

## The ameliorative effect of Heme Oxygenase System on early Diabetic Nephropathy in Streptozotocin-Induced Diabetic Rats

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Received: Jul 21<sup>st</sup> 2014  
Accepted: Sept 25<sup>th</sup> 2014  
Available online: 1 Jan 2015

### Keywords

- Diabetic nephropathy
- Heme oxygenase
- Hemin
- Chromium
- Mesoporphyrin

### Abstract

**Background:** Diabetic nephropathy (DN) is one of the most common and severe microvascular complications of diabetes mellitus (DM) and the leading cause of end-stage renal disease. Although hemoxygenase (HO) is cytoprotective, the potential renoprotective mechanisms of HO-1 induction remain to be explored. **Aim:** The aim of this study is to evaluate the possible protective effect of HO induction on streptozotocin (STZ)-induced early DN and various mechanisms underlie this effect in rats. **Materials & methods:** Single intraperitoneal (i.p.) injection of STZ(60mg/kg) was administered to induce early DN in rats. Four weeks after STZ injection, the diabetic rats were divided into four groups (10 rats each), namely, STZ -diabetic, Hemin treated diabetic, Hemin and chromium-mesoporphyrin (CrMP) treated diabetic and CrMP treated diabetic group. The normal rats were chosen as control group. The diabetic rats received either hemin(15 mg/kg,ip) or CrMP(4  $\mu$ mol/kg ,ip) alone or combined treatment of both , twice weekly for 4 weeks. DN was assessed, eight weeks of STZ injection, by measuring plasma adiponectin, serum insulin, glucose and creatinine levels, renal hemoxygenase -1(HO-1)concentration , monocyte chemoattractant protein - 1(MCP-1),intercellular adhesion molecule - 1(ICAM-1), nuclear factor - Kappa beta(NF- $\kappa$ B), tumor necrosis factor - alpha (TNF- $\alpha$ ), hydroxyprolin, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and urinary albumin , collagen, creatinine and 8- isoprostane and 8- hydroxyguanosine (8-OHDG) levels.

**Results:** Hemin – induced HO upregulation, in STZ -diabetic rats suppressed the hyperglycemia with increased adiponectin and insulinotropic effect. Correspondingly, hemin reduced the markers of kidney dysfunction including albuminuria, collagen and creatinine excretion together with reduced serum creatinine level, suggesting improved kidney function. Similarly, hemin, suppressed renal proinflammatory chemokines, MCP-1, ICAM-1 with parallel reduction in renal proinflammatory cytokine TNF- $\alpha$  and the inflammatory/oxidative transcription factor, NF- $\kappa$ B, in STZ-diabetic rats. Moreover hemin therapy was associated with marked reduction in STZ induced elevation in renal fibrotic marker, hydroxyprolin. Furthermore, hemin significantly decreased the enhanced renal NADPH oxidase with parallel reduction of urinary 8- isoprostane and 8-OHDG, surrogate urinary markers of oxidative stress. Contrarily, coadministering the HO inhibitor, CrMP with the HO-inducer, hemin nullified the antidiabetic and renoprotective effects, whereas administering CrMP alone abrogated HO level, aggravated hyperglycemia, further increased renal MCP-1, ICAM-1, NF- $\kappa$ B, TNF- $\alpha$ , hydroxyprolin with deterioration of oxidative stress markers and exacerbating renal dysfunction in diabetic animals.

**CONCLUSION:** These findings suggest that the reduction of renal injury in diabetic rats upon induction of HO-1 was associated with decreased renal oxidative stress, fibrosis and inflammation, implicating the role of HO-1 induction as a future treatment of DN.

## INTRODUCTION

Diabetes mellitus is a multisystem disorder that affects various organs. Almost 30% of diabetic patients develop DN, despite control of blood glucose and/or blood pressure (1). The underlying molecular mechanisms of DN are not fully established. DN has been traditionally considered to be caused by metabolic and hemodynamic alterations, but recent studies suggest that inflammatory processes with chronic low grade inflammation and aberrant immune responses are also involved in the development and progression of the disease (2). Increased levels of some proinflammatory factors such as ICAM-1 and MCP-1 have been found in diabetic patients with nephropathy. MCP-1 and ICAM-1 have been identified as key players in monocyte/macrophage infiltration and leukocyte adhesion in diabetic animal models (3).

A common denominator of diabetes is the enhanced level of TNF- $\alpha$ , which has been largely implicated in the inflammatory cascade which in turn activates the NF- $\kappa$ B pathway, creating a vicious cycle that exacerbates diabetes and its renal complications (4).

Long-standing hyperglycemia, a common cardinal feature in diabetes, is known to stimulate renal cells to produce several humeral mediators and growth factors (5), that form complex cross-links over time and induce several structural alterations such as interstitial extracellular matrix (ECM) expansion, that is initially triggered by increased cellular components which is followed by an increase in interstitial fibrillary collagen, that appears to be critical for final progression of DN (6).

Increased oxidative stress has been implicated in the pathogenesis of diabetes. NADPH oxidase, the major source of superoxide production, has been shown to be activated in the kidney of diabetic animal models, with enhanced expression in the glomerulus and distal tubules, constituting a fundamental link between hyperglycemia and oxidative stress and ultimately leads to deteriorated function (7).

Heme oxygenase (HO) is a ubiquitous microsomal rate-limiting enzyme involved in the oxidative degradation of heme to biliverdin (BV), which is rapidly converted into bilirubin (BR) by biliverdin reductase. During this step, iron is released from the heme ring and carbon monoxide (CO) is generated. To date, three distinct isoforms of HO have been identified: HO-1, an inducible form; HO-2, a constitutive form; and HO-3, probably a pseudogene (8). Among these isoforms, HO-1 has been the most extensively studied HO isoenzyme, seems to provide higher cytoprotection and is known to be upregulated in the kidney under various physical, chemical, and pathophysiological stimuli including oxidative and inflammatory insults, as well as metabolic and hemodynamic factors such as high glucose, elevated blood pressure and lipids. HO-1 induction is considered to be an adaptive cellular response to stresses. Therefore, HO-1 may be considered a sensitive index that is triggered in the onset of pathophysiological changes. Since the pathophysiological activation of HO-1 may fall below the threshold necessary to activate important signaling components through which the HO system elicits its effects of restoring tissue homeostasis. This can be achieved by pharmacological agents capable of inducing HO

like some metalloprotoporphyrin such as hemin (ferric protoporphyrin IX chloride) (9).

The importance of HO-1 in dictating the outcome of many diseases are the observations that pharmacological induction or overexpression of HO-1, as well as administration of the different end-products of heme catabolism by HO-1, all have significant beneficial or therapeutic effects in a large number of pathologic conditions.

