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SULFASALAZINE PREVENTS DIABETIC NEPHROPATHY IN TYPE-I DIABETIC RATS

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ABSTRACT

Aim: *Diabetic nephropathy is major cause of end stage renal disease affecting 20-30% patients with diabetes. Inflammation plays important role in diabetic nephropathy. Nuclear factor kappa B(NF-κB) is transcription factor involved in pathogenesis of diabetic nephropathy. Sulfasalazine is nuclear factor kappa B inhibitor. The objective of present study was to evaluate effect of sulfasalazine a known NF-κB inhibitor in prevention of diabetic nephropathy of type I diabetic rats.* ***Methods:*** *Type I diabetes was induced by single i.p. injection of streptozotocin (65 mg/kg). After four weeks of streptozotocin injection rats were divided into six groups: Normal control group, Normal rats given sulfasalazine, Diabetic control group, diabetic rats treated with insulin, diabetic rats treated with sulfasalazine alone, diabetic rats treated with insulin in addition to sulfasalazine. The treatment was given from 4th week to 8th week. Renal function, oxidative stress and histopathology of kidneys were assessed at the end of 8th week.* ***Results:*** *Renal dysfunction and oxidative stress was significantly lowered in all treatment groups with the maximum protective effect in rats treated with insulin in addition to sulfasalazine. Histopathological reports strongly suggest protective effect of sulfasalazine on kidneys.* ***Conclusion:*** *The results suggest that sulfasalazine showed significant Reno protective effects.*

KEY WORDS

Diabetic nephropathy, Nuclear factor-kappa B (NF- κB), Sulfasalazine

INTRODUCTION

Diabetic nephropathy is a long term microvascular complication of type I as well as Type II diabetes mellitus caused considerable morbidity and mortality [1].

Diabetic nephropathy characterized by structural and functional changes of kidney in diabetic patients. The structural changes in early diabetic nephropathy characterized by glomerular hypertrophy, thickening of basement membrane, mesangial expansion followed by diffuse and nodular glomerular sclerosis [2,3]. Functional changes include glomerular hyperfiltration and normal urinary albumin excretion in silent phase followed by proteinuria, systemic hypertension and eventual loss of kidney function in later phases of diabetic nephropathy. [4,5]. Hyperglycemia induced overproduction of reactive oxygen species (ROS) is main cause of initiation and progression of diabetic nephropathy [6]. Overproduction of ROS activates nuclear factor kappa B (NF- κB) within diabetic kidney. [7]. Activated NF- κB translocated to nucleus and promotes the expression of many inflammatory genes and cell adhesion molecules, such as the interleukins, tumor necrosis factor-α (TNF- α), transforming growth factor- β, monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule 1 (ICAM-1), and vascular adhesion molecule 1 (VCAM-1) [8-12]. These

inflammatory molecules cause glomerular hypertrophy, glomerulosclerosis, mesangial expansion, tubular inflammation, and cell proliferation [12]. Sulfasalazine has been reported to have protective effects on kidneys in gentamycin induced injury through inhibition of NF-κB [13]. Sulfasalazine is reported NF- κB inhibitor can prevent renal damage in diabetic kidney through inhibition of expression of inflammatory genes. The present study was carried out to evaluate effect of sulfasalazine a known NF- κB inhibitor in prevention of diabetic nephropathy in type I diabetic rats.

MATERIALS AND METHODS

Animals

Healthy Wistar strain male rats weighing 200-250 g were procured from zydus cadila, ahmedabad. The animals were housed, three each in polypropylene cage, maintain under standard laboratory condition of 12:12 light and dark cycles at an ambient temperature of 24±3°C and 55±5% relative humidity. The rats were fed standard rat pellet diet (commercial rat feed from Pranav Agro Industries Ltd., Baroda, India) and water *ad libitum*. The study was approved by the Animal Ethical Committee of the Institute and carried out in the laboratory of B. S. Patel Pharmacy College, Linch. All the protocols and experiments were conducted in compliance with ethical principles and guidelines provided by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

Drugs and reagents

Streptozotocin (purchased from Enzo life sciences, USA) was prepared in citrate buffer and Sulfasalazine (purchased from Sigma-Aldrich) was prepared in saline. A glucose oxidase peroxidase diagnostic enzyme kit was purchased from Span Diagnostic Chemicals, India. Measurement of serum and urinary creatinine, urea and urinary protein was also done using kit purchased from Span Diagnostic Chemicals, India. All other chemicals used for biochemical estimations are of analytical grade.

