Renoprotective Effect of Ace Inhibitor-Lisinopril and Heme Oxygenase-1 Inducer-Hemin Combination against Streptozotocin Induced Advanced Diabetic Nephropathy in Rats

1Yogesh Kumar Paliwal, 2Sidharth Mehan, 1Krishna Reddy V. Bijjem and 1Pyare I. Sharma
1Department of Pharmacology, ISF College of Pharmacy, Moga, Punjab, 142001, India
2Department of Pharmacology, Rajendra Institute of Technology and Sciences, Sirsa, Haryana, 125055, India

ABSTRACT
Background: Diabetic nephropathy is a microvascular complication of diabetes mellitus, characterized by glomerular hypertrophy, accumulation of extracellular matrix protein and vascular endothelial dysfunction, progressively leading to glomerulosclerosis, tubulointerstitial fibrosis and proteinuria. The present study investigated the combined effect of lisinopril (ACE inhibitor) and Hemin-Heme oxygenase (HO-1 inducer) in experimental model of advanced diabetic nephropathy. Materials and Methods: Streptozotocin (STZ) (50 mg kg⁻¹ i.p., once) was administered to induce diabetes mellitus. STZ-diabetic rat was subjected to renal ischemia of 30 min to induce advanced nephropathy assessed by measuring serum creatinine, Blood Urea Nitrogen (BUN) and proteinuria. In addition, dyslipidemia, renal oxidative stress, renal lipid profile and also Mean Arterial Blood Pressure (MABP) were noted. Results: Treatment with Hemin (5, 10 and 20 mg kg⁻¹ i.p.) dose-dependently and Lisinopril (1 and 5 mg kg⁻¹ p.o.) significantly but not dose-dependently, attenuated the elevated levels of creatinine, BUN, proteinuria and also improved the both serum and renal lipid profile. Moreover, combination of Lisinopril (1 mg kg⁻¹ p.o.) plus Hemin (5 mg kg⁻¹ i.p.); Lisinopril (5 mg kg⁻¹ p.o.) and Hemin (5 mg kg⁻¹ i.p.) and Lisinopril (5 mg kg⁻¹ p.o.) plus Hemin (20 mg kg⁻¹ i.p.); for 2 weeks was markedly attenuated the increased serum creatinine, BUN, proteinuria, renal oxidative stress, abnormal lipid profiles and MABP, as compared to high dose Lisinopril or Hemin treatment alone, in renal I/R subjected diabetic rat. Conclusion: Thus, it may be concluded that concurrent administration of Hemin and Lisinopril at different dose combination produced a synergistic ameliorative effects in the experimental model of severe diabetic nephropathy which may be due to more effective inhibition of serum and renal lipids, renal oxidative stress and MABP.

Key words: Diabetic nephropathy, streptozotocin, dyslipidemia, lisinopril, hemin

Pharmacologia 5 (2): 60-75, 2014

INTRODUCTION
Diabetic nephropathy, a condition of progressive damage of kidney, has become a major cause of end stage of renal disorders worldwide (Schiffrin et al., 2007; Atkins and Zimmet, 2010). Although, persistent hyperglycemia and hypertension has been implicated as a primary risk factors responsible for the progression of nephropathy (Phillips et al., 2001; Abrass, 2004; Giunti et al., 2006) but growing evidences clearly suggests that elevated circulating lipids may also contribute to renal disease progression in patient with diabetes mellitus (Abrass, 2004; Wang et al., 2005; Trevisan et al., 2006). Growing evidences have indicated that ROS plays a key intermediate role in the development of diabetic nephropathy (Kedziora-Kornatowska et al., 2000; Vasavada and Agarwal, 2005). Heme Oxygenase (HO) is a rate-limiting enzyme that catalyses the conversion of heme into biliverdin, carbon monoxide (CO) and iron (Tenhunen et al., 1979; Abraham and Kappas, 2008). Pharmacological induction or over expression of HO-1 has shown to produce anti-inflammatory, anti-oxidant, anti-apoptotic, anti-fibrotic and antihypertensive effects (Schaaf et al., 2002; Nath, 2006; Olszonecki et al., 2007). Nrf2, a Cap'n'Collar transcription factor, is primarily involved in the regulation of expression of HO-1 gene (Alam et al., 1999). Recently, in-vivo studies demonstrated that glomeruli of human diabetic nephropathy patients are associated with elevated Nrf2 levels. In addition, STZ-induced diabetic kidney obtained from Nrf2 (-/-) mice exhibits an higher ROS production and oxidative damage, along with severe renal injury as compared with Nrf2 (+/+) mice (Jiang et al., 2010). Hemin has been reported to selectively induce the expression and activity of HO (Zenke-Kawasaki et al., 2007). Where, hyperglycemia has been reported to repress the both
expression and activity of HO (Abraham et al., 2003; Quan et al., 2004a; Sacerdotti et al., 2005). Pharmacological upregulation of HO-1 by Hemin and cobalt protoporphyrin has shown to attenuate mercuric chloride-induced nephropathy (Yoneya et al., 2000), cationic bovine serum albumin induced membranous nephropathy (Wu et al., 2008). Clinically, renoprotective effect of Lisinopril (ACE inhibitor) has been well reported to decreased serum lipid levels and reduction in mean arterial blood pressure in diabetic patients (Tarnow et al., 2000; Ruggenenti et al., 2003; Paliwal et al., 2013). Treatment with either an ACE inhibitor or angiotensin (AT1) receptor blockers may only, effectively delay but not prevent the development of end-stage renal disease in most of the patients (Lewis et al., 1993; Amann et al., 2003), may due to failure of optimal inhibition of the RAAS (Sanowski, 2009). Up regulation of HO-1 has shown to prevent the Ang-II induced superoxide generation and ameliorated the Ang-II induced tubulointerstitial injury and hypertension (Quan et al., 2004b; Pradhan et al., 2006; Kelsen et al., 2008).