Although many therapeutic interventions have been shown to delay the development or retard the progression of DN, currently no intervention has been able to halt or reverse its progression (10). Therefore, better therapeutic modalities are urgently needed. The HO system may constitute a novel approach that could be explored against diabetes and its related renal and metabolic complications. This study highlights the potential therapeutic benefit of HO-1 induction, through hem into protect the kidney from diabetic renal injury.

### **Materials and Methods**

**Animals:** Fifty male albino rats weighing 150–180g were used after one week of proper acclimatization to the animal house conditions (12h lighting cycle and  $25 \pm 2$  °C temperature). The rats were housed four per cage, had free access to standard rodent chow and water. Procedures involving animals and their care were conducted in conformity with the protocols of the Research Advisory Ethical Committee of Faculty of Medicine, Tanta University, Egypt.

**Experimental Induction of Diabetes,** Diabetes was induced in overnight fasted rats by single i.p. injection of freshly prepared STZ (60mg/kg, dissolved in 0.1M cold citrate buffer; pH 4.5) (11). STZ was purchased from Sigma-Aldrich (St.

Louis, MO, USA). The STZ-treated animals were allowed to drink 5% glucose solution instead of drinking water for the first 24 h after STZ challenge to overcome initial drug-induced hypoglycemic mortality. Three days after STZ injection, blood samples were collected and blood glucose levels were measured using a glucometer (OneTouch Horizon, LifeScan, Johnson & Johnson, CA, USA). Animals with blood glucose above 250 mg/dL were used. After maintenance for four weeks, the blood and urine samples from these rats were again tested for hyperglycemia and proteinuria. The urine protein was estimated by Biuret's method using a commercial kit (Diamond Diagnostic, Egypt) (12). Rats with hyperglycemia ( $\geq 250$  mg/dl) and proteinuria ( $\geq 8.0$  mg/dl) at the end of four weeks post STZ injection were selected for further study.

**Experimental Procedures:** The diabetic rats were left untreated, for four weeks after induction of diabetes, to induce early DN (11), and were assigned to receive treatments with either hemin or CrMP or combined Hemin and CrMP. The animals were divided into 5 groups of 10 rats each, the control normal group: received i.p. injection of 0.1 mol/l citrate buffer, pH 4.5 (Vehicle for STZ), diabetic group (STZ injected and vehicle treated), Hemin treated diabetic group: Hemin (15 mg/kg ip, Sigma Aldrich, UT, USA) , Hemin and CrMP treated diabetic group: Hemin (15mg/kg ip) and CrMP (4  $\mu$ mol/kg ip, Sigma Aldrich, UT, USA) , and CrMP treated diabetic group (4  $\mu$ mol/kg ip, Sigma Aldrich, UT, USA). Both Hemin and CrMP were administered twice weekly for 4 weeks.

At the end of eight weeks after STZ injection, the rats were placed in metabolic cages (Nalgene Corp. Rochester, NY) for 24-hour urine collection

before they were killed. Blood samples were collected and centrifuged at 3000 g for 10 min to obtain clear sera. The kidneys of all rats were then immediately excised, decapsulated, and divided longitudinally into two equivalent sections. One section was retained with dissection of the renal cortices, that were snap frozen in liquid nitrogen, stored at  $-80^{\circ}\text{C}$ , and subsequently homogenized in cold potassium phosphate buffer (0.05M, pH 7.4) for various biochemical analyses. The protein content of the renal samples was measured by the method of Bradford (13), using crystalline BSA as standard.

**Assessment of STZ induced diabetic state:** Using commercially available colorimetric kits, serum glucose (Diamond Biodiagnostic Comp, Egypt) was estimated. Serum insulin was estimated using rat insulin enzyme-linked immunosorbent assay (ELISA) kit (American Diagnostica Inc., South San Francisco, CA, USA).

**Assessment of STZ induced DN: The extent of DN was assessed biochemically.** Serum and urinary creatinine levels were estimated by standard alkaline-picrate method using commercially available creatinine estimation kit (14) (Cayman Chemical, Ann Arbor, MI). Urinary albumin was measured by ELISA using microalbumin estimation kit (BioSystems, Spain), and collagen (Exocell, Philadelphia, PA) excretion levels were determined as indices of renal injury.

**Urinary 8-isoprostane and 8-OHdG assay.** Urinary 8-isoprostane, as a marker of lipid peroxidation, was estimated by ELISA according to the method of Milne et al., (15), using kit purchased from (Cayman chemical company, Ann Arbor, USA). 8-OHdG, marker of oxidative DNA damage, was estimated by the method described

by Kim et al., (16). The concentration of urinary 8-OHdG was calculated from the standard curve and divided by the urinary creatinine.

**Renal nuclear protein extraction and NF- $\kappa$ B p65 subunit assay:** Nuclear protein was isolated from the kidneys as described by Lee et al., and Zhou et al., (17,18). The nuclear level of p65 correlate positively with the activation of NF- $\kappa$ B pathway (19). The NF- $\kappa$ B/p65 ActivELISA (Imgenex, San Diego, CA) kit was used to quantify NF- $\kappa$ B free p65 in the nuclear fraction of kidney tissue homogenate. The analysis was done according to the manufacturer's instructions.