Induction and assessment of Diabetes mellitus

Hyperglycaemia was induced in adult wistar strain male albino rats by a single intraperitoneal injection of 55 mg/kg streptozotocin prepared in citrate buffer (pH 4.4, 0.1 mol/L) [14,15]. The age-matched normal control rats received an equal volume of citrate buffer and used along with diabetic animals. Diabetes was confirmed after 48 h of streptozotocin injection, the blood samples were collected through tail vein and plasma glucose was estimated by Auto span diagnostic kit method [16]. The rats with serum glucose level above 250 mg/dL were selected in the experiment [17].

Treatment schedule

After 4 weeks of STZ injection, control and diabetic rats were randomly divided into six groups consisting of 8 animals each and treated up to 8 weeks: I) A Normal control group given saline, II) Normal rats were administered sulfasalazine (75 mg/kg) diluted with saline, i.p , III) diabetic rats given saline, IV) The diabetic rats were administered insulin (10 IU/kg), s.c., V) The diabetic rats were administered sulfasalazine (75 mg/kg) diluted with saline, i.p and VI) The diabetic rats were administered sulfasalazine (75 mg/kg) diluted with saline, i.p. in addition to insulin 10 (IU/kg), s.c. Body weight was observed every week, food intake, water intake, and urine output was measured at 4 weeks and 8 weeks.

Sample collection

At the end of the 8thweek, rats were kept individually in metabolic cages for 24 h to collect urine for the measurement of urine output and renal function. At the end of experimental period, the rats were anesthetized by an injection of ketamine (100 mg/kg, i.m) and diazepam (5 mg/kg, i.m). The blood was collected by carotid bleeding and serum was separated. The kidneys were rapidly removed. The right kidney was fixed in 10% phosphate buffered formalin for histopathological evaluation. The left kidney was used for estimation of oxidative stress [18].

Biochemical Analysis

Renal function parameters

Renal function was assessed by measuring plasma and urine levels of creatinine, urea and urine albumin excretion using semi-auto-analyser (Elico, SL218, Hyderabad, India). Creatinine and urea clearance were measured as an index of glomerular filtration rate (GFR). Creatinine clearance (reflecting glomerular filtration) was calculated using the formula:

Creatinine clearance (ml/24 hr) =
Urine creatinine × 24-h urine volume/serum creatinine.

Assessment of renal oxidative stress

After sacrificing the rats by carotid bleeding under deep anaesthesia, the left kidney was dissected, rinsed with cold isotonic saline. Entire kidney tissue was minced properly, and the homogenate was prepared (1g/10 ml of PBS, pH 7.8) with the help of homogenizer and centrifuged at 2^0C to 8^0C (15000 rpm for 10 minutes). The supernatant was used for estimation of Superoxide dismutase (SOD), catalase and total nitrite level.

1) Superoxide dismutase activity: SOD activity was assayed according to the method of Misra et al [19]. The spontaneous oxidation of adrenaline to adrenochrome was inhibited by SOD at 480nm. Briefly the assay mixture consisted of 0.1 ml supernatant of kidney sample, 0.5 ml of carbonate buffer, 0.1 ml EDTA and 1.0 ml of epinephrine and the optical density of formed adrenochrome were read for 3 min. The result was calculated as U/mL, where one unit of SOD inhibits the rate of increase in absorbance at 480 nm by 50% under the condition of assay.
2) Catalse activity: Catalase activity was assayed by method of Aebi HE [20]. The breakdown of hydrogen peroxide (H_2O_2) was measured at 240 nm. The reaction mixture consists of 150 µL H_2O_2 diluted with potassium phosphate buffer (pH 7.0) to this added 50 µL supernatant of kidney sample and change in absorbance was read at 240 nm. The amount of catalase activity was calculated using extinction coefficient of H_2O_2, 0.041/nmole/cm^2 and has been reported as µmoles of H_2O_2 utilized/min/mg/protein.
3) Nitrite level: Renal tissue nitrite levels were estimated using Greiss reagent, which served as an indicator of NO production [21]. One mL of Greiss reagent (1:1 solution of 1% sulfoanilamide in 5% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloric acid in water) was added to 1 mL of postmitochondrial supernatant of renal tissue and absorbance was measured at 546 nm. Nitrite concentration was calculated using a standard curve for sodium nitrite and nitrite levels were expressed as% of control.

Histopathological studies

After the animals were sacrificed right kidneys were fixed in 10% neutral buffered formalin solution and embedded in paraffin. Renal sections (5 µm) were cut with automatic microtome (Leica RM 2255), deparaffinized, hydrated, and stained with hematoxylin-eosin. and subjected to further processing for histopathological evaluation.

