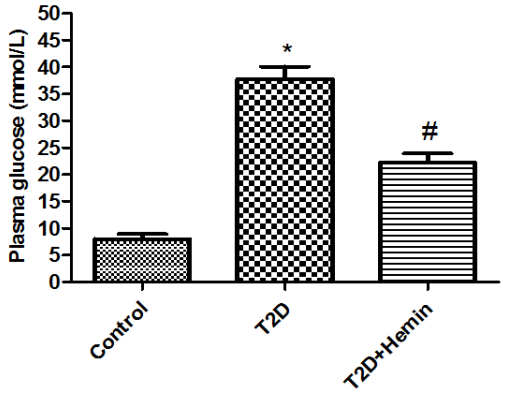

Fig. 1. Effect of Hemin treatment on plasma glucose level

**: Significant from control group #: significant from diabetic untreated group*

3.3 Hemin Therapy Reduced Insulin Resistance

HOMA-IR analysis was performed to detect the effect of Hemin treatment on insulin sensitivity. Fig. 3 demonstrates that Hemin treatment significantly reduced insulin resistance in Hemin treated diabetic rats from 10.28±0.74 to 6.05±0.66 to nearly control values 5.46±0.67.

3.4 Hemin Therapy Potentiated HO Activity

Effect of Hemin therapy on HO activity was measured as an increase in bilirubin level (mmol/L). In Fig. 4 Hemin treatment significantly increased plasma bilirubin level 12.88±0.98 compared to control 9.91±0.72 and T2D group 4.9±.41.

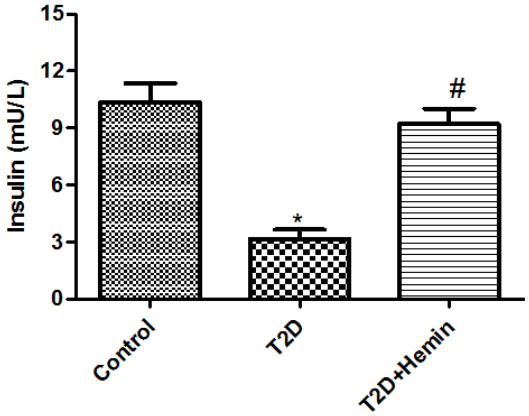

Fig. 2. Effect of Hemin treatment on Plasma insulin level

**: Significant from control group #: significant from diabetic untreated group*

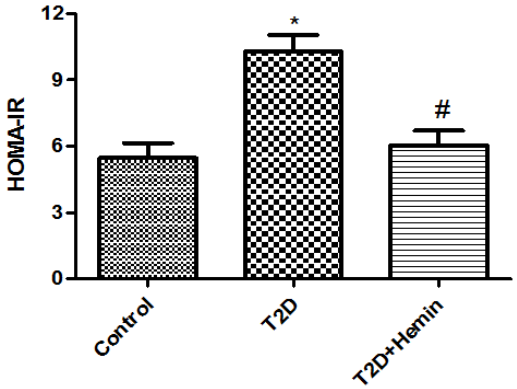

Fig. 3. Effect of Hemin treatment on HOMA-IR

**: Significant from control group #: significant from diabetic untreated group*

3.5 Hemin Therapy Decreased the Elevated MDA Levels

Hyperglycaemia is reported as a major inducer for oxidative stress so we investigated the effects of Hemin treatment on lipid peroxidation products (MDA) (µmol/L). The level of MDA was markedly elevated in T2D group 22.23±1.69 compared to control group 0.54±0.08. Hemin therapy reduced the elevated MDA level 1.85±0.24 as compared to T2D group (Fig. 5).

3.6 Hemin Therapy Decreased the Elevated Basal Levels of TNF-α

Our results indicate that the basal level of TNF-α (inflammatory cytokine) (pg/ml) in T2D group was

significantly elevated 58.95±5.29 as compared to control group 20.05±2.77. Treatment with Hemin attenuated TNF-α elevated level 35.17±3.52 (Fig. 6).