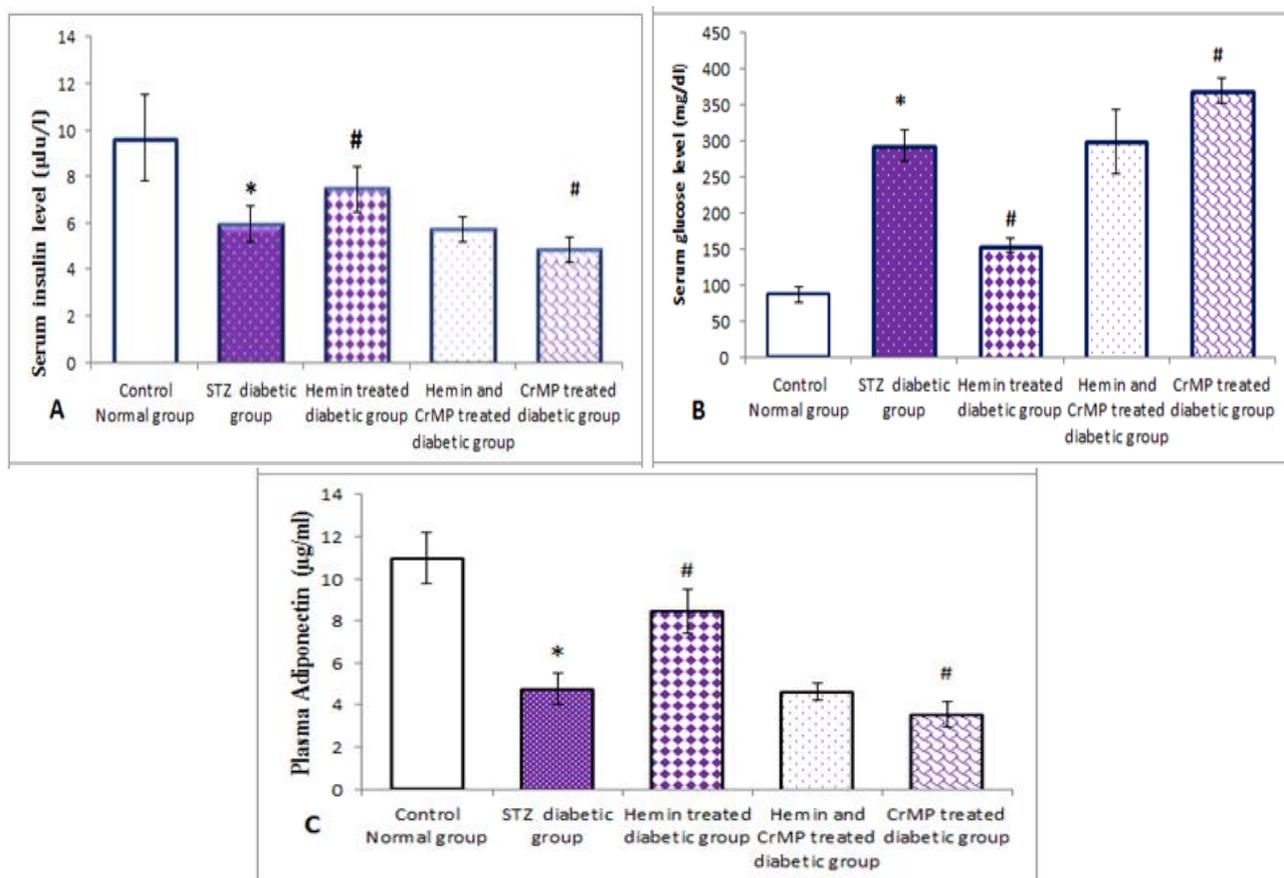
**Renal NADPH Oxidase and (TNF-  $\alpha$ ) activity Assay.** NADPH activity was measured in renal cortical samples as previously described (20). Average sample counts (cpm) were normalized to  $\mu\text{g}$  protein. Renal (TNF-  $\alpha$ ) assay was performed with rat TNF- $\alpha$  ELISA kit (Ray- Biotech, Inc., GA, USA) according to supplier's instructions.

**Renal HO-1 and hydroxyproline levels Assay:** HO-1 concentration was measured in renal cortical samples using a commercially available ELISA according to manufacturer's instructions (Enzo Life Sciences Inc., Farmingdale, NY). Hydroxyproline level, as an index of basement membrane collagen, was measured according to the method described by Woessner (21).

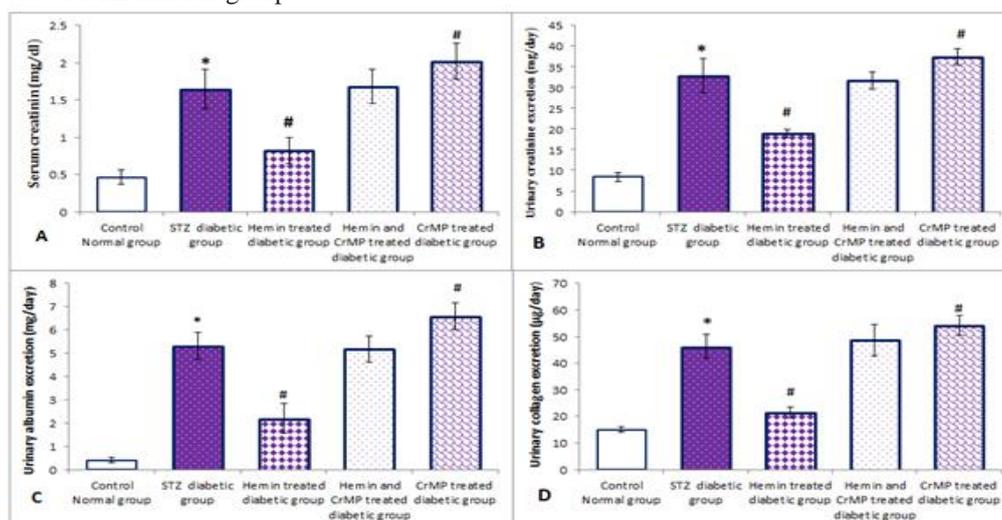
**Renal cortical MCP-1 and sICAM-1 Assay:** Renal cortical MCP-1 levels were assessed using a commercially available ELISA according to manufacturer's instructions (BD Biosciences, Bedford, MA) (22). Renal soluble ICAM-1 levels were also determined using a commercially available ELISA according to manufacturer's instructions (R&D Systems, Minneapolis, MN) (23).

**Statistical Analysis:** The data are expressed as mean  $\pm$  standard deviation (SD). Statistical analysis was performed using unpaired t test and one-way

ANOVA for multiple comparisons with  $P < 0.05$  being considered as statistically significant.



**Fig. 1:** Effects of the HO inducer, hemin and HO inhibitor, CrMP on serum insulin (A) and glucose (B) and plasma adiponectin (C) levels in STZ – induced early DN in rats. Data are mean  $\pm$  SD of 10 rats. \* $P < 0.05$  vs control normal group; # $P < 0.05$  vs STZ diabetic group



**Fig. 2:** Effects of the HO inducer, hemin and HO inhibitor, CrMP on renal Functions, Serum creatinine (A), urinary creatinine (B), albuminuria (C) and collagen excretion (D) in STZ – induced early DN in rats. Data are mean  $\pm$  SD of 10 rats. \* $P < 0.05$  vs control normal group; # $P < 0.05$  vs STZ diabetic group.

## Results

### *Effect of hemin therapy on serum insulin, glucose and adiponectin levels in STZ diabetic rats.*

Induction of diabetes with STZ, resulted in marked hyperglycemia ( $292.243 \pm 21.288$  vs  $86.717 \pm 11.288$  mg/dl) associated with significant reduction of both adiponectin ( $4.761 \pm 0.748$  vs  $10.973 \pm 1.203$   $\mu$ g/ml) and insulin levels ( $5.943 \pm 0.753$  vs  $9.644 \pm 1.861$   $\mu$ Iu/l) in control normal group (figure 1). Hemin therapy of STZ diabetic rats resulted in significant lowering in fasting glucose level ( $154.253 \pm 9.851$  mg/dl,) coincided with increased levels of plasma adiponectin ( $8.468 \pm 1.042$   $\mu$ g/ml) and insulin ( $7.449 \pm 0.988$   $\mu$ Iu/l), while coapplication of Ho inhibitor, CrMP with hemin obliterated the hemin effects, to be similar to STZ diabetic group, whereas CrMP therapy alone exacerbated hyperglycemia ( $369.197 \pm 17.967$  mg/dl), further reduced adiponectin ( $3.564 \pm 0.6239$ ) and insulin ( $4.822 \pm 0.558$   $\mu$ Iu/l) levels compared to STZ diabetic.

