

Low Dose Irbesartan Has a Renoprotective Effect as High Dose Ramipril in Diabetic Rats Treated with Insulin

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Abstract

Objectives: The aim of this study is to compare the efficacy of the anti-angiotensinic drug, ramipril and irbesartan on the vascular protection of kidneys of streptozotocin (STZ)-induced diabetic rats (DR). **Methods:** 110 male albino rats were divided into 7 main groups. Group-1 (10 normal control rats; NC). Group-2 (10 rats) was injected intra-peritoneally with STZ (Diabetic Rats; DR). Group-3 (10 DR) is controlled by insulin. Groups 4 to 7 (20 DR), each is subdivided into two subgroups that received either low or high dose of ramipril or irbesartan with or without insulin. Two months post treatment, rat-tail blood was collected to measure: Fasting blood sugar, HbA1c, total serum proteins, albumin and lipid profiles. Urine was collected to measure albuminuria. Kidneys were isolated for histopathological study. **Results:** Biochemically, both ramipril and irbesartan (without insulin) lowered albumin concentration in urine samples especially at high doses. Histopathologically, there is no beneficial response of both drugs without insulin. Combination of insulin together with either drug has beneficial effects biochemically and histopathologically at high doses. Low dose irbesartan only has renoprotective effect in DR treated with insulin. The other biochemical parameters showed negligible response to both drugs. **Conclusion:** Low dose irbesartan and high doses of both drugs have renoprotective effect in DR treated with insulin.

Keywords

Diabetic Nephropathy, Albuminuria, Ramipril, Irbesartan

1. Introduction

Diabetic nephropathy occurs in 20% - 40% of patients with diabetes and is the single leading cause of end stage renal disease (ESRD). Persistent albuminuria in the range of 30 - 299 mg/24h has been shown to be an early stage of diabetic nephropathy in type 1 diabetes (T1D) and a marker for development of nephropathy in type 2 diabetes (T2D). It is a well-established marker of increased cardiovascular disease (CVD) risk [1]. Patients with persistent albuminuria (30 - 299 mg/24hrs.) who progress to more significant levels (300 mg/24hrs.) are likely to progress to ESRD [2]. A numbers of interventions have been demonstrated to reduce the risk and slow the progression of renal disease. Intensive diabetes management with the aim of achieving near-normoglycemic level has been shown to delay the onset and progression of albuminuria in patients with T1D [3] and T2D [4] [5]. The UKPDS provided strong evidence that control of high blood pressure can reduce the development of nephropathy [6].

In addition, large prospective randomized studies in patients with T1D have demonstrated that achievement of lower levels of systolic blood pressure (<140 mmHg) resulting from treatment using angiotensin converting enzyme (ACE) inhibitors provides a selective benefit over other classes of antihypertensive drugs in delaying the progression of albuminuria and can slow the decline in glomerular filtration rate (GFR) in patients with higher levels of albuminuria [7] [8]. In T2D with hypertension and normal urinary albumin, renin angiotensin aldosterone system (RAAS) inhibition has been shown to delay the onset of elevated albuminuria [9] [10]. In the latter study, there was an unexpected higher rate of fatal cardiovascular events with olmesartan among patients with preexisting chronic heart disease. ACE inhibitors have been shown to reduce major CVD outcomes such as myocardial infarction (MI), stroke and death, in patients with diabetes [11], thus further supporting the use of ACE inhibitors in patients with elevated albuminuria. Angiotensin receptor blockers (ARBs) have conflicting evidences in preventing onset of elevated albuminuria in normotensive patients with T1D and T2D [12] [13]; however, ARBs have been shown to reduce the progression rate of albumin levels excretion as well as ESRD in patients with T2D [14]. In patient with nephropathy, ARBs have a smaller magnitude of rise in potassium compared with ACE inhibitors [15].

In the absence of side effects or adverse events (e.g., hyperkalemia or acute kidney injury), it is suggested to titrate up to the maximum approved dose for the treatment of hypertension. Combinations of drugs that block the RAAS (e.g., an ACE inhibitor plus an ARB, a mineralocorticoid antagonist, or a direct renin inhibitor) provide additional lowering of albuminuria [16]. However, such combinations have been found to provide no additional cardiovascular benefit and have higher adverse event rates [17].