However, Lisinopril and Hemin produce their renoprotective action by different mechanisms; it is likely that their combination would result in renoprotective action. Thus, this study was designed to explore this hypothesis in experimental diabetic nephropathy produced by subjecting the renal ischemia of 30 min in STZ induced diabetic rats.

MATERIALS AND METHODS

Animals: The experimental protocol used in the present study was approved by the Institutional Animal Ethics Committee. Age matched young male wistar rats weighing 200-260 g were employed in the present study. Rats were fed on standard chow diet and water ad libitum. They were maintained as per the CPCSEA (India) guidelines for care and use of laboratory animals.

Experimental protocol: Male Wistar rats weighing approximately 200-260 g were rendered diabetic by a single injection of STZ (50 mg kg⁻¹ i.p.). The experimental rats were subsequently divided into 14 groups and each group contains six animals.

- Group 1: Sham control: Rat were maintained on standard food and water and no treatment was given
- Group 2: I/R control: Renal ischemia of 30 min was given in normal rats and these rats were maintained on standard food and water and no treatment was given
- Group 3: Lisinopril per se: The normal I/R subjected rats were administered Lisinopril (5 mg kg⁻¹ p.o.) for 2 weeks
- Group 4: Hemin per se: The normal I/R subjected rats were administered Hemin (20 mg kg⁻¹ i.p.) for 2 weeks
- Group 5: Diabetic control: Rats were administered STZ (50 mg kg⁻¹ i.p. once) dissolved in citrate buffer (pH 4.5)
- Group 6: Diabetic I/R control: Renal ischemia of 30 min was given to rats after 2 weeks of STZ administration to produce advanced diabetic nephropathy
  - Group 6A: Hemin treated: The I/R subjected diabetic rats were treated with Hemin (5 mg kg⁻¹ i.p.) for last 2 weeks
  - Group 6B: Hemin treated: The I/R subjected diabetic rats were treated with Hemin (10 mg kg⁻¹ i.p.) for last 2 weeks
  - Group 6C: Hemin treated: The I/R subjected diabetic rats were treated with Hemin (20 mg kg⁻¹ i.p.) for last 2 weeks
  - Group 6D: Lisinopril treated: The I/R subjected diabetic rats were treated with Lisinopril (1 mg kg⁻¹ p.o.) for last 2 weeks
  - Group 6E: Lisinopril treated: the I/R subjected diabetic rats were treated with Lisinopril (5 mg kg⁻¹ p.o.) for last 2 weeks
  - Group 6F: Lisinopril+Hemin treated: The I/R subjected diabetic rats were treated with Lisinopril (5 mg kg⁻¹, p.o.) and Hemin (5 mg kg⁻¹, i.p.) concurrently for 2 weeks
  - Group 6G: Lisinopril+Hemin treated: The I/R subjected diabetic rats were treated with Lisinopril (1 mg kg⁻¹, p.o.) and Hemin (5 mg kg⁻¹, i.p.) concurrently for 2 weeks
  - Group 6H: Lisinopril+Hemin treated: The I/R subjected diabetic rats were treated with Lisinopril (5 mg kg⁻¹, p.o.) and Hemin (20 mg kg⁻¹, i.p.) concurrently for 2 weeks

For the entire duration of the experiments the animals were kept in plastic cages and fed standard rat chow and water ad libitum.

Drugs and chemicals: Lisinopril was purchased from Ranbaxy Pvt. Ltd, Haryana, India. Hemin was purchased from Central Drug House (P) Ltd., Punjab, India. Streptozotocin (STZ) was purchased from Sigma Chemicals Co., St. Louis, USA. All other reagents in the study were of analytical grade from Loba Chemicals (P) Ltd., Mumbai, India.

Induction of advanced diabetic nephropathy in STZ-induced diabetic rats: The experimental diabetes mellitus was induced in rats by single injection of STZ
(50 mg kg\(^{-1}\) i.p.) dissolved in freshly prepared ice cold citrate buffer of pH 4.5. After 1 week of STZ administration animals having random serum glucose more than 240 mg dL\(^{-1}\) were considered as diabetic. To induce advanced nephropathy, diabetic rats after two weeks of STZ administration subjected to unilateral Renal Ischemia (RI) of 30 min duration after anesthetized with ketamine 70 mg kg\(^{-1}\) i.p. and diazepam (2 mg kg\(^{-1}\), i.p.). A flank incision was made and the left renal artery was located and dissected free from its surrounding structures. The renal ischemia was produced by clamping the renal artery for 30 min. The extent of diabetic nephropathy in both non-treated and treated animals was assessed after 4 weeks of renal ischemia in diabetic animals (Melin et al., 1997, 2002).