### *Effect of hemin therapy on renal functions in STZ diabetic rats*

Serum and urinary creatinine, albumin and collagen excretions were measured to assess renal functions and injury in DN. Eight weeks after induction of diabetes with STZ, the diabetic rats exhibited marked deterioration in renal functions, as indicated by significant elevation in serum creatinine level ( $1.648 \pm 0.262$  vs  $0.462 \pm 0.097$  mg/dl) associated with increased creatinine excretion ( $32.889 \pm 4.149$  vs  $8.433 \pm 1.123$  mg/day) together with marked albuminuria ( $5.304 \pm 0.578$  VS  $0.402 \pm 0.122$  mg/day) and

marked increase in collagen excretion ( $46.276 \pm 4.642$  VS  $14.939 \pm 0.977$   $\mu$ g/day) in control normal group (fig 2). Treatment with Ho inducer, hemin resulted in marked improvement in renal parameters as indicated by significant reduction in Serum creatinine ( $0.826 \pm 0.174$  mg/dl) and urinary creatinine ( $18.919 \pm 0.978$  mg/day), albumin ( $2.202 \pm 0.635$  mg/day) and collagen excretion ( $21.467 \pm 1.898$   $\mu$ g/day) compared to STZ diabetic animals, while concomitant treatment with both hemin and HO inhibitor, CrMP blunted the hemin effects and CrMP alone further deteriorating the renal parameters.

### *Effect of hemin therapy on renal MCP-1, ICAM-1, TNF- $\alpha$ and NF- $\kappa$ B in STZ diabetic rats*

We investigated the effects of enhancing HO system, through hemin therapy, on these inflammatory markers, in STZ-diabetic rats. As shown in Fig 3, the STZ diabetic rats exhibited significant increase in the renal levels of ICAM-1 ( $21.066 \pm 3.208$  vs  $6.575 \pm 0.8779$  ng/mg protein), MCP-1 ( $136.947 \pm 8.315$  vs  $88.312 \pm 7.730$  pg/mg protein) and TNF- $\alpha$  ( $550.859 \pm 85.199$  vs  $143.18 \pm 15.751$  pg/mg protein) in control normal group. Interestingly, the administration of hemin therapy to STZ diabetic rats significantly abated the elevated renal ICAM-1 level ( $15.31 \pm 1.072$  ng/mg protein), MCP-1 ( $110.619 \pm 9.460$  pg/mg protein), associated with marked reduction in renal TNF- $\alpha$  ( $283.896$  ,pg/mg protein), whereas coadministration of hemin and CrMP to diabetic rats nullified the hemin induced effects, while CrMP treatment of diabetic rats alone further deteriorated these proinflammatory markers.

we further investigated whether the inhibitory effects of Ho upregulation in diabetic kidney,

through hemin, on renal MCP-1 and ICAM-1 levels are through the inhibition of NF- $\kappa$ B activation. As shown in figure (3B), STZ induced diabetes was associated with marked increase in renal NF- $\kappa$ B compared to normal control group. Hemin therapy markedly attenuated the elevated NF- $\kappa$ B in diabetic rats ( $27.79 \pm 5.067$  vs  $43.17 \pm 6.886$  ng/mg protein). On the other hand CrMP together with hemin administration to diabetic rats blunted the hemin effect, while CrMP alone exacerbated the elevated NF- $\kappa$ B in diabetic rats. These results suggest that inflammation is critically involved in the pathogenesis of DN and HO system could play a central role.

#### ***Effect of hemin therapy on renal NADPH Oxidase and Oxidative Stress***

Renal cortical NADPH oxidase activity was significantly elevated in STZ diabetic rats compared to control normal group (Fig 4(a)). The increased NADPH oxidase activity was also associated with elevation in the urinary oxidative stress markers, 8-isoprostane and 8-OHdG excretion levels in STZ diabetic versus control normal group (Fig4 (b) and 4(c)). Induction of HO-1 with hemin significantly inhibited NADPH oxidase activity and reduced urinary excretion levels of 8-isoprostane and 8-OHdG in diabetic rats by 1.4, 1.5 and 1.7 fold respectively. Whereas coapplication of hemin and CrMP abolished the hemin effects and restored similar levels of these

oxidative stress markers as in STZ diabetic group. On the other hand, treatment of diabetic animals with CrMP alone resulted in further accentuation in renal NADPH oxidase, urinary 8-isoprostane and 8-OHdG by 1.2, 1.1 and 1.2 fold compared to STZ diabetic animals, suggesting that induction of HO-1 act as antioxidant in diabetic kidney.

***Effect of hemin renal Ho-1 level in STZ-induced diabetic rats*** As shown in (fig 5), our results demonstrated that STZ-induced upregulation of HO-1 activity in diabetic kidney by 1.2-fold compared to control normal group. Hemin therapy induced further upregulation of HO-1 activity by 1.6-fold, while CrMP induced reduction in HO-1 activity by 1.7-fold compared to diabetic group. Hemin and CrMP coapplication abolished hemin effects.

#### ***Effects of hemin on STZ-induced renal hydroxyproline level***

The renal hydroxyproline level in STZ induced diabetic rats was significantly higher compared to control normal group (Fig6). Induction of HO-1 with hemin markedly reduced the elevated renal hydroxyproline content by about 1.6-fold, an effect which is reversed by CrMP treatment and blunted when hemin combined with CrMP, to be comparable to that of diabetic group. The results suggest that HO upregulation could efficiently prevent renal fibrosis in diabetic rats.