Statistical analysis

All the data are expressed as mean ± S.E.M. The significant difference was analysed using one-way analysis of variance, followed by the tukey multiple comparison test, with the help of GraphPad Instat software (version 6.0, GraphPad Software Inc, La Jolla, USA). A *P* value less than 0.05 was considered significant.

RESULTS

Effect on body weight and hyperglycemia

At the end of 8^{th} week, the body weight of normal control group and normal control group treated with sulfasalazine was increased as compare to initial body weight. However, there was decrease in body weight of all diabetic rats as compare to initial body weight. In diabetic control group there was significant increase in plasma glucose level compared to normal control group. At the end of 8^{th} week there was significant decrease in plasma glucose in treatment groups as compare to diabetic control group. (Table 1)

Effect on kidney function parameters

Diabetic control group exhibited polyuria, increased urinary albumin excretion, increased serum creatinine and blood urea nitrogen as compared to normal control group. Treatment with insulin, sulfasalazine alone and sulfasalazine in addition to insulin significantly decrease polyuria and urinary albumin excretion compared to diabetic control group. The serum creatinine clearance and urea clearance were also improved in all treatment groups compared to diabetic control group. The maximum improvement of kidney function was in diabetic rats treated with sulfasalazine in addition to insulin. There was no significant change in kidney functions of normal rats treated with sulfasalazine compared to normal control group. (Table 1, Figure 1 and 2)

Effect on oxidative stress markers

Effect on SOD and catalase activity

At the end of 8^{th} week diabetic rats showed significant decrease in SOD and catalase activity as compared to normal control group. Administration of insulin, sulfasalazine alone, sulfasalazine in addition to insulin significantly increases SOD and catalase activity as

compared to diabetic control group. The maximum increase in SOD and catalase activity was observed in group treated with sulfasalazine in addition to insulin. There was no significant change in SOD and catalase activity of normal rats treated with sulfasalazine compared to normal control group. (Table 2, Figure 3)

Effect on renal nitrite level

There was marked increase in nitrite level in diabetic rats compared to normal rats. Treatment with insulin, sulfasalazine alone, sulfasalazine in addition to insulin reverses increase nitrite levels compared to normal control group. There was no significant change in nitrite level of normal rats treated with sulfasalazine compared to normal control group. (Table 2, Figure 3)

Histopathological studies

The kidneys of control group showed normal glomeruli and epithelial cells. The kidney of diabetic rats showed thickening of glomerular basement membrane, interstial fibrosis and necrosis. These pathological alterations were significantly reduced in all treatment groups with the maximum effect in group treated with sulfasalazine in addition to insulin. (Figure 4)

Table 1: General characteristics and kidney function parameters in normal, diabetic and treated rats

I Normal	II Norm +Sulfa	III Diabetic	IV D-Insulin	V D-Sulfasa	VI D-Sul+Insul
Body weight (g) 227.06 ±2.61	227.80 ± 3.45 ^{ns}	166.03 ± 3.34 ^{a*}	182.35 ± 2.68 ^{b*}	197.65 ± 3.67 ^{b*}	191.25 ± 3.61 ^{b*}
Serum glucose (mg/dL) 103.14 ± 0.63	102.57± 0.84 ^{ns}	411.71± 1.4 ^{a*}	180.57± 1.08 ^{b*}	298.14± 3.8 ^{b*}	149.86± 1.8 ^{b*}
Urine Volume (mL/24 h) 9.71 ± 0.08	9.43 ± 0.19 ^{ns}	46.00 ± 0.48 ^{a*}	24.43 ± 0.64 ^{b*}	23.34 ± 0.56 ^{b*}	17.07 ± 0.49 ^{b*}
Proteinuria (mg/dL) 3.45 ± 0.55	3.50 ± 0.46 ^{ns}	11.59 ± 0.58 ^{a*}	7.91 ± 0.64 ^{b*}	6.67 ± 0.17 ^{b*}	6.01 ± 0.34 ^{b*}
Serum creatinine (mg/dL) 0.33 ± 0.0064	0.31 ± 0.0050 ^{ns}	1.43 ± 0.0076 ^{a*}	0.82 ± 0.0009 ^{b*}	0.74 ± 0.0102 ^{b*}	0.58 ± 0.0081 ^{b*}
Serum urea (mg/dL) 25.59 ± 0.13	25.23 ± 0.07 ^{ns}	59.41 ± 0.15 ^{a*}	46.09 ± 0.19 ^{b*}	38.61 ± 0.13 ^{b*}	32.37 ± 0.14 ^{b*}
Creatinine clearance (mL/24 h) 0.72 ± 0.0086	0.71 ± 0.0082 ^{ns}	0.25 ± 0.006 ^{a*}	0.51 ± 0.0062 ^{b*}	0.58 ± 0.0083 ^{b*}	0.63 ± 0.0075 ^{b*}
Urea clearance (mL/24 h) 0.88 ± 0.0096	0.87 ± 0.0105 ^{ns}	0.32 ± 0.0081 ^{a*}	0.61 ± 0.0091 ^{b*}	0.67± 0.0077 ^{b*}	0.76 ± 0.0082 ^{b*}