Assessment of STZ-induced diabetes mellitus and lipid profile: The blood sugar level was monitored once after one week of administration of STZ in select the diabetic animals. Then, at the end of experimental protocol (6 weeks after STZ administration), the blood samples were collected and serum was separated. The serum samples were frozen until analyzing the biochemical parameters. The serum glucos concentration was estimated by glucose oxidase-peroxidase (GOD-POD) method (Kinoshita et al., 1979; Lott and Turner, 1975) using commercially available kit (Coral clinical system, Goa, India). The total cholesterol was estimated by cholesterol oxidase peroxidase (CHOD-PAP) method (Allain et al., 1974) using commercially available kit (Coral clinical system, Goa, India). The serum triglyceride was estimated by glycerophosphate oxidase peroxidase (GOD-PAP) method (Kinoshita et al., 1979; Bucolo and David, 1973) using commercially available kit (Coral clinical system, Goa, India). The HDL was estimated by PEG (Polyethylene glycol) precipitation method (Allain et al., 1974) using commercially available kit (Coral clinical system, Goa, India).

Assessment of diabetic nephropathy: The extent of diabetic nephropathy was estimated biochemically by estimating BUN, serum creatinine and proteinuria in urine.

- **Estimation of BUN:** The BUN was estimated by Berthelot method (Fawcett and Scott, 1960) using the commercially available kit (Coral clinical system, Goa, India)

- **Estimation of serum creatinine:** The serum creatinine concentration was estimated by alkaline picrate method (Bonsnes and Taussky, 1945) using commercially available kit (Coral clinical system, Goa, India)

- **Estimation of proteins in urine:** The proteinuria was estimated by pyrogallol red method (Watanabe et al., 1986) using the commercially available kit (Coral clinical system, Goa, India). Thousand microliter of reagent (pyrogallol dye) was added to 10 μL of urine sample, 10 μL of standard protein and 10 μL of purified water to prepare test, standard and blank, respectively. All the test tubes were mixed and incubated for 10 min at 37°C. The absorbance of test and standard sample were measured against blank at 600 nm spectrophotometrically. When the pyrogallol red-molybedate complex binds to basic amino groups of protein molecules, there is a shift in reagent absorbance. The absorbance is directly proportional to protein concentration in the sample. The urinary protein was calculated using the following equation:

\[
\text{Urinary protein concentration (mg dL}^{-1}\text{)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 100 \\
\]

Total microprotein excreted (mg 24 h) = Urinary protein concentration (mg dL\(^{-1}\)) × 10 × 24 h urine volume in L

Assessment of renal oxidative stress: The development of oxidative stress in the kidney was assessed by estimating renal thiobarbituric acid reactive substances (TBARS) and reduced form glutathione content (GSH).

- **Preparation of renal homogenate:** The kidney was dissected and washed with ice cold isotonic saline and weighed. The kidney was then minced and a homogenate (10% w/v) was prepared in chilled 1.15% KCl. The homogenate was used for estimating TBARS, GSH and total protein

- **Estimation of TBARS:** The renal TBARS, an index of lipid peroxidation, was estimated according to the method described earlier (Ohkawa et al., 1979)

- **Estimation of reduced glutathione:** The GSH level in the kidney was estimated by the method as described earlier (Ellman, 1959)

- **Estimation of total protein:** The renal protein content was estimated by Lowry method (Lowry et al., 1951)

**Estimation of total renal cholesterol and renal triglycerides:** Total tissue lipid was extracted and washed with Folch wash reagent (Folch et al., 1957; Cho, 1983; Deepa and Varalakshmi, 2005). For determination of renal triglycerides and renal cholesterol, dried total lipid extract was dissolved in peroxide-free dioxane/isopropanol and aliquots were
taken for estimation total renal cholesterol was estimated by cholesterol oxidase peroxidase (CHOD-PAP) method (Allain et al., 1974) renal tissue triglycerides were estimated by glycerophosphate oxidase peroxidase (GODPAP) method (Kinoshita et al., 1979) using commercially available kit (Coral clinical system, Goa, India).

**Estimation of total renal cortical protein:** The total renal proteins were estimated by biuret method using commercially available kit (Coral clinical system, Goa, India). The absorbance of test and standard samples was noted against blank spectrophotometrically at 540 nm. Renal triglycerides and renal cholesterol were expressed in terms of µg mg⁻¹ protein of renal cortex.

**Measurement of mean arterial BP (MABP)**

**Non invasive tail cuff method:** The mean arterial blood pressure was recorded in rats by tail-cuff apparatus (NIBP MP100, Biopac) containing sensitive photoelectric sensors. The indirect Mean Arterial Pressure (MAP) was determined from the cuff pressure when the pulse volume oscillations were maximal. To create sufficiently large pulse volume oscillations, the rats were heated for about 12 min at 38°C prior to recording the pressure. The heating increased the mean arterial pressure by an average of 4+/−2 mm Hg, as indicated by direct measurement of pressure. Three different sizes of cuffs were tested, with the result that the indirect measurements were nearly identical to those obtained directly when an appropriate cuff size was selected. The MAP determined at maximum pulse volume oscillations coincides fairly well with the true mean arterial pressure (Sakamaki et al., 1987).

**Statistical analysis:** Values were expressed as Mean±SD (N=6 animals/groups). The data obtained from various groups were statistically analyzed using one-way ANOVA followed by Tukey's multiple comparisons test. P-value of less than 0.05 was considered to be statistically significant.