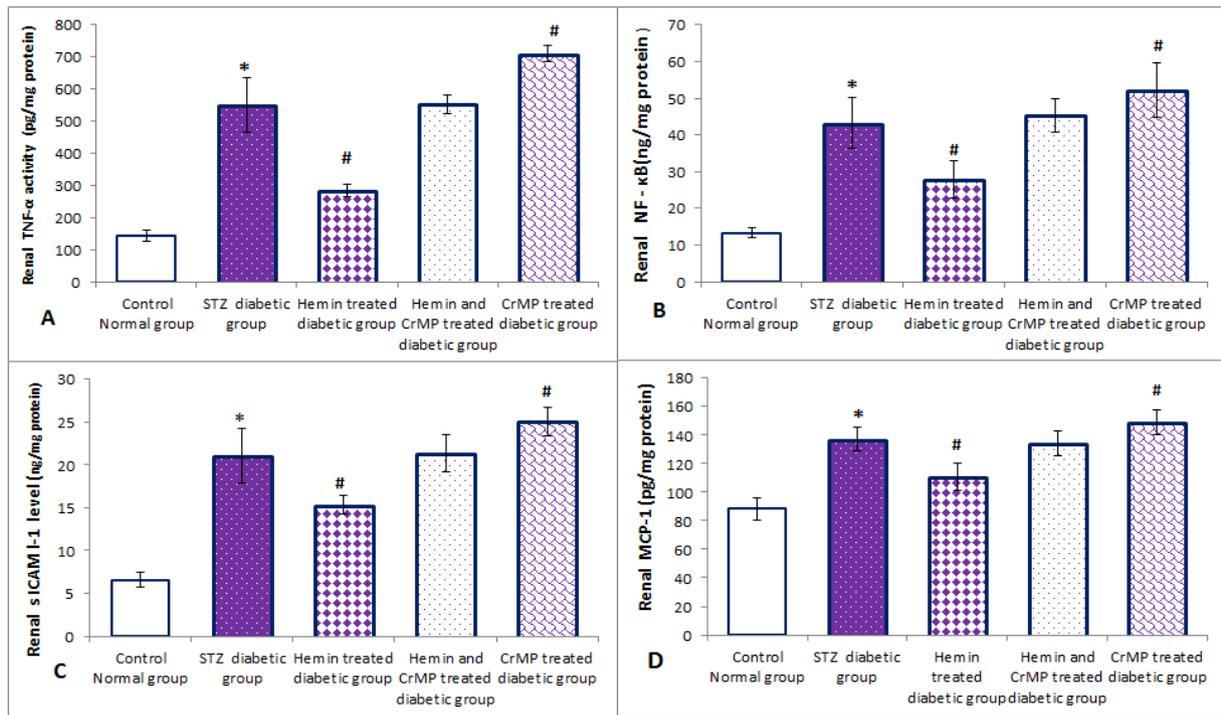


Fig.3: Effects of the HO inducer, hemin and HO inhibitor, CrMP on renal proinflammatory makers, renal TNF- $\alpha$  (A), NF- $\kappa$ B (B), s ICAM-1 (C) and MCP-1 (D) in STZ – induced early DN in rats . Data are mean  $\pm$  SD of 10 rats. \*P < 0.05 vs control normal group; #P < 0.05 vs STZ diabetic group

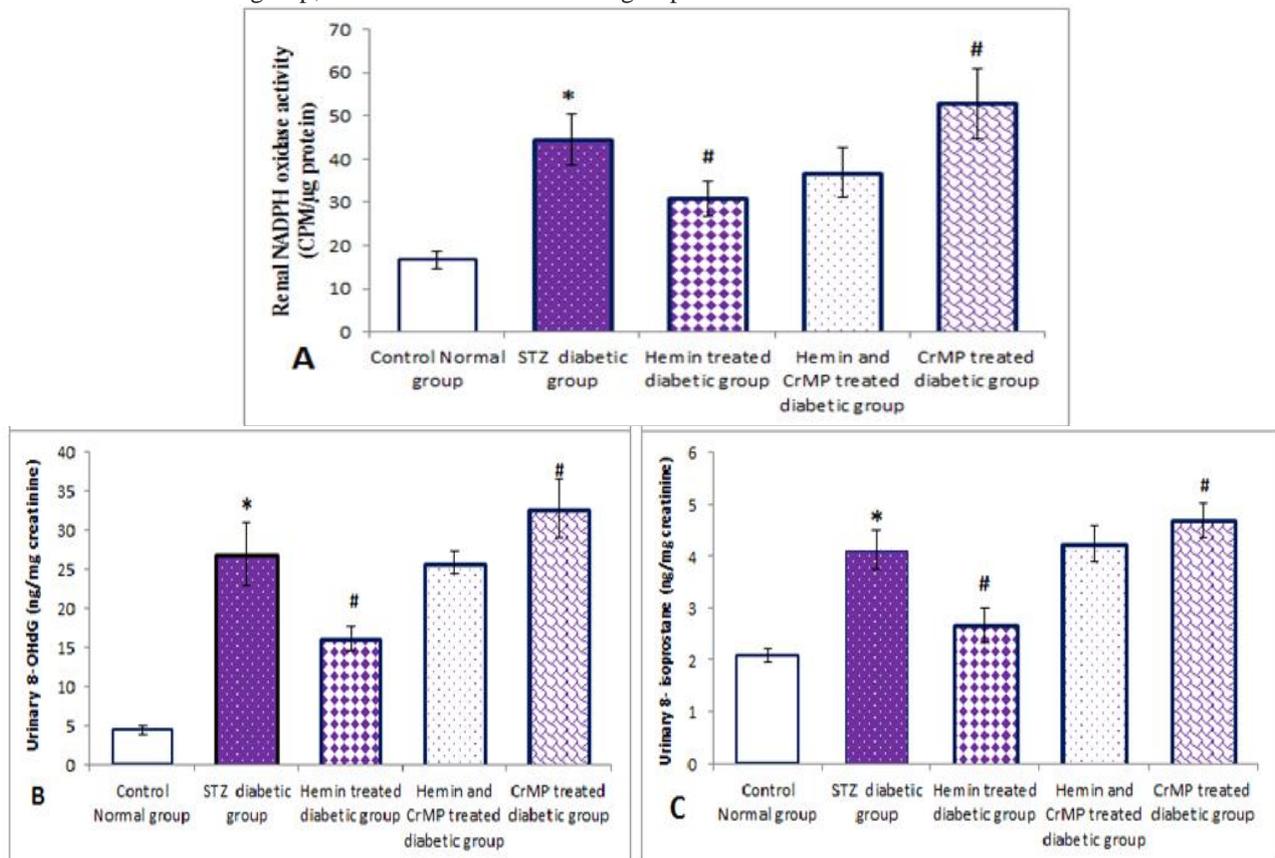


Fig. 4: Effects of the HO inducer, hemin and HO inhibitor, CrMP on renal oxidative stress markers, renal NADPH oxidase activity(A), urinary 8- isoprostane(B) and urinary 8- OHdG (C) in STZ – induced early DN in rats. Data are mean  $\pm$  SD of 10 rats. \*P < 0.05 vs control normal group; #P < 0.05 vs STZ diabetic group.

### Discussion

In the present study, we validated the efficacy of HO-1 induction in treatment of DN and the blockade of its associated signaling pathways underlying the pathophysiology of DN.

Many studies have underscored the cytoprotective effects of the HO system. The mechanisms underlying the renoprotective effect of hemin in diabetic rats are complex, challenging and not fully understood. Substantial evidences indicate that HO-1 provides the provenance for pathways that can interrupt virtually all major mechanisms of diabetic kidney injury, including reduction of the high blood glucose, that represent central core of DN.