The aim of this work is to compare the vascular protective effects of the ACE-inhibitor, Ramipril, versus the ARB, Irbesartan, in low and high doses on kidneys of diabetic rat model treated or non-treated with insulin.

2. Methods

2.1. Induction of DM

DM in rats was induced by intra-peritoneal (i.p.) administration of nicotinamide (NAD) (230 mg/kg), 15 min prior to the single dose of streptozotocin (STZ) (65 mg/kg, i.p.) [18]. Control animals were received an equal volume of saline. The STZ solution contained STZ dissolved in saline with a sodium citrate buffer, pH 4.0. The blood glucose levels (by using standard diagnostic kits) were recorded to monitor the degree of diabetes.

2.2. Groups and Treatments

One hundred and ten male albino rats were used in this study. They were divided into 7 main groups. Group (1) was served as control, which received no medications. Group (2) was diabetic induced rats as mentioned above. Group (3) was controlled by insulin after induction of DM. The first three groups consisted of 10 rats each.

Groups from the fourth to the seventh consisted of 20 diabetic induced rats, and further subdivided as follows: group (4a) consisted of 10 rats and treated orally with low dose of ramipril (0.3 mg/Kg) for 60 days. Group (4b) consisted of 10 rats and treated orally with low dose of ramipril (0.3 mg/Kg) and insulin for 60 days. Group (5a) consisted of 10 rats and treated orally with low dose of irbesartan (15 mg/Kg) for 60 days. Group (5b) consisted of 10 rats and treated orally with low dose of irbesartan (15 mg/Kg) and insulin for 60 days. Group (6a) consisted of 10 rats and treated orally with high dose of ramipril (1.0 mg/Kg) for 60 days. Group (6b) consisted of 10 rats and treated orally with high dose of ramipril (1.0 mg/Kg) and insulin for 60 days. Group (7a) consisted

of 10 rats and treated orally with high dose of irbesartan (30 mg/Kg) for 60 days. Group (7b) consisted of 10 rats and treated orally with high dose of irbesartan (30 mg/Kg) and insulin for 60 days. The dose of insulin was calculated according to the weight of each rat and the blood glucose level.

2.3. Biochemical Measurements

Two months post treatment, rat-tail blood was collected to measure: fasting blood sugar level (FBS), glycosylated hemoglobin (HbA1c), total and free serum proteins (Albumin & Globulin) and lipid profile. Twenty four hours urine samples were collected for complete urine analysis and albuminuria. Measurements of glucose, HbA1c, serum total protein and albumin, total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) were done using the standard procedures and available commercial kits in a fully automated system (COBAS integra 400 plus). Cholesterol CHOD-PAP, triglycerides GPO-PAP, LDL-C plus second generation, HDL-C plus third generation and reagents (Roche Diagnostics, Indianapolis, IN) were used on the Chemistry Analyzer to determine levels of TC, TG, LDL-C and HDL-C, respectively. All assays were done following the recommended procedures for instrument operation, calibration, quality control, and assay guidelines. The instrument was calibrated using calibrator for automated systems (Roche Diagnostics) for glucose, TC and TG, and calibrator for automated systems lipids (Roche Diagnostics) for LDL-C and HDL-C. Results were expressed for all parameters in mg/dl. Except for HbA1c, where it was expressed as percentage of glycosylated hemoglobin [19].

Estimation of microalbuminuria (MA) levels by immunoassay classic quantitative assay for detection of antigen antibody reaction using a labeled substance to measure the binding of the unlabeled substance to a specific antibody or other receptor. The auto analyzer measures the change in absorbency at 380 nm spectral wavelength, and the value obtained is proportional to the urine albumin sample.

2.4. Histopathological Examination

All specimens were fixed in 10% formalin, embedded in paraffin and stained with heamatoxylin and eosin (H&E) stain and periodic acid Schiff (PAS) staining. Representative sections from each case was examined by light microscope for glomerular basement membrane (GBM) thickening throughout the entire length & mesangial matrix along mesangial cell proliferation and presence or absence of nodular glomerulosclerosis.