**RESULTS**

Administration of Hemin (20 mg kg⁻¹ i.p.) and Lisinopril (5 mg kg⁻¹ p.o.) for 2 weeks to normal rats did not produce any significant per se effects on various parameters of diabetic nephropathy used in the present study. Administration of single injection of streptozotocin (50 mg kg⁻¹, i.p.) produced hyperglycemia and after 7 days of STZ administration, the rats showed blood glucose level greater than 200 mg dl⁻¹ were selected as diabetic rats. Hemin (5, 10 and 20 mg kg⁻¹ i.p., 2 weeks) and Lisinopril (1 and 5mg kg⁻¹, p.o., 2 weeks) were administered to diabetic rats after 2 weeks after renal ischemia and their treatment were continued for 2 weeks. All the parameters were assessed at the end of 6th weeks in normal and diabetic rats with or without drug treatment.

**Effect of lisinopril or hemin treatment alone and in various combinations on elevated serum glucose level observed in I/R subjected diabetic rats:** The marked increase in serum glucose was noted in diabetic rats as compared to sham control rats. Further, I/R (30 min) subjected diabetic rats have also exhibited a marked increase in serum glucose level (Fig. 1). However, treatment with Hemin (5, 10 and 20 mg kg⁻¹ i.p., 2 weeks) dose dependently and partially reduced the glucose level in I/R subjected diabetic rats. Treatment with Lisinopril (1 and 5 mg kg⁻¹ p.o., 2 weeks) did not affect the elevated serum glucose concentration both in diabetic and I/R subjected diabetic rats. On the other hand, the glucose lowering effect of Hemin in I/R subjected diabetic rats was not altered by its combination with Lisinopril.

**Effect of lisinopril or hemin treatment alone and in various combinations on altered lipid profiles observed in I/R subjected diabetic rats:** The increase in serum Total Cholesterol (TC) and triglycerides (TGs) and decrease in HDL level were observed in diabetic rats. Further, inducing I/R in diabetic rats has been significantly enhanced the altered lipid profile (Fig. 2-4) observed in diabetic rats. Treatment with both Lisinopril (1 and 5 mg kg⁻¹ p.o., 2 weeks) and Hemin (5, 10 and 20 mg kg⁻¹ i.p., 2 weeks) dose dependently attenuated the altered lipid level in I/R subjected diabetic rats. Further, combined treatment with Lisinopril (5 mg kg⁻¹, p.o.) and Hemin (5 mg kg⁻¹, i.p.) for 2 weeks significantly reduced the increased serum TC and TGs and increased the decreased HDL level as compared to Lisinopril (5 mg kg⁻¹, p.o.) treatment alone in I/R subjected diabetic rat. In addition, combined treatment with Lisinopril (1 mg kg⁻¹, p.o.) and Hemin (5 mg kg⁻¹, i.p.) for 2 weeks significantly reduced the increased serum TC and TGs and increased the decreased HDL level as compared to both Lisinopril (1 mg kg⁻¹, p.o.) or Hemin (5 mg kg⁻¹, i.p.) treatment alone in I/R subjected diabetic rat. Moreover, combined treatment with Lisinopril (5 mg kg⁻¹, p.o.) and Hemin (20 mg kg⁻¹, i.p.) for 2 weeks significantly reduced the increased serum concentration of total cholesterol and triglycerides and increased the decreased HDL level as compared to Hemin (20 mg kg⁻¹, i.p.) treatment alone in I/R subjected diabetic rat.
Fig. 1: Effect of lisinopril or hemin treatment alone and in various combinations on serum glucose level in I/R subjected diabetic rats. All values are expressed as Mean±SD, a = p<0.05 vs. sham control, b = p<0.05 vs. diabetic control, c = p<0.05 vs. diabetic I/R control, I/R = Ischemia repurfusion, Lisino = Lisinopril

Fig. 2: Effect of lisinopril or hemin treatment alone and in various combinations on serum cholesterol level in I/R subjected diabetic rats. All values are expressed as Mean±SD, a = p<0.05 vs. sham control, b = p<0.05 vs. diabetic control, c = p<0.05 vs. diabetic I/R control, d = p<0.05 vs. hemin (5 mg kg⁻¹), e = p<0.05 vs. lisinopril (5 mg kg⁻¹), f = p<0.05 vs. lisinopril (1 mg kg⁻¹), g = p<0.05 vs. hemin (5 mg kg⁻¹), h = p<0.05 vs. lisinopril (5 mg kg⁻¹), i = p<0.05 vs. hemin (20 mg kg⁻¹), I/R = Ischemia repurfusion, Lisino = Lisinopril
Fig. 3: Effect of lisinopril or hemin treatment alone and in various combinations on serum TGs level in I/R subjected diabetic rats. All values are expressed as Mean±SD, a = p<0.05 vs. sham control, b = p<0.05 vs. diabetic control, c = p<0.05 vs. diabetic I/R control, d = p<0.05 vs. hemin (5 mg kg⁻¹), e = p<0.05 vs. lisinopril (5 mg kg⁻¹), f = p<0.05 vs. lisinopril (1 mg kg⁻¹), g = p<0.05 vs. hemin (5 mg kg⁻¹), h = p<0.05 vs. lisinopril (5 mg kg⁻¹), i = p<0.05 vs. hemin (20 mg kg⁻¹), I/R = Ischemia reperfusion, Lisino = Lisinopril