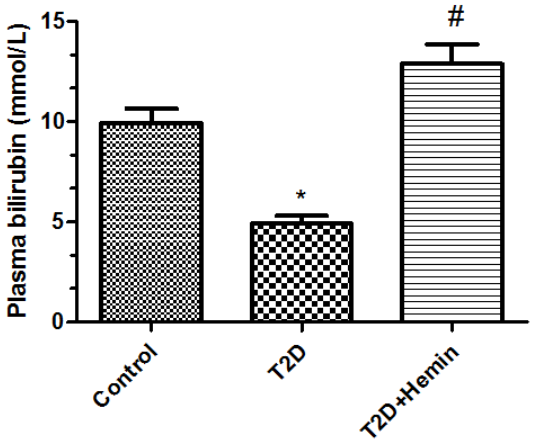

Fig. 4. Effect of Hemin treatment on HO activity

**: Significant from control group #: significant from diabetic untreated group*

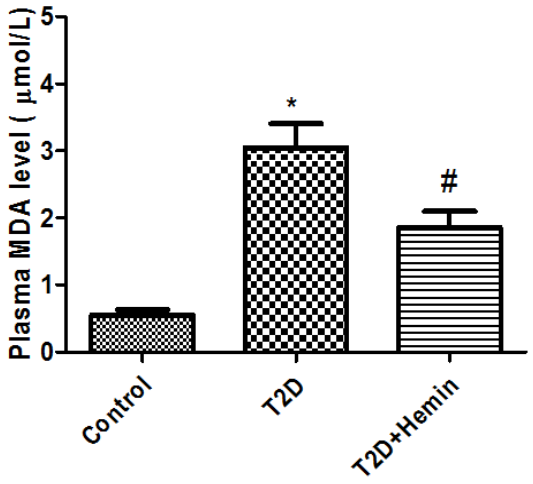

Fig. 5. Effect of Hemin treatment on MDA level

**: Significant from control group #: significant from diabetic untreated group*

3.7 Hemin Therapy Increased Total Antioxidant Capacity

The HO system is known to enhance the antioxidant system; we measured the total antioxidant capacity. The plasma TAC level (µm/mg) was significantly depressed in T2D 22.38±2.75 compared to control group 55.45±4.42 (Fig. 7). Hemin therapy greatly increased TAC levels even higher than in the control group 70.33±5.29.

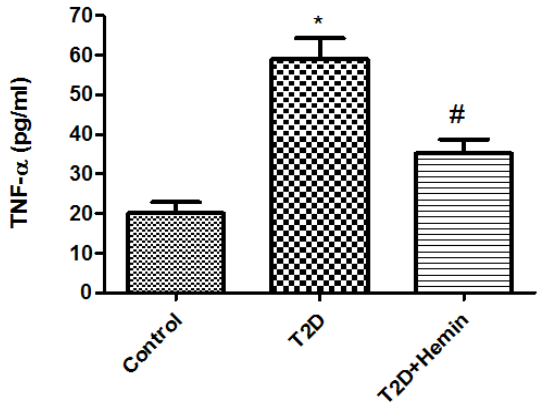

Fig. 6. Effect of Hemin treatment on TNF-alpha

**: Significant from control group #: significant from diabetic untreated group*

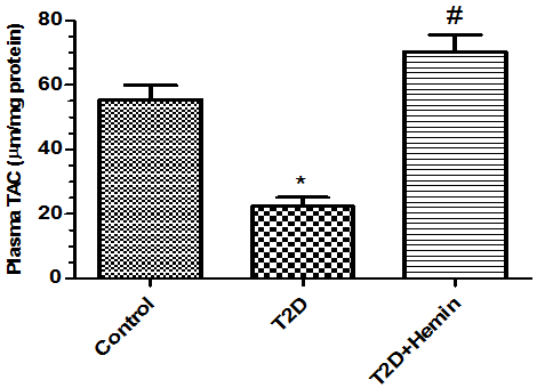

Fig. 7. Effect of Hemin treatment on total antioxidant capacity

**: Significant from control group #: significant from diabetic untreated group*

3.8 Hemin Therapy Reduced Histopathological Lesions of Pancreas

Severe inflammation was evident in diabetic rats with loss of pancreatic architecture, there were significant elevation of mononuclear cell infiltration, increased interstitial edema, and marked vacuolization. Hemin therapy preserves pancreatic architecture and reduced the inflammatory lesions and greatly reduced vacuolization in diabetic rats (Fig. 8).