2.5. Statistical Analysis

All statistical analyses were performed using the SPSS program (version 16). All quantitative data are described as the mean + SD. Comparisons between the control group and all other groups were performed using the Student's t test or the Mann-Whitney U test, depending on the distribution of the data. The Kruskal-Wallis H test was used when comparing all groups, as some of the data were not normally distributed. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Fasting Blood Sugar

Table 1 and **Figure 1** show that FBS in diabetic rats or that received low or high dose ramipril or irbesartan have significant rise in FBS as compared to normal rats ($P < 0.05$). Diabetic rats that received both drugs in low or high doses and treated with insulin did not show any significant difference from control group. Treated diabetic rats with insulin significantly lowered blood glucose levels as compared to diabetic rats ($P < 0.05$) and administration of either drug (alone without insulin) have insignificant effect on blood glucose levels.

3.2. Glycosylated Hemoglobin

Table 1 and **Figure 2** Show that HbA1c% in diabetic rats; taking low or high doses of ramipril or irbesartan without insulin have significantly higher levels of HbA1c as compared to normal non-diabetic rats. Treated diabetic rats with insulin and on low dose ramipril had little significant elevation of HbA1c as compared to normal non-diabetic rats. However treatment of diabetic rats with insulin whether taking low or high dose of either drug significantly lowered the % of HbA1c as compared to control diabetic rats. It appears that administration of

Table 1. Measurements of biochemical parameters in all Group (n = 10 in each group).

Groups	NC	DR	LDR	HDR	LDI	HDI	LDR1	HDR1	LDI1	HDI1	LDI2	HDI2	DRI
FBS (mg/dl)	101 ± 8	303 ± 139*	347 ± 135*	339 ± 145*	288 ± 89*	360 ± 200*	184 ± 33*	157 ± 31*	149 ± 45*	158 ± 35*	149 ± 45*	158 ± 35*	150 ± 42*
HbA1c (%)	6.0 ± 0.7	11.9 ± 3.3*	10 ± 3.6*	11.4 ± 2.3*	10.9 ± 2.8*	10.3 ± 3.3*	7.5 ± 1.6**	6.4 ± 1.0*	6.3 ± 1.1*	6.1 ± 0.9*	6.3 ± 1.1*	6.1 ± 0.9*	7.9 ± 0.8**
Total proteins (g/dl)	6.7 ± 0.6	7.8 ± 2.8	7.3 ± 0.61	6.4 ± 1.7	5.1 ± 2.3	6.0 ± 0.9	7.5 ± 0.6	7.6 ± 0.3	7.4 ± 0.6	7.2 ± 0.9	7.4 ± 0.6	7.2 ± 0.9	6.8 ± 0.7
Albumin (g/dl)	3.6 ± 0.21	4.2 ± 1.5	3.9 ± 0.52	3.8 ± 0.28	2.91.3	3.2 ± 0.46	3.8 ± 0.21	3.8 ± 0.13	4 ± 0.17	3.6 ± 0.5	4 ± 0.17	3.6 ± 0.5	3.7 ± 0.5
TG (mg/dl)	132 ± 32	70 ± 38	67 ± 29.1	67.6 ± 52.6	49 ± 36.4	98 ± 34	115 ± 65	133 ± 37.6	109 ± 49	122 ± 37.5	109 ± 49	122 ± 37.5	81 ± 25
TC (mg/dl)	144 ± 20	213 ± 75	199 ± 52	126 ± 38	68 ± 35	91 ± 33	172 ± 28	178 ± 21	162 ± 25	165 ± 32	162 ± 25	165 ± 32	137 ± 38
LDL-C (mg/dl)	45.4 ± 14.5	156 ± 54*	144 ± 32.8*	83 ± 29*	37 ± 22.3	47 ± 30	107 ± 22*	108 ± 19*	103 ± 22*	103 ± 22*	103 ± 22*	103 ± 22*	86 ± 32*
HDL-C (mg/dl)	74 ± 9.6	43 ± 15*	42 ± 13.4*	30.3 ± 7.5*	22 ± 10*	24 ± 6.6*	42 ± 8.7*	43 ± 6*	37.1 ± 5.2*	38 ± 8.4*	37.1 ± 5.2*	38 ± 8.4*	30.6 ± 12*
Micro-albuminuria (mg/dl)	32 ± 25	405 ± 261.2*	354.7 ± 114.8*	164.4 ± 21.8**	236.3 ± 73.6**	146.8 ± 39.8**	264.2 ± 53.4**	102.6 ± 37.1**	155.8 ± 41.5**	70.2 ± 42.4**	155.8 ± 41.5**	70.2 ± 42.4**	279.4 ± 58.3**