Data are expressed as mean ± SEM, n= 8. Norm +Sulfa: Normal rats given sulfasalazine (75 mg/kg), D-Insulin: Diabetic group treated by insulin (10 IU/kg), D-sulfasa: Diabetic group treated by sulfasalazine (75 mg/kg), D-sul+Insul: Diabetic group treated with sulfasalazine (75 mg/kg) and insulin (10 IU/kg) both.

^{ns}: not significant compare to normal, ^{a*} : Significant compare to normal p<0.001,

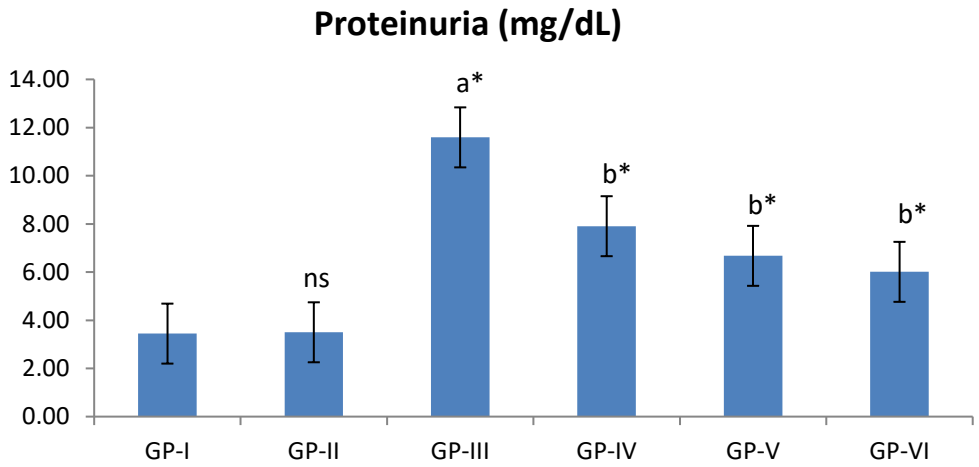
^{b*}: Significant compare to diabetic p<0.001

Table 2: Oxidative stress markers in normal, diabetic and treated rats

I Normal	II Norm +Sulfa	III Diabetic	IV D-Insulin	V D-Sulfasa	VI D-Sul+Insul
SOD (U/mL) 8.97 ± 0.026	9.0± 0.017 ^{ns}	4.08 ± 0.013 ^{a*}	6.45 ± 0.013 ^{b*}	7.86 ± 0.024 ^{b*}	8.22 ± 0.034 ^{b*}
Catalase activity (µmol/min/mg) 3.99 ± 0.013	3.98± 0.015 ^{ns}	0.94 ± 0.012 ^{a*}	1.97 ± 0.016 ^{b*}	2.60 ± 0.0083 ^{b*}	3.28 ± 0.016 ^{b*}
Total nitrite (uM) 100.8 ± 0.48	103.0± 0.96 ^{ns}	272.1 ± 0.74 ^{a*}	237.0 ± 0.63 ^{b*}	166.6 ± 0.66 ^{b*}	113.4 ± 0.76 ^{b*}

Data are expressed as mean ± SEM, n= 8. Norm +Sulfa: Normal rats given sulfasalazine (75 mg/kg), D-Insulin: Diabetic group treated by insulin (10 IU/kg), D-sulfasa: Diabetic group treated by sulfasalazine (75 mg/kg), D-sul+Insul: Diabetic group treated with sulfasalazine (75 mg/kg) and insulin (10 IU/kg) both.