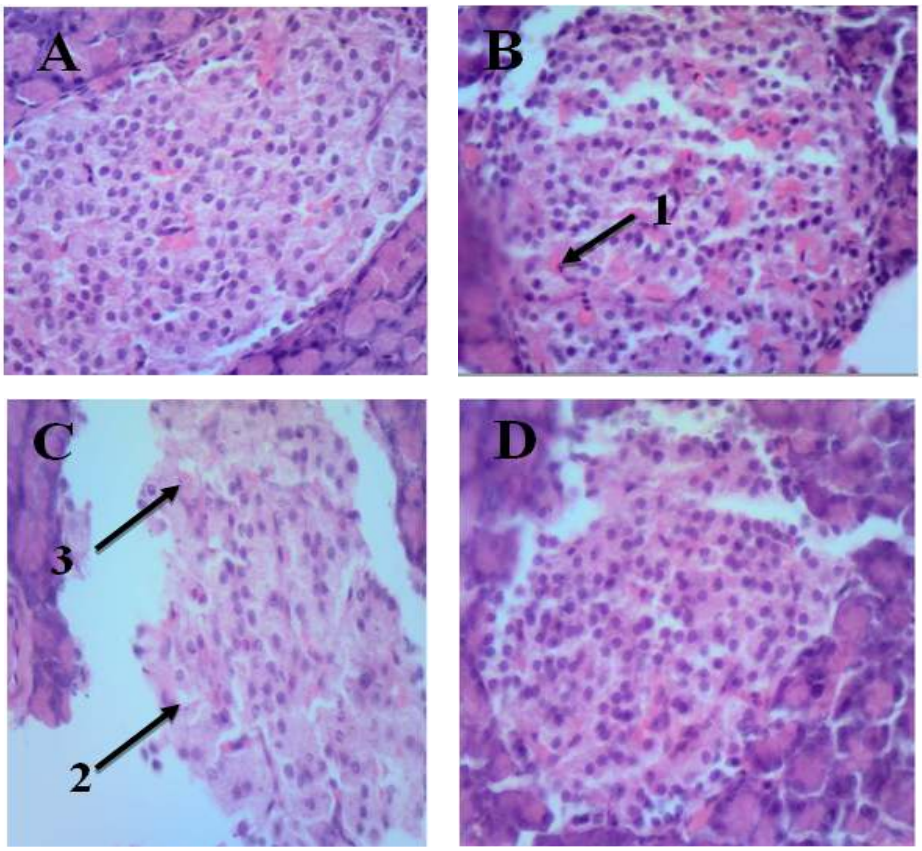

Fig. 8. (H&E x400): Panel (A) shows a photomicrograph of normal islets. The exocrine portion consists of pancreatic acini while the endocrine part consists of anastomosing cords of cells with fenestrated capillaries. Panel B&C: show photomicrographs of a diabetic rat pancreas showing many eosinophilic cells with small dark nuclei (arrow 1), enlarged beta cells with faint vacuolated cytoplasm (arrows 2) and. Loss of architecture with decrease number of cells is also present (arrow 3). Panel (D): Hemin treated pancreas shows restoration of architecture of pancreatic islets with nearly normal density of cells (number), Decrease number of eosinophilic cells with less vaccuolations

4. DISCUSSION

The present study demonstrates the obvious antidiabetic effect of hemin against hyperglycemia in Type 2 diabetic rats. The mechanisms involved include upregulation of the HO system, along with enhancement of the total antioxidant capacity. Correspondingly, markers of oxidative injury like MDA levels were reduced. Hemin treatment also improved pancreatic tissue morphology in the form of restoration of normal pancreatic islets architecture, decreased number of eosinophilic cells with less cellular vaccuolations.