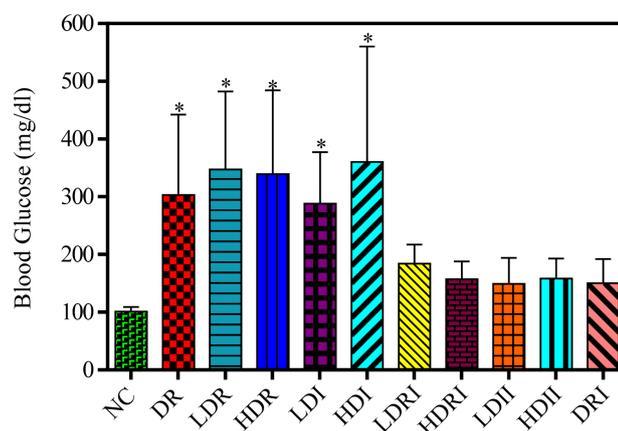


Figure 1. Measurement of FBS in diabetic rats receiving low or high dose of ramipril, or low or high dose of irbesartan in the presence or absence of insulin and compared with control groups ($n = 10$).

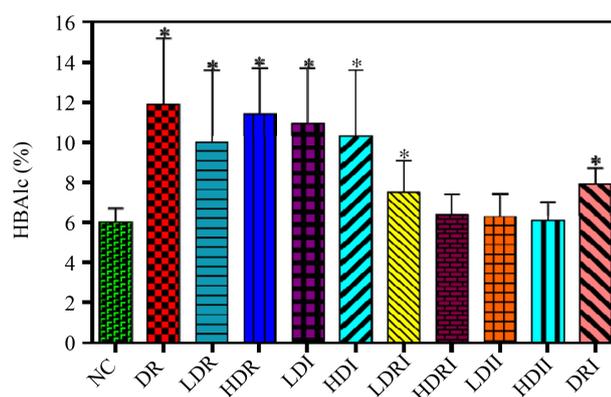


Figure 2. Percentage of HbA1c in diabetic rats receiving low or high dose of ramipril, or low or high dose of irbesartan in the presence or absence of insulin and compared with control groups ($n = 10$).

either drug alone without insulin has no lowering effect on HbA1c in diabetic rats.

3.3. Albuminuria

Table 1 and **Figure 3** showed that biochemically, both ramipril and irbesartan (without insulin) lowered albumin concentration in urine samples especially at high doses. Histopathologically, showed no beneficial response of both drugs without insulin. Combination of insulin together with either drug has beneficial effects biochemically and histopathologically at high doses. Low dose irbesartan only has renoprotective effect in DR treated with insulin as compared to normal non-diabetic rats ($P < 0.05$). Non-insulin treated diabetic rats treated with low or high dose of ramipril or irbesartan have significantly lower urine albuminuria as compared to diabetic rats ($P < 0.05$). Similarly, diabetic rats treated with insulin and low or high dose of ramipril or irbesartan have significantly lower urine albuminuria as compared to diabetic rats ($P < 0.05$). It appears that low dose irbesartan in non-insulin treated diabetic rats and low and high doses of either drug in insulin treated diabetic rats have more significant lowering effects on urine MA as compared to diabetic rats taking low dose ramipril without insulin. **Figure 4** shows that FBS and urine albuminuria are apparently matching each other (*i.e.* the higher the level of FBS, the higher the level of albuminuria and vice versa). Low or high dose irbesartan has more significant lowering effect on urine albuminuria especially in insulin treated groups. Low dose ramipril in non-insulin treated rat appears to have less lowering effect on albuminuria as compared to all other insulin treated and