^{ns}: not significant compare to normal, ^{a*}: Significant compare to normal p<0.001,^{b*}: Significant compare to diabetic p<0.001



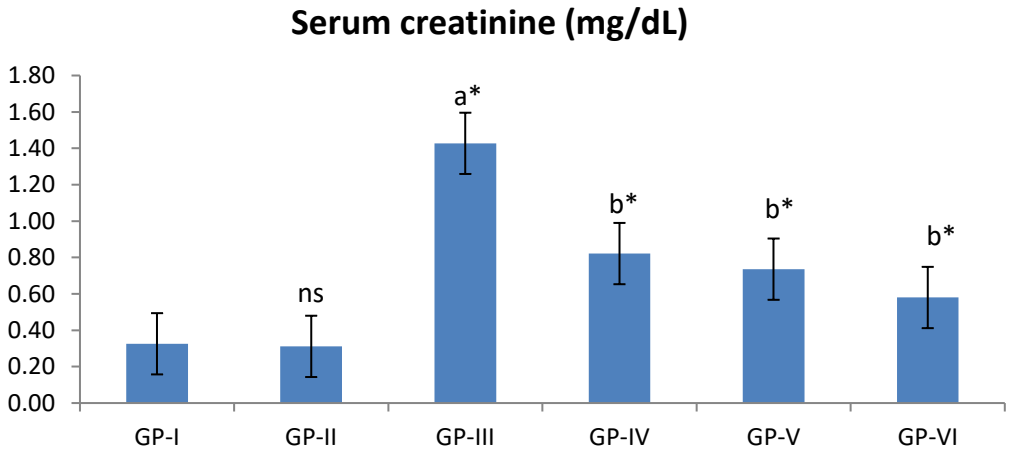
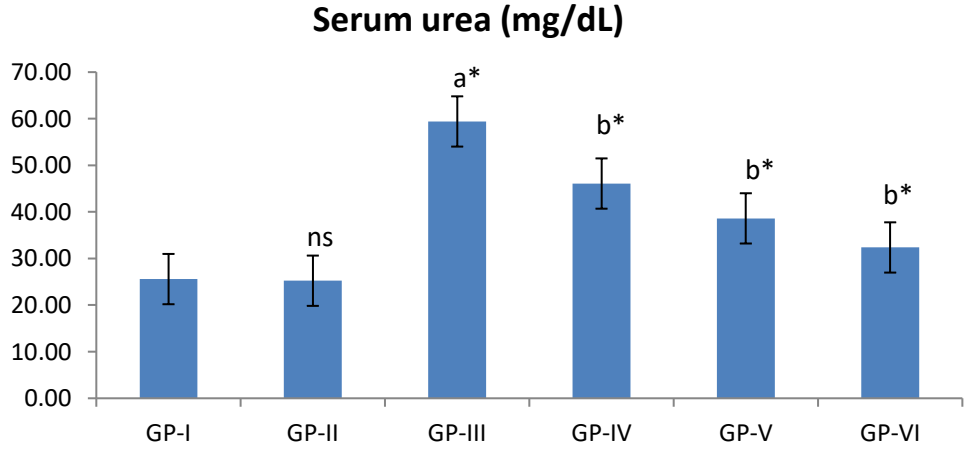


Figure 1: Preoteinuria and serum creatinine level. Data are expressed as mean ± SEM, n= 8.

Group I: Normal, Group II: Normal rats given sulfasalazine (75 mg/kg), Group III: Diabetic Group IV: Diabetic group treated by insulin (10 IU/kg), Group V: Diabetic group treated by sulfasalazine (75 mg/kg), Group VI: Diabetic group treated with sulfasalazine (75 mg/kg) and insulin (10 IU/kg) both.

^{ns}: not significant compare to normal, ^{a*}: Significant compare to normal p<0.001,