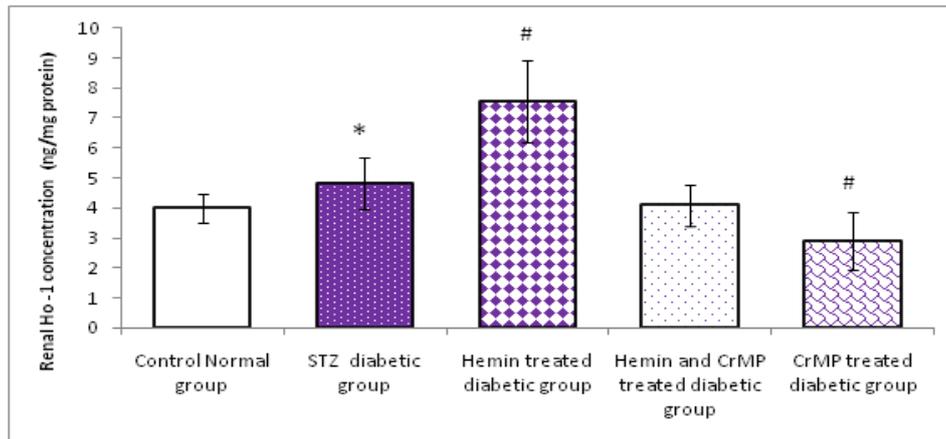
The results obtained in our study proved the antidiabetic role of Ho induction in STZ-induced diabetes (Fig1), as evidenced by significant insulinotropic effect, that was associated with lowering of glucose and improved adiponectin levels. Our data are consistent with those previously confirmed in different diabetic models, including nonobese Goto-Kakizaki (GK) rats (24) and Zucker diabetic fatty rats (25), a genetically obese leptin receptor-deficient model (26), and in STZ-induced diabetic model (27).

Several possible mechanisms have been postulated to explain antidiabetic effect of HO induction. Donget al (28), demonstrated that BR administration improved hyperglycemia and obesity by increasing insulin sensitivity. It has been widely reported that HO upregulation has been proved to mediate insulin release by pancreatic cells, possibly through CO (29), or the abating c-jun N-terminal kinase (JNK) activity, which has been implicated in blocking insulin biosynthesis (30), conferring a protective effect.

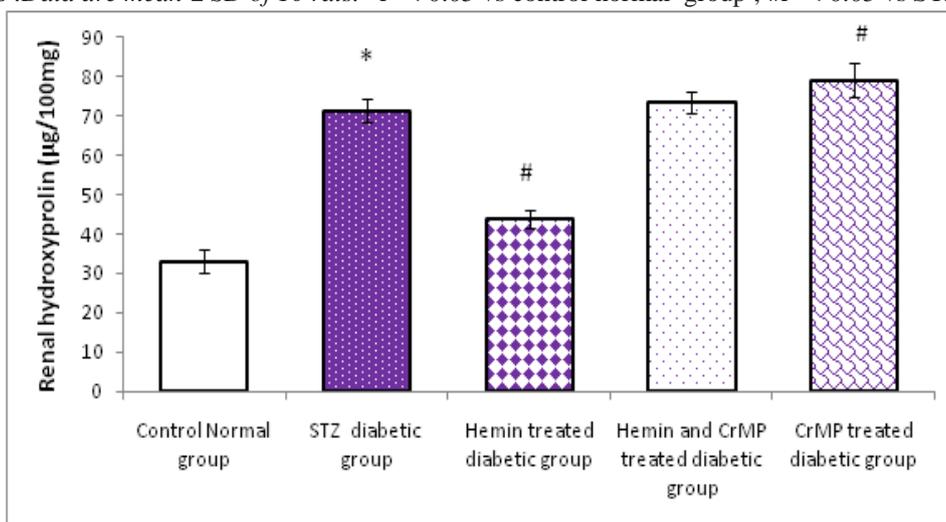
The reduced hyperglycemia observed in hemin treated diabetic rats could be attributed to the hemin-mediated increase of insulin and adiponectin (fig 1), which would greatly improve glucose metabolism, through enhanced adenosine monophosphate-activated protein kinase (AMPK) - dependent glucose transporter 4 (GLUT4) expression and translocation (31) with concomitant potentiation of insulin-sensitizing pathways as cyclic guanosine monophosphate (cGMP), cyclic adenosine monophosphate (cAMP), and activate peroxisome proliferator-activated receptor alpha (PPAR- $\alpha$ ) in the liver, alongside decreased oxidative stress and inflammatory markers (32).

Beyond glucose-lowering and insulin-sensitizing effects of adiponectin that have been observed both in humans and animal diabetic models. It has been proved that adiponectin has antifibrotic effect, at least in part, through antagonizing the effects of Ang II or TGF- $\beta$ , two key profibrotic factors in DN, anti-inflammatory effect via inhibiting the activation of ERK1/2 and NF- $\kappa$ B-dependent pathways, and vasoprotective effect in DN (33).

The concept that DN is essentially a micro-inflammatory disease and the role of microinflammation-mediated activation of intrinsic immune system has been recently emerging, where inflammatory cascade is triggered by the interactions between various chemokines secreted from resident glomerular cells such as (MCP)-1 and adhesion molecules such as (ICAM)-1, leading to the underlying pathological changes in DN (34). This observation



**Fig. 5:** Effects of the HO inducer, hemin and HO inhibitor, CrMP on renal Ho -1 concentration , in STZ – induced early DN in rats .Data are mean  $\pm$  SD of 10 rats. \* $P < 0.05$  vs control normal group ; # $P < 0.05$  vs STZ diabetic group.



**Fig. 6:** Effects of the HO inducer, hemin and HO inhibitor, CrMP on renal hydroxyprolin level, in STZ – induced early DN in rats. Data are mean  $\pm$  SD of 10 rats. \* $P < 0.05$  vs control normalgroup; # $P < 0.05$  vs STZ diabetic group

has been confirmed using the ICAM-1 and MCP-1 gene knockout diabetic mice, both exhibited markedly reduced kidney monocytes/macrophages accumulation, that was associated with lowered albuminuria and marked attenuation of renal injury(35).

Our findings indicated that, MCP-1 and ICAM-1 levels were significantly increased in the kidneys of diabetic rats compared with control group with marked reduction in their levels under hemin therapy(fig 3), which may explain, at least in part, the beneficial effect of hemin on DN.

In agreement with our results, Pan Y et al (36), observed that ICAM-1, and MCP-1

expressions were increased in experimental diabetic renal injury, which were associated with inflammatory cell infiltration and adhesion, and renal fibrosis.

Supporting the role of NF- $\kappa$ B activation and inflammation in DN, we found increased renal NF- $\kappa$ B activity and TNF- $\alpha$  level in diabetic rats. This is consistent with previous reports indicated that the expression of NF- $\kappa$ B was significantly increased in the renal tissues of STZ-induced DN rats, and positively correlated with renal interstitial MCP-1 and ICAM-1 protein and mRNA expression and proteinuria (37).

The availability of binding sites for NF- $\kappa$ B on both MCP-1(38) and the HO-1 genes promoter suggests greater interaction between this transcription factor and the HO system and inflammation(39). Supportive of this notion our results of reduced NF- $\kappa$ B level in hemin-treated animals, that was increased with blockade of the HO system, with subsequent elevated inflammatory markers. This is consistent with previous reports indicating that HO-1 induction produced a reduction of NF- $\kappa$ B induced inflammation in GK and STZ-induced diabetic rats (24,27).