Fig. 4: Effect of lisinopril or hemin treatment alone and in various combinations on serum HDL level in I/R subjected diabetic rats. All values are expressed as Mean±SD, a = p<0.05 vs. sham control, b = p<0.05 vs. diabetic control, c = p<0.05 vs. diabetic I/R control, d = p<0.05 vs. hemin (5 mg kg⁻¹), e = p<0.05 vs. lisinopril (5 mg kg⁻¹), f = p<0.05 vs. lisinopril (1 mg kg⁻¹), g = p<0.05 vs. hemin (5 mg kg⁻¹), h = p<0.05 vs. lisinopril (5 mg kg⁻¹), i = p<0.05 vs. hemin (20 mg kg⁻¹), I/R = Ischemia reperfusion, Lisino = Lisinopril
Effect of lisinopril or hemin treatment alone and in various combinations on serum creatinine, blood urea nitrogen and urinary protein in I/R subjected diabetic rats: The serum creatinine, blood urea nitrogen and urinary protein level were noted to be markedly increased in diabetic rats. Further, it has been observed that induction of 30 min I/R in diabetic rats has further significantly increased serum creatinine, blood urea nitrogen and urinary protein as compared to diabetic rats (Fig. 5-7). Treatment with Lisinopril (1 and 5 mg kg\(^{-1}\), p.o., 2 weeks) or Hemin (5, 10 and 20 mg kg\(^{-1}\) i.p., 2 weeks) significantly attenuated the increased serum creatinine, blood urea and urinary protein level in I/R subjected diabetic rats. In addition, combined treatment with low dose Lisinopril (1 mg kg\(^{-1}\), p.o.) and low dose Hemin (5 mg kg\(^{-1}\), i.p.) for 2 weeks significantly reduced the increased serum creatinine, blood urea nitrogen and urinary protein as compared to Lisinopril (1 mg kg\(^{-1}\), p.o.) or Hemin (5 mg kg\(^{-1}\), i.p.) treatment alone. Further, combined treatment with high dose Lisinopril (5 mg kg\(^{-1}\), p.o.) and low dose Hemin (5 mg kg\(^{-1}\), i.p.) as well as high dose Lisinopril (5 mg kg\(^{-1}\), p.o.) and high dose Hemin (20 mg kg\(^{-1}\), i.p.) for 2 weeks markedly attenuated the increased serum creatinine, blood urea nitrogen and urinary protein as compared to high dose Lisinopril or Hemin, respectively treatment alone in I/R subjected diabetic rat.

Effect of lisinopril or hemin treatment alone and in various combinations on renal TBARS and reduced glutathione in I/R subjected diabetic rats: The marked increase in renal tissue TBARS concentration and decrease in reduced form of glutathione (GSH) were noted in kidney of STZ administered diabetic rats as compared to sham operated rats. Further, I/R subjected diabetic rats after 6 weeks exhibited significant increase in renal TBARS (Fig. 8) and decrease in reduced glutathione (Fig. 9), as compared to diabetic rats. Treatment with Lisinopril (1 and 5 mg kg\(^{-1}\) p.o., 2 weeks) or Hemin (5, 10 and 20 mg kg\(^{-1}\) i.p., 2 weeks) dose dependently reduced the increased renal TBARS level and decreased GSH level in I/R subjected diabetic rats. Further, combined treatment with low dose Lisinopril (1 mg kg\(^{-1}\), p.o.) and low dose Hemin (5 mg kg\(^{-1}\), i.p.) for 2 weeks significantly attenuated the increased renal TBARS and increased the reduced renal glutathione as compared either treatment alone. In addition, combined treatment with Lisinopril (5 mg kg\(^{-1}\), p.o.) and Hemin (20 mg kg\(^{-1}\), i.p.) for 2 weeks significantly reduced the increased renal TBARS and increased the reduced renal glutathione level as compared to Hemin (20 mg kg\(^{-1}\), i.p.) treatment alone in I/R subjected diabetic rat. Moreover, combined treatment with Lisinopril (5 mg kg\(^{-1}\), p.o.) and Hemin (5 mg kg\(^{-1}\), i.p.) for 2 weeks significantly reduced the

![Fig. 5: Effect of lisinopril or hemin treatment alone and various combinations on serum creatinine level in I/R subjected diabetic rats. All values are expressed as Mean±SD, a = p<0.05 vs. sham control, b = p<0.05 vs. diabetic control, c = p<0.05 vs. diabetic I/R control, d = p<0.05 vs. hemin (5 mg kg\(^{-1}\)), e = P<0.05 vs. hemin (10 mg kg\(^{-1}\)), f = p<0.05 vs. lisinopril (5 mg kg\(^{-1}\)), g = p<0.05 vs. lisinopril (1 mg kg\(^{-1}\)), h = p<0.05 vs. hemin (20 mg kg\(^{-1}\)), I/R = Ischemia repuffusion, Lisino = Lisinopril](image-url)
Fig. 6: Effect of lisinopril or hemin treatment alone and in various combinations on serum BUN level in I/R subjected diabetic rats. All values are expressed as Mean±SD, a = p<0.05 vs. sham control, b = p<0.05 vs. diabetic control, c = p<0.05 vs. diabetic I/R control, d = p<0.05 vs. hemin (5 mg kg⁻¹), e = p<0.05 vs. lisinopril (5 mg kg⁻¹), f = p<0.05 vs. lisinopril (1 mg kg⁻¹), g = p<0.05 vs. hemin (5 mg kg⁻¹), h = p<0.05 vs. lisinopril (5 mg kg⁻¹), i = p<0.05 vs. hemin (20 mg kg⁻¹), I/R = Ischemia repurfsion, Lisino = Lisinopril