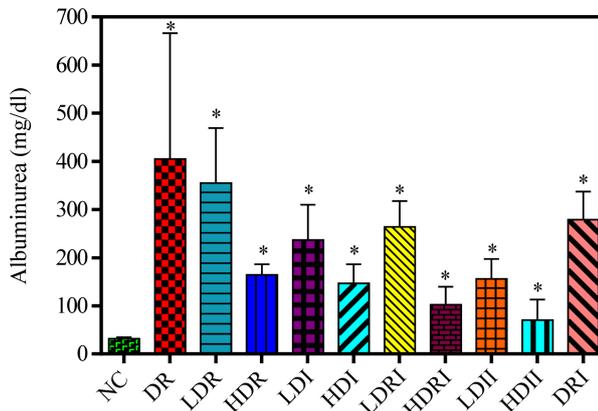


Figure 3. Measurement of albuminuria in diabetic rats receiving low or high dose of ramipril, or low or high dose of irbesartan in the presence or absence of insulin and compared with control groups ($n = 10$).

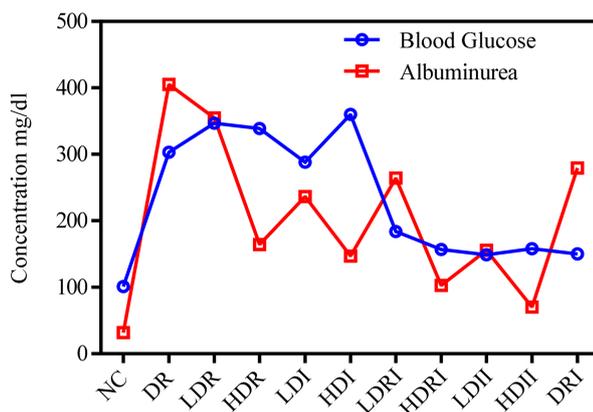


Figure 4. Correlation between the FBS and albuminuria in control groups and in diabetic rats receiving low or high dose of ramipril, or low or high dose of irbesartan in the presence or absence of insulin ($n = 10$).

non-insulin treated rats.

3.4. Lipid Profile

Only LDL-C and HDL-C showed significant changes in lipid profile pattern of the rats studied. LDL-C sig. increased in diabetic rats treated with insulin or not and either taking ramipril or irbesartan or not except rats treated with low and high doses of irbesartan without insulin, as compared to normal control rats. HDL-C sig. decreased in diabetic rats treated with insulin or not, taking either drug in low or high dose or not as compared to normal control rats in most of the groups studied (**Table 1, Figure 5**).

3.5. Total Serum Proteins and Albumin

Table 1 and **Figure 6** show that neither induction of diabetes in rats nor their treatment with insulin or the addition of either drug, affect serum protein or albumin levels in all groups studied as compared to normal non-diabetic rats.

3.6. Histopathology

Histopathological study of all groups shows that light microscopic examination of LDR group demonstrated

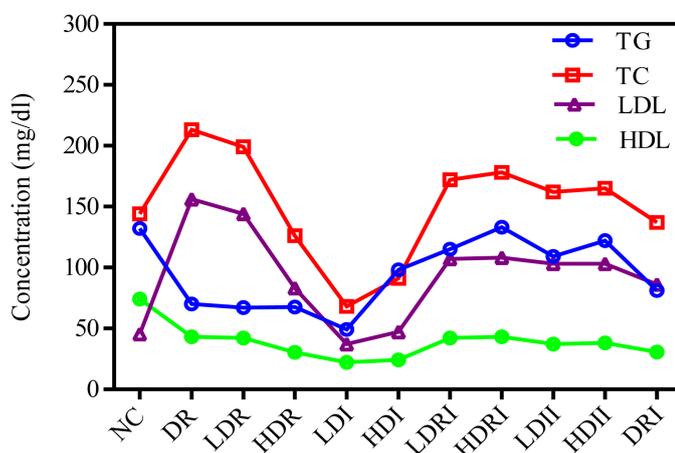


Figure 5. Level of TG, TC, LDL-C and HDL-C in control groups and in diabetic rats receiving low or high dose of ramipril, or low or high dose of irbesartan in the presence or absence of insulin ($n = 10$).