^{b*}: Significant compare to diabetic p<0.001



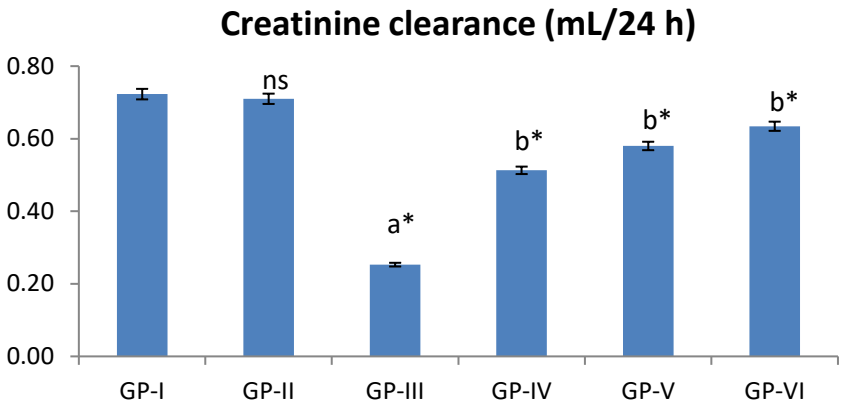
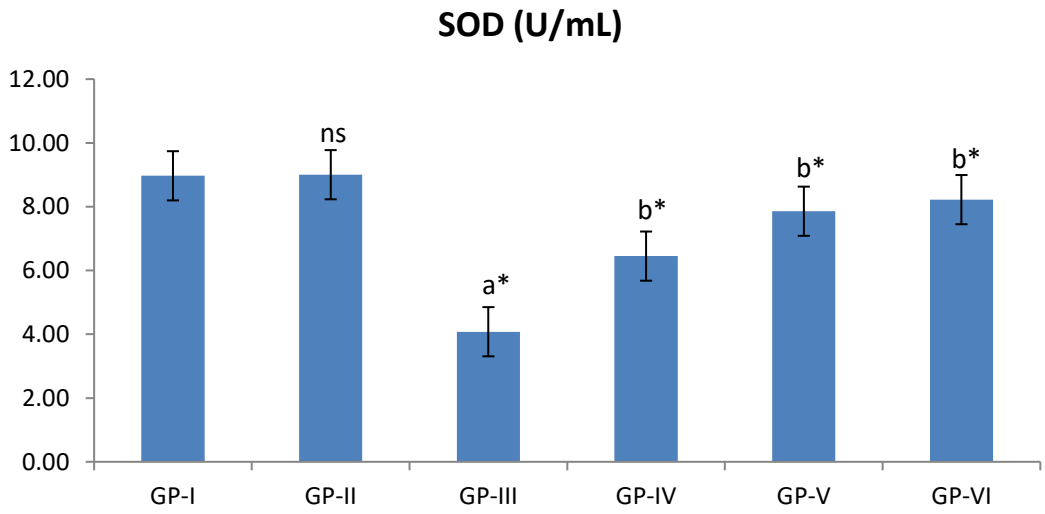
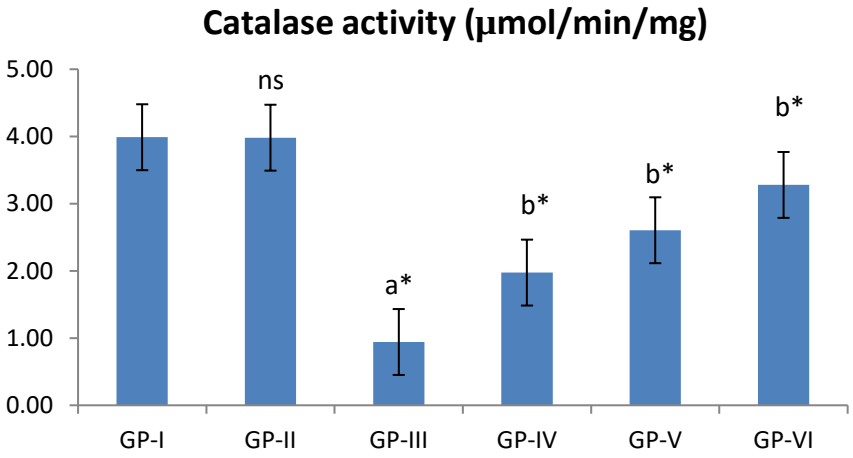


Figure 2: Serum urea and creatinine clearance level. Data are expressed as mean ± SEM, n= 8.
Group I: Normal, Group II: Normal rats given sulfasalazine (75 mg/kg), Group III: Diabetic Group IV: Diabetic group treated by insulin (10 IU/kg), Group V: Diabetic group treated by sulfasalazine (75 mg/kg), Group VI: Diabetic group treated with sulfasalazine (75 mg/kg) and insulin (10 IU/kg) both.
^{ns}: not significant compare to normal, ^{a*}: Significant compare to normal p<0.001,
^{b*}: Significant compare to diabetic p<0.001





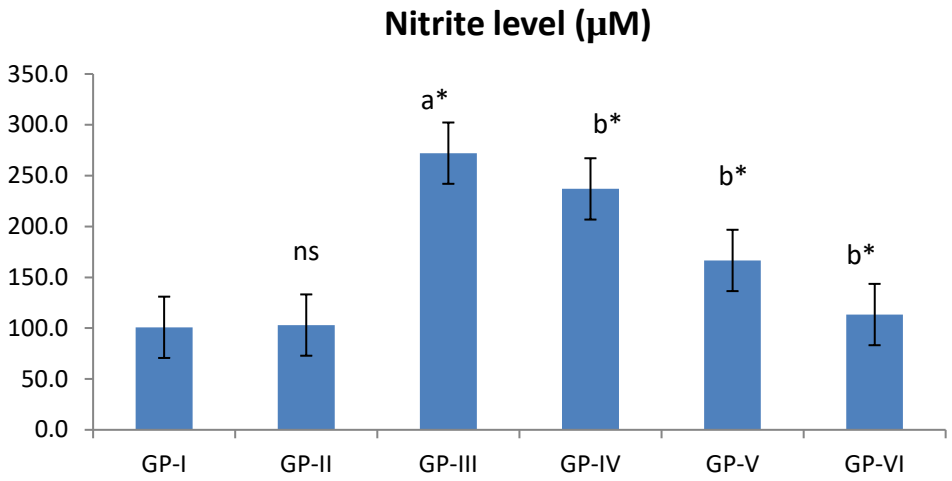


Figure 3: Oxidative stress markers. Data are expressed as mean ± SEM, n= 8.