Fig. 7: Effect of lisinopril or hemin treatment alone and various combinations on urine microprotein level in I/R subjected diabetic rats. All values are expressed as Mean±SD, a = p<0.05 vs. sham control, b = p<0.05 vs. diabetic control, c = p<0.05 vs. diabetic I/R control, d = p<0.05 vs. hemin (5 mg kg⁻¹), e = p<0.05 vs. hemin (10 mg kg⁻¹), f = p<0.05 vs. lisinopril (5 mg kg⁻¹), g = p<0.05 vs. Lisinopril (1 mg kg⁻¹), h = p<0.05 vs. hemin (20 mg kg⁻¹), I/R = Ischemia repurfsion, Lisino = Lisinopril
Fig. 8: Effect of lisinopril or hemin treatment alone and in various combinations on renal TBARS level in I/R subjected diabetic rats. All values are expressed as Mean±SD, a = p<0.05 vs. sham control, b = p<0.05 vs. diabetic control, c = p<0.05 vs. diabetic I/R control, d = p<0.05 vs. hemin (5 mg kg⁻¹), e = p<0.05 vs. lisinopril (5 mg kg⁻¹), f = p<0.05 vs. lisinopril (1 mg kg⁻¹), g = p<0.05 vs. hemin (5 mg kg⁻¹), h = p<0.05 vs. lisinopril (5 mg kg⁻¹), i = p<0.05 vs. hemin (20 mg kg⁻¹), I/R = Ischemia repufusion, Lisino = Lisinopril

Fig. 9: Effect of lisinopril or hemin treatment alone and in various combinations on renal glutathione level in I/R subjected diabetic rats. All values are expressed as Mean±SD, where a = p<0.05 vs. sham control, b = p<0.05 vs. diabetic control, c = p<0.05 vs. diabetic I/R control, d = p<0.05 vs. hemin (5 mg kg⁻¹), e = p<0.05 vs. lisinopril (5 mg kg⁻¹), f = p<0.05 vs. lisinopril (1 mg kg⁻¹), g = p<0.05 vs. hemin (5 mg kg⁻¹), h = p<0.05 vs. hemin (20 mg kg⁻¹), I/R = Ischemia repufusion, Lisino = Lisinopril
Fig. 10: Effect of lisinopril or hemin treatment alone and in various combinations on renal TGs level in I/R subjected diabetic rats. All values are expressed as Mean±SD a = p<0.05 vs. sham control, b = p<0.05 vs. diabetic control, c = p<0.05 vs. diabetic I/R control, d = p<0.05 vs. hemin (5 mg kg⁻¹), e = p<0.05 vs. lisinopril (5 mg kg⁻¹), f = p<0.05 vs. lisinopril (1 mg kg⁻¹), g = p<0.05 vs. hemin (5 mg kg⁻¹), h = p<0.05 vs. lisinopril (5 mg kg⁻¹), i = p<0.05 vs. hemin (20 mg kg⁻¹), I/R = Ischemia reperfusion, Lisino = Lisinopril

Increased renal TBARS and increased the reduced renal glutathione as compared to Lisinopril (5 mg kg⁻¹ p.o., 2 weeks) treatment alone in I/R subjected diabetic rat.

**Effect of lisinopril or hemin treatment alone and in various combinations on renal TGs and renal cholesterol in I/R subjected diabetic rats:** The diabetic rats exhibited a significant rise in renal TGs and renal cholesterol as compared to sham control rats. Further, it has been observed that induction of I/R for 30 min in diabetic rats further increased renal TGs (Fig. 10) and renal cholesterol (Fig. 11), as compared to diabetic rats. However, treatment with Lisinopril (1 and 5 mg kg⁻¹, p.o., 2 weeks) or Hemin (5, 10 and 20 mg kg⁻¹, i.p., 2 weeks) dose dependently attenuated the altered renal TG and renal cholesterol in I/R subjected diabetic rats. The combined treatment with Lisinopril (5 mg kg⁻¹, p.o.) and Hemin (5 mg kg⁻¹, i.p.) for 2 weeks significantly reduced the increased renal TGs and renal cholesterol as compared to Lisinopril (5 mg kg⁻¹ p.o., 2 weeks) treatment alone in I/R subjected diabetic rat. Combined treatment with low dose Lisinopril (1 mg kg⁻¹, p.o.) and low dose Hemin (5 mg kg⁻¹, i.p.) for 2 weeks significantly attenuated the increased renal TGs and renal cholesterol as compared to either treatment alone. Moreover, combined treatment with high dose Lisinopril (5 mg kg⁻¹, p.o.) and high dose Hemin (20 mg kg⁻¹, i.p.) for 2 weeks also significantly reduced altered renal lipid levels as compared to either treatment alone. In addition, combination of low dose Hemin with high dose Lisinopril also has significantly attenuated altered lipid levels even compared with high dose Lisinopril treated group.