The abrogated renal inflammatory markers observed in diabetic rats under hemin therapy (fig 3) might be mediated by HO- induced inhibition of NF- $\kappa$ B activation. It has been previously demonstrated that NF- $\kappa$ B blockage could be a candidate way of prevention of diabetic renal damage based on ablating the NF- $\kappa$ B induced underlying inflammatory and oxidative stress in DN (40).

There were a supporting lines of evidences, *in vivo*(34)and *in vitro*( 41), suggesting that the pathological actions of MCP-1 in DN could exceed its role in macrophage recruitment to the possibility that MCP-1 could directly promote damage in intrinsic kidney cells or induce additional responses such as macrophage activation or the recruitment and activation of T cells.

TNF- $\alpha$  is cytotoxic to glomerular, mesangial and epithelial cells and competent to induce direct renal injury through the generation of reactive free radicals, through c-Src/NADPH oxidase, which in turn initiates NF- $\kappa$ B activation (42).

Recently, several potential mechanisms have been described the metabolic effects of TNF- $\alpha$ . TNF- $\alpha$

initiates the activation of caspase 8 via its binding to the death receptor, TNF-R1, resulting in apoptosis (43).Moreover TNF- $\alpha$  stimulates serine kinases, such as c-Jun NH2-terminal kinase(JNK) and p38 mitogen-activated protein kinase (MAPK), results in serine phosphorylation of IRS-1 and IRS-2, which in turn reduces the downstream insulin signaling (44). Moreover, TNF- $\alpha$  induces insulin resistance by reducing the expression and translocation of (GLUT4) by inhibiting the phosphatidylinositol 3 kinase/protein kinase B(P13K/Akt) and peroxisome proliferator-activated receptor- $\gamma$ (PPAR- $\gamma$ ) signaling (45).So it is tempting to speculate that the HO mediated suppression of TNF- $\alpha$  and NF- $\kappa$ B constitutes not only an important anti-inflammatory mechanism to limit tissue insult in DN but also a mechanism that could be explored to improve insulin sensitivity and glucose metabolism.

The anti-inflammatory effect of HO-1 has been also highlighted in HO-1 deficient human cases and HO-1- knockout mice, where both exhibiting a pro-inflammatory phenotype, with overexpression of several pro-inflammatory mediators, including (MCP-1), (ICAM-1) and (NF- $\kappa$ B) activation (46).

The anti-inflammatory mechanisms of HO-1 generate considerable research activity but remain unclear, although animal model clues exist, such as a relationship between HO-1 and cytokines, where HO upregulation has been reported to modulate the inflammatory response by inhibiting the production of the inflammatory markers such as MCP-1or TNF- $\alpha$  by monocyte and macrophage(47),or inhibiting the expression of adhesion molecules, such as (ICAM-1), *in vitro* and *in vivo*, (48) that could be mediated by depleting heme, which has several pro-

inflammatory activities (49), generating antioxidants (i.e., BV=BR), or Possibly to be CO mediated(50). Confirming these observations, Rucker et al.,(51) have shown selective inhibition of HO-1 activity aggravated expression of ICAM-1, that was reversed by HO upregulation.

HO upregulation capable of selectively modulating the polarization of macrophages towards the anti-inflammatory M2-phenotype, which suppress the release of pro-inflammatory mediators and provoke the formation of anti-inflammatory mediators, such as IL-10, and concomitantly reducing the pro-inflammatory M1-phenotype and its related secretagogues like TNF- $\alpha$ , MCP-1, suggesting another anti-inflammatory mechanism of the HO-system, as proved previously, *in vitro*, by Weis N et al., (52).

We observed a significant increase in the levels of renal hydroxyproline content in STZ-induced diabetic rats; which in turn increases the severity of the kidney lesions and fibrosis in experimental animals, as reflected from deteriorated renal functions. Our data consistent with those reported in previous studies (6, 23).

Although the pathogenesis of tubular damage including tubulointerstitial fibrosis undoubtedly is multifactorial, it is well accepted that oxidative stress is a principal regulator, with activation of transcription factors and release of inflammatory markers that lead to excessive collagen deposition in the diabetic kidney due to disruption of the balance between processes of synthesis and degradation (53).

Hemin therapy, on the other hand, effectively diminished the renal hydroxyproline level, suggesting its anti-fibrotic efficacy, in diabetic condition. The anti-fibrogenic properties of HO-1

and its products have been extensively studied. Increased HO activity in human hepatic myofibroblasts correlates with decreased proliferation and procollagen I mRNA expression, which was attributed to BR(54), while HO-1 inhibition, in a rat hypoxia model, increased collagen (type I, type III) and TGF- $\beta$ 3 expression, an effect could be attributed to decreased CO level(55).

Our findings indicated that hemin induced HO upregulation not only plays a vital role in the modulation of the inflammatory process, but also has obvious antioxidant property thought decreased renal NADPH oxidase activity, the only mammalian enzyme dedicated to ROS generation (56), alongside with reduced urinary 8- isoprostane and 8-OHDG levels in diabetic rats.

Hyperglycemia is strongly associated with increased production of ROS, inducing DNA, proteins and lipids damage and triggering renal cell injury (7). Therefore, HO upregulation, through having both hypoglycemic and antioxidant properties might be considered a protective agent against DN.

The antioxidant effect of HO-1 has been confirmed by the phenotypic consequences of HO-1-deficient mice and a patient with HO-1 deficiency, while HO-1 up-regulation has been shown to decrease ROS and NADPH oxidase activity *in vitro* and *in vivo*, with BR and Co have been suggested as potential mediators and protect against oxidative stress-induced cell death (57). Datlaet al., (58) proved that up-regulation of HO-1 gene expression was shown to decrease the availability of the heme-containing gp91 subunit necessary for NADPH oxidase activity.

In the current study HO-1 decreased albuminuria in hemin treated diabetic rats. The decreased podocyte number in diabetic kidney has been identified as a leading cause of proteinuria in DN, with renal apoptosis has been implicated in podocyte loss(59).

Several studies have suggested that one possible pathway by which HO-1 confers protection in DN ,is via its ability to impart antiapoptotic activity, which is largely mediated by augmented iron efflux or CO (60), where HO induction upregulating the expression of the anti-apoptotic proteins p-AKT, Bcl-XL and p21(61) and decreased glomerular caspase 3 expression (62). At the same time HO appears to have a role in reducing the pro apoptotic effects of (TNF- $\alpha$ ), hyperglycemia, and iron. At the intracellular level, this may involve expression of MAPK enzymes and possibly the activation of NF -  $\kappa$ B(63).