Group I: Normal, Group II: Normal rats given sulfasalazine (75 mg/kg), Group III: Diabetic Group IV: Diabetic group treated by insulin (10 IU/kg), Group V: Diabetic group treated by sulfasalazine (75 mg/kg), Group VI: Diabetic group treated with sulfasalazine (75 mg/kg) and insulin (10 IU/kg) both.

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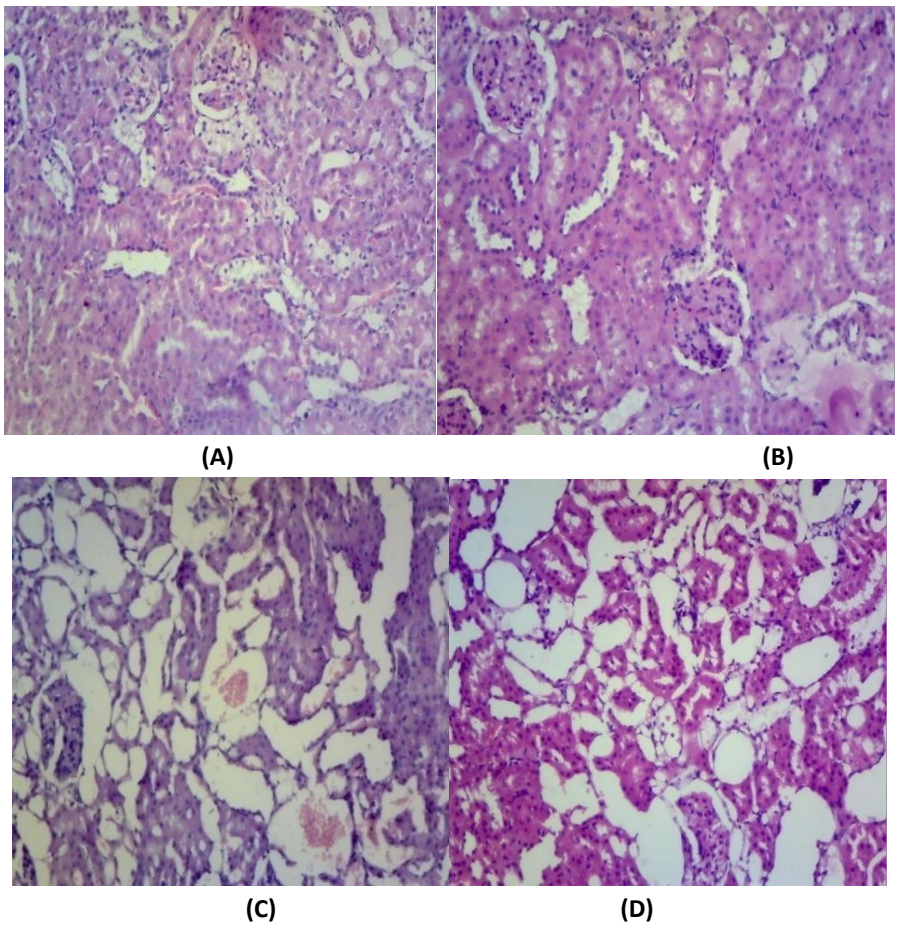

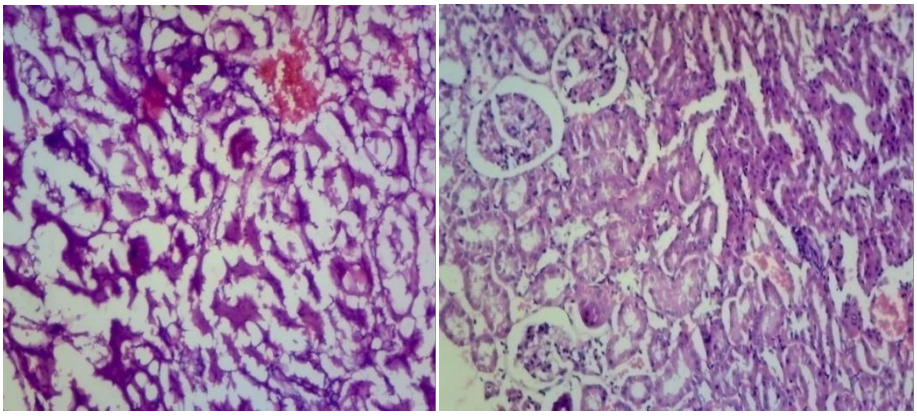

(E) **(F)**

Figure 4: (A) and (B) : Normal glomeruli and epithelial cell, (C): Diabetic control showing thickening of basement membrane, mesengial expansion, necrosis, (D),(E) and (F):Showing diminished kidney lesion.