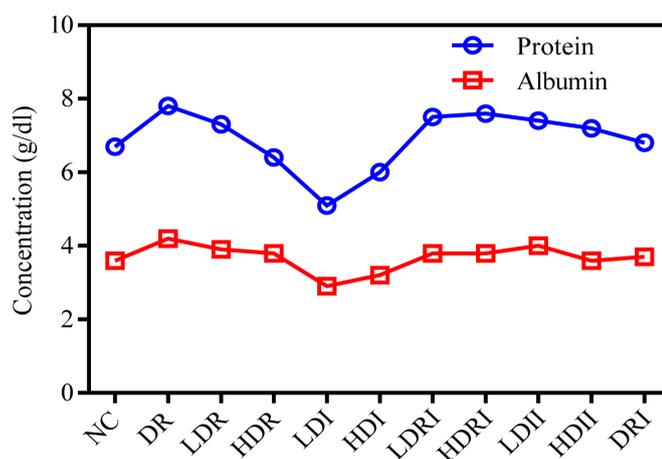


Figure 6. Level of serum proteins and albumin in control groups and in diabetic rats receiving low or high dose of ramipril, or low or high dose of irbesartan in the presence or absence of insulin ($n = 10$).

glomerular basement membrane (GBM) thickening throughout the entire length of diffuse increase in mesangial matrix along mesangial cell proliferation with no improvement in diabetic changes (**Figure 7(a)**). Similarly, HDR group shows GBM thickening throughout the entire length of diffuse increase in mesangial matrix along mesangial cell proliferation with no marked improvement in diabetic changes (**Figure 7(b)**). LDRI group shows slight GBM thickening throughout the entire length and some increases in mesangial matrix along mesangial cell proliferation with little improvement in diabetic changes (**Figure 7(c)**). HDRI group shows slight GBM thickening throughout the entire length and slight increases in mesangial matrix along mesangial cell proliferation with an improvement in diabetic changes (**Figure 7(d)**).

Histopathological examination of LDI and HDI groups shows GBM thickening throughout the entire length and diffuse increase in mesangial matrix along mesangial cell proliferation with no marked improvement in diabetic changes (**Figure 7(e)** and **Figure 7(f)**). Interestingly, LDII and HDII groups show absence of GBM thickening throughout the entire length and there is no increase in mesangial matrix along mesangial cell proliferation with marked improvement in diabetic changes (**Figure 7(g)** and **Figure 7(h)**).

4. Discussion

Despite being increasingly recognized as a CV risk factor, the definition of MA was based on its ability to predict