Although several studies focused on HO-1 induction as a potential therapeutic target in DN, other works have directed attention to its product molecules as a protective strategy against injury. Increased HO-1 activity results in degradation of the heme moiety, a toxic prooxidant (64). Indeed, BV and BR can directly scavenge ROS and interact with the free radical NO and inhibits lipid peroxidation (65).In addition, ferritin is coinduced with HO-1, allowing safe sequestration of redox-active iron (66). Moreover, CO has vasodilatory effects mediated via cGMP and potassium channels (57).These signaling pathways exert broad-based, far ranging cellular effects, that may interact and be ultimately integrated in protecting renal damage in diabetes.

### **Conclusion**

The studies on HO-1 and DN, including those surveyed here, have demonstrated that Ho upregulation could effectively exert protective roles against STZ-induced early DN, Such protective effects were probably carried out through suppression of the underlying inflammatory , oxidative stress and fibrotic pathway with improved glycemic control ,which support a therapeutic potential of DN based on HO-1 induction , that may open the way to translate such potential into a therapeutic reality , with further investigation.

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تأثير نظام الهيم- أوكسيجينز على تحسن إعتلال الكلى المبكر لمرض السكري المستحدث بالستربتوزوتوسين

## في الفئران

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### الملخص العربي

**الخلفية العلمية والهدف من البحث:** يعد إعتلال الكلى السكري هو إحدى مضاعفات الأوعية الدموية الدقيقة الشديدة الخطورة و الأكثر شيوعاً لداء السكري ، و السبب الرئيسي للفشل الكلوي في المرحلة المتأخرة للمرض. و على الرغم من أن الهيم أوكسيجينز له أثر وقائي ، إلا أنه لا يزال يتعين استكشاف المزيد من الآليات المحتملة لحماية الكلى و الناتجة من تحفيز الهيم أوكسيجينز 1.

**الهدف من هذه الدراسة** هو تقييم التأثير الوقائي المحتمل الناتج من تحفيز الهيم أوكسيجينز على إعتلال الكلى المبكر لمرض السكري المستحدث بالستربتوزوتوسين و الآليات المختلفة الكامنة وراء هذا التأثير في الفئران.

**طرق البحث:** تم إستحداث إعتلال الكلى المبكر لداء السكري عن طريق حقن الستربتوزوتوسين بجرعة (٦٠مجم/كجم) مرة واحدة بالتجفيف البريتوني . بعد مرور أربعة أسابيع من الحقن ، تم تقسيم الفئران المصابة بالسكري إلى أربع مجموعات تحتوي كل منها على عشر فئران. وهي المجموعة المصابة بالسكري ، المجموعة المعالجة بالهيمين ، المجموعة المعالجة بالهيمين و الكروميوم ميزوبورفرين و المجموعة المعالجة بالكروميوم ميزوبورفرين ، وتم إختيار الفئران الطبيعية كمجموعة ضابطة. أما الفئران المصابة بالسكري فتم علاجها إما بالهيمين ( بجرعة ١٥ مجم/كجم من وزن الجسم بالحقن البريتوني) أو بالكروميوم ميزوبورفرين ( بجرعة ٤ ميكرو مول/كجم بالحقن البريتوني) أو كلاهما معاً ، مرتين أسبوعياً لمدة أربعة أسابيع . وبعد مرور ثمانية أسابيع من حقن الستربتوزوتوسين ، تم تقييم الإعتلال الكلوي السكري و ذلك بقياس مستوى الأديبونيكتين في البلازما ، الأنسولين ، الجلوكوز و الكرياتينين في الدم. و كذلك التركيز الكلوي لكل من الهيم أوكسيجينز 1 ، البروتين الكيميائي الجاذب للوحيدات- ١ ، جزيء الالتصاق ١ بين الخلايا، العامل النووي كابا بيتا، عامل نخر الورم ألفا، الهيدروكسي بروتين، والأنزيم المؤكسد لنيكوتيناميد الأدينين ثنائي النوكليوتيد فوسفات . كما تم قياس مستوى الزلال، الكولاجين، الكرياتينين، اللايزوبروستان ٨ و الهيدروكسي جوانوزين ٨ في البول.

**نتائج البحث:** أدى تحفيز الهيم أوكسيجينز عن طريق الهيمين في الفئران المصابة بالسكري إلى إنخفاض مستوى السكر في الدم و زيادة الأديبونيكتين و الأنسولين . ، كذلك أدى الهيمين إلى إنخفاض دلالات الخلل الوظيفي للكلية بما في ذلك إفراز الزلال ، و الكولاجين و الكرياتينين في البول فضلاً عن إنخفاض مستوى الكرياتينين في الدم وهو ما يشير إلى تحسن وظائف الكلى. و بالمثل أدى الهيمين إلى إنخفاض في دلالات الإلتهاب الكيميائية و الخلوية للكلية بما ذلك البروتين الكيميائي الجاذب للوحيدات- ١ ، جزيء الالتصاق ١ بين الخلايا، عامل نخر الورم ألفا العامل النووي كابا بيتا. فضلاً عن أن علاج- الفئران المصابة بالسكري - بالهيمين أدى إلى إنخفاض ملحوظ في الهيدروكسي بروتين ، وهو إحدى دلالات التليف الكلوي . علاوة على ذلك أدى الهيمين إلى إنخفاض ملحوظ في الأنزيم المؤكسد لنيكوتيناميد الأدينين ثنائي النوكليوتيد فوسفات بالكلية مع إنخفاض مواز لكل من اللايزوبروستان ٨ و الهيدروكسي جوانوزين ٨ في البول، و هما من دلالات زيادة الأوكسدة. وفي المقابل أدى إعطاء كل من- الكروميوم ميزوبورفرين (المثبط للهيم أوكسيجينز) و الهيمين - إلى إلغاء تأثير الهيمين الوقائي للكلية و المضاد لداء السكري . بينما أدى إعطاء الكروميوم ميزوبورفرين وحده إلى إنخفاض في مستوى الهيم أوكسيجينز مما أدى إلى تفاقم الإرتفاع في سكر الدم و مستوى البروتين الكيميائي الجاذب للوحيدات- ١ ، جزيء الالتصاق ١ بين الخلايا ، عامل نخر الورم ألفا ، العامل النووي كابا بيتا و الهيدروكسي بروتين في الكلى. مع تدهور في دلالات زيادة الأوكسدة و الخلل الوظيفي للكلية في الفئران المصابة بداء السكري.

**الإستنتاج:** وتشير هذه النتائج إلى أن الإنخفاض في إصابة الكلى في الفئران المصابة بداء السكري -عند تحفيز الهيم أوكسيجينز ١- كان مرتبطاً بإنخفاض في مستوى الأوكسدة، التليف و الإلتهاب بالكلية مما يشير إلى إمكانية إستخدام تحفيز الهيم أوكسيجينز ١ كعلاج محتمل لإعتلال الكلى المبكر لداء السكري في المستقبل .