DISCUSSION:

Diabetes is major cause of end stage renal disease (ESRD). No satisfactory treatment is available for diabetic nephropathy except tight glycemic control and blood pressure control. Many factors contribute in pathogenesis of diabetic nephropathy. Bohle et al reported presence of T cells, Macrophage, fibroblast in diabetic kidney [22]. Inflammation plays role in progression of diabetic nephropathy. [23,24]. NF-κB is major regulator of inflammatory process. Current work was to evaluate effect of sulfasalazine, known NF-κB inhibitor in prevention of diabetic nephropathy.

In present study, STZ induced diabetic rats showed hyperglycemia, weight loss, polyuria and polydipsia while insulin and insulin plus sulfasalazine treated rats showed decrease in blood sugar, reduction in decrease in body weight and other symptoms of diabetes. Sulfasalazine treated group did not show decrease in blood sugar significantly compared to insulin treated group as it is not hypoglycaemic agent.

Diabetic rats showed polyuria, characterized by increase in 24-hour urine volume. In Diabetic nephropathy increased glomerular blood pressure may lead to increase in glomerular filtration rate (GFR) and increase urine output. Treatment with insulin, sulfasalazine and sulfasalazine in addition to insulin decreased 24-hour urine volume.

Proteinuria in diabetic nephropathy is due to effacement of processes of podocytes forming filtration membrane of glomeruli [25,26]. Proteinuria is marker for progression of diabetic nephropathy [27]. Diabetic rats showed marked proteinuria, treatment with insulin, sulfasalazine and insulin plus sulfasalazine showed decreased proteinuria. Insulin decrease proteinuria possibly due to its hypoglycaemic effect while sulfasalazine also decrease proteinuria without significant decrease in blood sugar.

Serum creatinine and creatinine clearance are main markers for estimating GFR and kidney function [28]. Diabetic rats showed increased serum creatinine and decreased creatinine clearance. All treatment groups showed decrease serum creatinine and improvement in creatinine clearance. Insulin a hypoglycaemic agent improved kidney functions. Sulfasalazine showed improvement in serum creatinine and creatinine clearance more than insulin treated group. The highest beneficial effect observed in rats treated with sulfasalazine in addition to insulin. Similarly effect on serum urea and increased urea clearance was higher in sulfasalazine treated group than in insulin treated group and combination of insulin to sulfasalazine had additive effect on urea and urea clearance.

Hyperglycaemia causes increase in oxidative stress in diabetic nephropathy [29]. Diabetic rats showed decreased SOD and catalase activity and increased nitrite level. Glycaemic control by insulin showed increased SOD and catalase activity and decreased nitrite level. Sulfasalazine non-hypoglycaemic agent also showed increased SOD and catalase activity and decrease nitrite level. Combination of insulin plus sulfasalazine had additive effect decrease in oxidative stress.

Normal rats given sulfasalazine showed all kidney and oxidative stress markers not significantly different to normal control group. Sulfasalazine did not produce any toxic effect to kidney of normal rats.

Histopathological studies strongly support the protective effect of sulfasalazine on kidneys. Diabetic rats showed glomerular basement membrane thickening, glomerulosclerosis and necrosis while sulfasalazine treated group showed diminished all these pathological changes. Sulfasalazine plus insulin treated group showed additive protective effect on kidney in histopathological studies.

Sulfasalazine have previously reported to have protective effect on kidneys in gentamicin induced toxicity to kidney through inhibition of NF-κB [13]. Sulfasalazine also showed protective effect in diabetic retinopathy [30]. Current work showed protective effect of sulfasalazine on diabetic kidney however more studies are required to finds mechanism of action of sulfasalazine whether or not protective effect is attributed through inhibition of NF- κB.

CONCLUSION:

From results of present study, it can be concluded that sulfasalazine has significant protective effect against diabetic nephropathy. Chronic treatment with sulfasalazine significantly reduced proteinuria, polyuria and increased serum creatinine and blood urea nitrogen in type I diabetic rats. Creatinine and urea clearance were also significantly improved following the administration of sulfasalazine

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