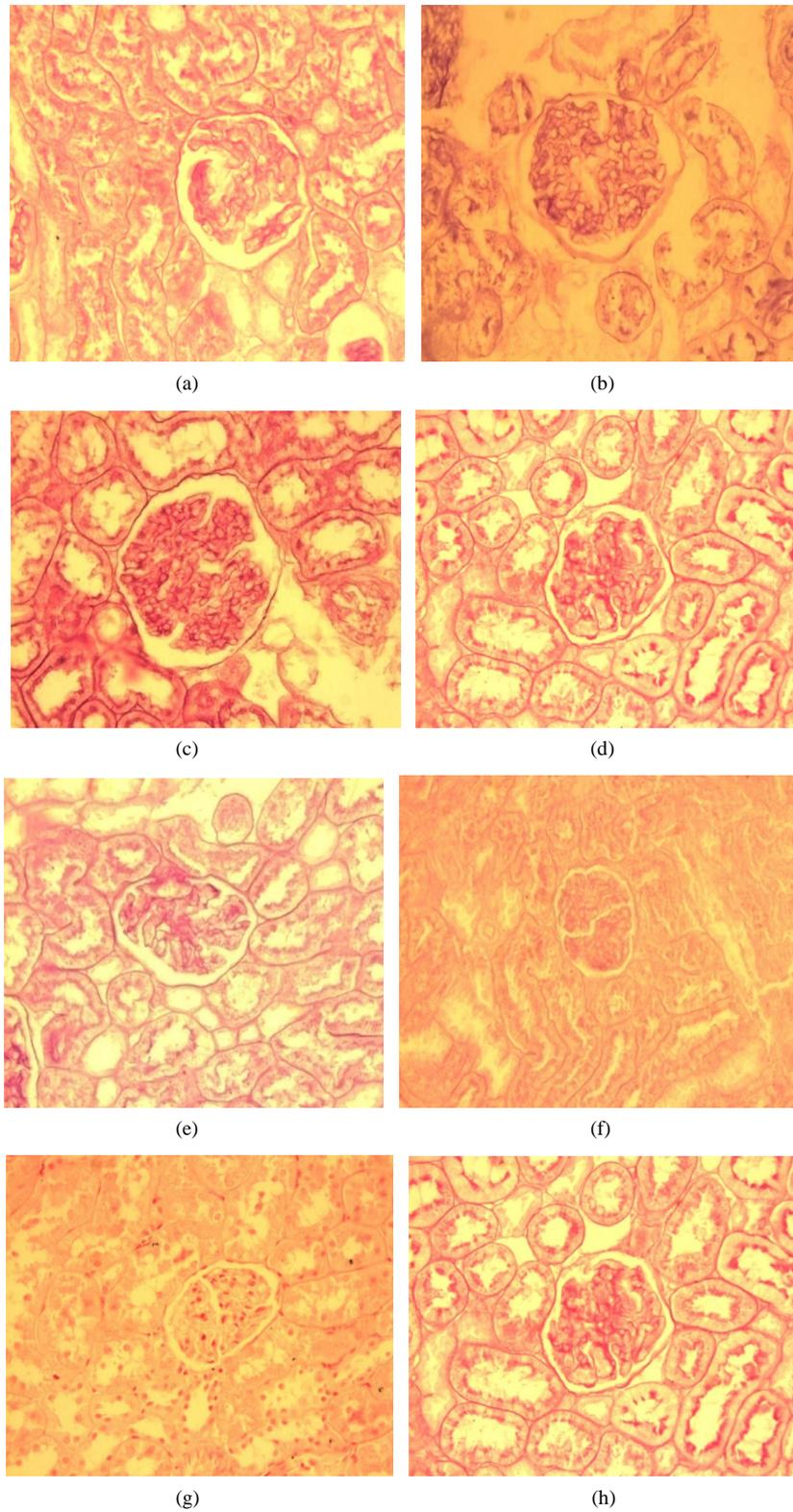


Figure 7. Histopathological examination by light microscope of diabetic rats receiving low or high dose of ramipril, or low or high dose of irbesartan in the presence or absence of insulin. Groups are shown as follows: LDR (a), HDR (b), LDR I (c), HDRI (d), LDR II (e), HDRII (f), LDII (g), HDII (h).

diabetic nephropathy (*i.e.*, MA or clinical proteinuria). Whether individuals with albumin excretion rates below the MA threshold are also at risk for CV disease or whether there is a progressive graded relationship between different degrees of albuminuria and CV events is unclear. Data also suggest that measurement of urinary albumin may help estimate the absolute risk of experiencing a CV event for individuals with or without DM (HOPE). Albuminuria is, however, linked with other risk factors that may themselves be causal or linked with causal processes. These include both diabetic and non-diabetic degrees of hyperglycemia [20], hypertension [21], renal dysfunction [22], dyslipidemia [23], hyperhomocysteinemia [24], dietary proteins [25], and smoking [26]. Albuminuria also reveals increased renal endothelial permeability [25].

Thus measurement of albuminuria as a marker of other CV factors, as well as existing renal endothelial dysfunction, is an easy tool that likely reflects underlying macro- and microvascular disease. Biochemically, our results confirmed the beneficial effect of low and high doses of both ramipril and irbesartan on urinary albumin excretion of diabetic rats as compared to non-treated diabetic rats. However, low dose irbesartan and high dose of both drugs seem to be more beneficial on urine albumin excretion. In contrast to the biochemical results, histopathological studies failed to demonstrate any beneficial effect of both drugs in diabetic rats not controlled with insulin. Experiments in rabbits have shown that atheromatous lesions were successfully prevented by a high dose of trandolapril, whereas a low dose had no effect [26], although plasma ACE activity was inhibited similarly. As for clinical investigation, the SECURE study has shown a strong dose dependency of atherosclerosis progression; a 2.5 mg dose of ramipril did not affect it more than placebo, whereas daily ramipril of 10 mg did [27]. Along the same lines, Parving *et al.* have shown a dose-response effect of the protection from ongoing kidney damage by the angiotensin receptor blocker, irbesartan, at 150 mg and 300 mg daily [15] [28]. The cardiovascular and renal protection by an ACE inhibitor is dose-dependent and has important implications for current clinical care and for research on prevention of cardiovascular disease by the inhibition of the renin-angiotensin system. It means that patients treated by once daily, usual doses of ACE inhibitors in hypertension trials may have experienced a lower cardiovascular benefit than could have been safely achieved if ACE inhibitors had been used at higher doses. Despite the well-known difficulties of interpretation generated by the results of “negative” randomized trials, we conclude that our results, interpreted in the context of other experimental and clinical data, show that high doses of ramipril and irbesartan are preferable to low doses in the prevention of renal events in patients at high risk of cardiovascular events [29]. However, our results showed much more beneficial effects of low and high dose irbesartan compared to ramipril especially in diabetic rats treated with insulin. The mechanism underlying the renoprotection by angiotensin II blockers may be complex, involving lowering the intraglomerular pressure [30], the beneficial effects of diminished proteinuria [31], and decreasing collagen formation [32], that may be related to decreased stimulation of transforming growth factor *b* by angiotensin II [33].