**Effect of lisinopril or hemin treatment alone and in various combinations on mean arterial blood pressure (MABP) in I/R subjected diabetic rats:** The significant increase in MABP was noted in diabetic rats as compared to sham control rats. This altered MABP was further significantly increased in I/R subjected diabetic rats as compared to diabetic rats (Fig. 12). Treatment with Lisinopril (1 and 5 mg kg⁻¹ p.o., 2 weeks) or Hemin (5, 10 and 20 mg kg⁻¹ i.p., 2 weeks) dose dependently attenuated the altered MABP in I/R subjected diabetic rats. Combined treatment with low dose Lisinopril (1 mg kg⁻¹, p.o.) and low dose Hemin (5 mg kg⁻¹, i.p.) for 2 weeks significantly reduced the increased MABP as compared to either treatment alone. Moreover, combined treatment with Lisinopril (5 mg kg⁻¹, p.o.) and Hemin (20 mg kg⁻¹, i.p.) for 2 weeks significantly reduced the increased MABP as compared to either treatment alone. In addition,
combination of low dose Hemin (5 mg kg$^{-1}$, i.p.) with high dose Lisinopril (5 mg kg$^{-1}$, p.o.) also has significantly attenuated altered MABP even compared with high dose Lisinopril treated group.

**DISCUSSION**

The data obtained in the present study shows that a concurrent administration of either low dose Hemin or low dose of Lisinopril synergistically attenuated the development of diabetic nephropathy. In addition, supplementation of both low dose and high dose of Hemin with high dose of Lisinopril provides additional renoprotective effects as compared to treatment with either drug at high dose alone. The development of the pathological changes like hyperglycemia, dyslipidemia and elevated serum creatinine, BUN, proteinuria has been well documented to occur in 6 to 8 weeks after STZ administration in rats (Gojo et al., 2007; Arora et al., 2010; Singh et al., 2010). Further, increase in serum creatinine, BUN and proteinuria have been documented to be index of nephropathy (Arora et al., 2010; Bohle et al., 1977; Perrone et al., 1992; Vaishya et al., 2008). However, occurrence of severe proteinuria and advanced renal lesions are not developed even after 8-9 months in STZ-induced diabetic rats, possibly due to spontaneous reduction in ambient BP (Anderson et al., 1989; Bidani et al., 2007; Tesch and Allen, 2007). Interestingly, it has been noted that induction of renal ischemia for 30 min produced severe diabetic nephropathy in STZ-diabetic rats (Melin et al., 1997, 2002). Therefore, in the present study this experimental model was used which is developed by subjecting renal ischemia for 30 min in diabetic rats 2 weeks after STZ. It was observed in this study that renal ischemia subjected diabetic rats exhibited a marked elevation in the serum levels of creatinine, BUN and proteinuria after six weeks as compared with both normal and diabetic control rats. These results suggest the development of more severe diabetic nephropathy in renal IR subjected STZ-diabetic rats as compared to STZ-diabetic rats.

Although, persistent hyperglycemia and hypertension has been implicated as primary risk factors responsible for the development of structural and functional changes of the diabetic kidney (Phillips et al., 2001; Giunti et al., 2006). Growing evidences clearly suggesting that elevation in circulating lipids may contribute to renal disease progression (Abras, 2004; Trevisan et al., 2006). Further, experimental studies clearly revealed that increase in lipid level is a major contributor in the development and progression of
Fig. 12: Effect of lisinopril or hemin treatment alone and in various combinations on mean atrial BP level in I/R subjected diabetic rats. All values are expressed as Mean±SD, a = p<0.05 vs. sham control, b = p<0.05 vs. diabetic control, c = p<0.05 vs. diabetic I/R control, d = p<0.05 vs. hemin (5 mg kg⁻¹), e = p<0.05 vs. lisinopril (5 mg kg⁻¹), f = p<0.05 vs. lisinopril (1 mg kg⁻¹), g = p<0.05 vs. hemin (5 mg kg⁻¹), h = p<0.05 vs. lisinopril (5 mg kg⁻¹), i = p<0.05 vs. hemin (20 mg kg⁻¹), I/R = Ischemia repertusion