The concomitant improvement of pancreatic morphology along with the higher insulin levels reflects increased insulin production [44,45]. Interestingly, the increased insulin production in hemin-treated animals was accompanied by a concomitant and paradoxical enhancement of insulin sensitization which was confirmed by the lower insulin resistance (HOMA-IR index) in hemin treated animals.

Hemin therapy enhanced HO activity in diabetic rats significantly. The enhancement obtained in diabetic group was higher than the level observed in the control group suggesting that more stimulation for HO system occurs in disease states to act as an intrinsic homeostatic and safeguard mechanism to fight against injurious insults. Also the concomitant increase in insulin secretion and insulin sensitivity suggest that the HO pathway in diabetes is very sensitive to pharmacological manipulation and hemin could have specific effects in diabetes.

The grater enhancement of HO activity observed in diabetic rats is consistent with previous reports that showed upregulation of the HO system in diabetes [46]. This greater enhancement of HO activity may be needed to initiate stronger signaling pathway to restore homeostatic state in diabetic rats [47].

The disturbed homeostasis that occurs with hyperglycemia in diabetes is a potent stimulant for oxidative stress [48,49]. The suppression of TNF-α was accompanied by corresponding reduction of markers of oxidative stress like MDA. Importantly, the hemin-dependent attenuation of oxidative stress could be linked to concomitant enhancement of antioxidants, including SOD, bilirubin, and ferritin, with subsequent potentiation of the total antioxidant status in T2D rats which was also confirmed by the results of the present study.

In tissues, the antioxidant system comprises different antioxidants such as SOD, catalase, glutathione peroxidase, ferritin, ascorbic acid, tocopherol, carotene, reduced glutathione, uric acid, biliverdin, bilirubin, etc [25,26,50,51,52], and the sum of endogenous and food-derived antioxidants represents the total antioxidant activity of the system [51]. The additive effect of all the different antioxidants provides greater protection against oxidative stress than any single compound alone. Therefore, in hemin-treated T2D rats, reduced oxidative stress coupled to enhanced antioxidant status may have led to improved insulin sensitivity observed in the present study.

The role of the HO system in the regulation of insulin release has been widely accepted [19,46,53]. Depressed HO status was detected in pancreatic islets of Goto-Kakizaki rats [46]. The defective HO system was corrected by hemin or CO, suggesting an important role of these substances in glucose metabolism [19,54]. Moreover, pancreatic cells produce CO to regulate insulin and glucagon secretion and thus glucose metabolism [19]. Similarly, glucose was shown to stimulate the production of CO by pancreatic islets, which in turn triggered insulin release [19], and to protect/preserve cell vitality [55].

Although several studies indicate that the HO system increases insulin levels [19,46,53], some conflicting observations have been reported. A recent study showed that the HO inducer CoPP did not increase insulin levels in obese diabetic mice [14]. Although the reason for this discrepancy remains unclear, further investigations are needed to verify whether the different effects of hemin and CoPP on insulin are strain dependent and/or specific to the type of HO inducers.

Previous studies showed that, high glucose levels are among one of the different stimuli that can induce HO-1 [29,30]. Also the presence of certain motifs for inflammatory/oxidative transcription factors like NF-kB, AP-1, AP-2, and a motif for glucocorticoid-responsive elements in the HO-1 gene promoter [17,18], suggest an important regulatory role for HO-1 in many cellular activities including defense and glucose metabolism [19,20]. However, further investigations are needed to clarify how HO-1 and the genes modified by it modulate insulin secretion and insulin sensitization and improve glucose metabolism in both type 1 and type 2 diabetes.

5. CONCLUSION

This study demonstrates a potent antidiabetic effect of hemin in obese diabetic rats as a model of T2D. Suggested mechanisms may include enhanced antioxidant status along with a decline in oxidative/inflammatory markers and subsequent improvement in pancreatic architecture in order to preserve the insulin-producing capability of β-cells. Correspondingly, increased insulin production alongside the paradoxical enhancement of insulin sensitivity could also be a contributing factor to the antidiabetic effect of HO-1 induction in such condition.

CONSENT

We did not use human subjects in this study so this section is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the Animal Care and Use Committee of Faculty of Medicine, Minia University, Egypt.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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