We cannot directly address the issue of whether the effects of ramipril and irbesartan would be equivalent in the treatment of patients with nephropathy due to T2D. It may seem reasonable to assume that agents that primarily reduce the generation or effect of angiotensin II would have similar clinical results. However, it is important to caution that ACE inhibitors and ARB are distinctly different classes of drugs and that one cannot assume equivalence between them.

The pharmacological activity of these agents is complex. The effect of ACE inhibition on renal hemodynamics could be limited by the non-ACE-dependent generation of angiotensin II that has been documented in patients in the hyperglycemic state [31].

The efficacy of ramipril in nephropathy study failed to demonstrate renoprotection in patients with nephropathy due to T2D who received ACE inhibitors [34]. Another study in patients with MA and T2D indicated that ACE inhibition slowed the progression of renal disease [35].

Our data as evidenced by histopathological results reveal that low and high doses of irbesartan combination with insulin is more renoprotective in diabetic rats and overt nephropathy and significantly slows the progression of glomerulopathy. These benefits appear to be independent of the other parameters studied. In contrast to our study, another one has reported that there was no difference in nephropathic changes and glycemic control among patients receiving irbesartan [36]. Nevertheless, another study emphasized that improvement in glycemic control slows the increase in the level of albuminuria and postpones the occurrence of overt diabetic nephropathy in patients with T2D [37].

Our study confirms and extends the finding that antihypertensive treatment has a renoprotective effect in patients with T2D and MA. There has been conflicting evidence regarding the existence of a specific renoprotective

tive effect—that is, a beneficial effect on kidney function beyond the hypotensive effect—of agents, such as ACE-inhibitors, that block the RAAS system in patients with T2D and MA [38] [39]. All the other parameters of the current study showed conflicting results which are not attributed to the inhibition of the RAAS system.

5. Conclusion

In conclusion, low dose irbesartan and high doses of both drugs have renoprotective effect in diabetic rats treated with insulin. In the light of the conflicting information from other reports; our drawn conclusion obtained from our results should be limited to the agents and dosages that we used in this study. According to the published guidelines for the treatment of diabetic kidney disease, routine screening of urine for MA should be performed in all patients with diabetes [39] [40] just as these patients are routinely screened for diabetic retinopathy.

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Declaration of Conflicting Interests

The authors declare that there is no conflict of interest.

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Abbreviations and Symbols

NC (Normal Control);

DR (Diabetic Rats);

DRI (Diabetic Rats Treated with insulin);

LDR (Low Dose Ramipril);

HDR (High Dose Ramipril);

LDI (Low Dose Irbesartan);

HDI (High Dose Irbesartan);

LDRI (Low Dose Ramipril Treated with insulin);

HDRI (High Dose Ramipril Treated with insulin);

LDII (Low Dose Irbesartan Treated with insulin);

HDII (High Dose Irbesartan Treated with insulin);

FBS (Fasting blood sugar);

HbA1c (glycosylated hemoglobin);

TG (triglycerides);

TC (total cholesterol);

LDL-C (low density lipoprotein cholesterol);

HDL-C (high density lipoprotein cholesterol);

Statistically significant ($P < 0.05$) as compared to control rats (*) and statistically significant ($P < 0.05$) as compared to diabetic rats (♥).