Lisino = Lisinopril

Diabetic nephropathy (Wang et al., 2005; Keane, 2000; Sun et al., 2000; Li et al., 2008). In addition, hyperlipidemia has been suggested to be an independent risk factor and major determinant of the progression of nephropathy in patients with diabetes mellitus (Vaziri et al., 2003; Brass, 2004). Besides sustained hyperglycemia, STZ-induced diabetic rats also shown to be associated with hypercholesterolemia and hypertriglyceridemia (Trevisan et al., 2006; Vaziri et al., 2003). Hyperglycemia and hyperlipidemia synergistically induce renal damage in mice with diabetic nephropathy (Spencer et al., 2004). Further, increased expression of lipolytic enzymes was documented in ACE−/− animals (Jayasooriya et al., 2008). In the present study, it has been observed that administration of Lisinopril do not produced any significant effect on altered serum lipid profiles observed in diabetic rats. On the other hand, combined treatment with both Hemin and Lisinopril at three different dose combinations employed significantly reversed the altered serum cholesterol, TG and HDL. It has been demonstrated in laboratory (Singh et al., 2010) and in others (Deepa and Varalakshmi, 2005) that STZ diabetic rats are associated with accumulation of renal triglycerides, renal cholesterol, renal total lipids. Interestingly, results obtained in the present study demonstrate for the first time that treatment with both Hemin as well as Lisinopril treatment alone has significantly attenuated the accumulation of renal TGs and cholesterol. In addition, combined treatment with both Hemin and Lisinopril at three different dose combinations employed in the present study synergistically reduced the renal TGs and cholesterol levels. Accumulating evidence suggests that Nrf2 may also have roles in lipid metabolism. The Nrf2−/− mice fed with high fat diet are associated with more serum cholesterol and a greater accumulation of hepatic lipids and lipid peroxidation by-products as compared to wild type mice (Tanaka et al., 2008). Induction of HO-1, by Hemin has shown to decrease hepatic steatosis by decreased expression and activity of SREBP1c, a key transcription factor, implicated in the
fatty acid and TG synthesis and increased expression and DNA binding activity of PPARs and its downstream targets (Eberle et al., 2004; Jump et al., 2005). It suggests that simultaneous modulation of HO-1 through administration of Hemin and ACE inhibition by Lisinopril treatment more effectively attenuated the both serum lipid profile and renal lipid accumulation even with two weeks pharmacological treatment employed in the present study.

Both acute and chronic administration of HO-1 inducers or HO degradation products produced significant decrease in blood pressure in SHR and renovascular hypertensive rats (Levere et al., 1990; Martasek et al., 1991; Botros et al., 2005). In consistency with this the results obtained in the present study demonstrate that treatment with Hemin dose-dependently reversed the increased systolic BP. Interestingly, concurrent administration of either low dose or high dose of Hemin with Lisinopril as resulted in marked reduction in systolic BP as compared to even high dose (5 mg kg⁻¹) of Lisinopril in I/R-subjected diabetic rats.

In this study, serum glucose level has not been modulated treatment with Hemin at the all the dose levels employed. Similarly, combined treatment with Hemin and Lisinopril at different dose combinations do not attenuated the hyperglycemia in renal I/R subjected diabetic rats. This is in consistency with previous study demonstrated that administration of Hemin at the dose (50 mg kg⁻¹ day⁻¹) for 30 days has not shown to decrease the hyperglycemia in STZ induced diabetic rats (Farhangkhooe et al., 2003). In contrast, treatment with Hemin has shown to enhance insulin sensitivity and glucose metabolism in STZ-induced type-I diabetic rats and type -2 diabetic Zucker fatty rats (Ndisang and Jadav, 2009; Ndisang et al., 2009). The discrepancies observed in these results may be due to the difference in the dose and duration of drug treatment schedule, severity of hyperglycemia.

The increased oxidative stress associated with diabetes mellitus has been documented to play a crucial role in the pathogenesis of diabetic complications (Abraham et al., 2003; Ahmad et al., 2005). Hyperglycemia has shown to repress expression and activity of HO-1 which is responsible for the increased glucose-mediated oxidative stress (Quan et al., 2004a; Sacerdotti et al., 2005). In addition, Koya et al., (2003) reported that a 16-fold increase in the expression of HO-1 mRNA and protein in diabetic glomeruli which is normalized by administration of vitamin E and probucol. Recently, in vitro studies demonstrated that glomeruli of human diabetic nephropathy patients are associated with elevated Nrf2 levels (Jiang et al., 2010). Therefore, it may suggest that HO-1 pathway may act as a retaliatory antioxidant mechanism to retard development of diabetic complications by scavenging the oxidative stress. In the present study Hemin treatment has dose-dependently decreased renal TBARS and increased level of reduced glutathione observed in diabetic rats.

The chronic use of ACE inhibitor or angiotensin (AT1) receptor blockers has been associated with activation of compensatory mechanisms (Ruggenenti et al., 2003; Sasoski, 2009; Muller and Luft, 2006). Both pharmacological induction or over expression of HO-1 has shown to prevent the Ang-II induced superoxide generation and renal cell death (Quan et al., 2004b; Kelsen et al., 2008) and ameliorated the Ang-II induced tubulointerstitial injury and hypertension (Pradhan et al., 2006; Vera et al., 2007). It is suggested that activation of HO-1 may effectively encounter the Ang-II mediated renal oxidative stress and cell death in diabetic kidney (Abraham et al., 2009). This speculation is further supported by the results observed in the present study that supplementation of Hemin has produced additional beneficial effects even with high dose of Lisinopril.

CONCLUSION

On the basis of above discussion, it may be concluded that concurrent administration Hemin and Lisinopril in different dose combinations has significantly attenuated the diabetic nephropathy, as compared to treatment with either drug treatment alone. These additional protective effects of Hemin with Lisinopril may be consequence of reduction of serum and renal lipid, combination of oxidative stress, MABP, without being modulated the hyperglycemia. Therefore, combination therapy targeting both ACE inhibition and HO-1 activation may provide additional therapeutic beneficial effects in the management of diabetic nephropathy.

ACKNOWLEDGMENT

We express our gratitude to Shri. Parveen Garg Ji, Honorable Chairman, ISF College of Pharmacy, Moga, Punjab, India for his inspiration and constant support for this study.

REFERENCES


