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Comparative effects of candesartan and losartan on mesangial expansion and glomerular volume in diabetes rat model receiving rosmarinic acid therapy

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ABSTRACT

Introduction: An oxidative stress and angiotensin II (Ang II) contribute significantly in the pathogenesis of diabetic nephropathy (DN). Therefore, interventions targeting these factors are anticipated to significantly contribute to inhibiting the progression of diabetic kidney disease.

Objectives: The aim of this study was to assess the impact of a combination of rosmarinic acid (RA) and angiotensin receptor blockers (ARBs) in preventing mesangial expansion and reducing glomerular volume in streptozotocin (STZ)-induced diabetic rats.

Materials and Methods: We observed experimental animals, 16 adults male Wistar rats, which were randomly grouped into 4 groups (n=4 per group): negative and positive control groups and diabetic rats treated with RA 75 mg/kg and candesartan 1 mg/kg (treatment group 1) and RA therapy 75 mg/kg and losartan 2.5 mg/kg (treatment group 2). Following 8 weeks of therapy, the renal histology was assessed to evaluate mesangial expansion and glomerular volume.

Results: In comparison to the positive control, treatment group 1 demonstrated a significant inhibition of mesangial expansion (MD: -19.92; 95% CI: -21.97, -17.87; $P<0.001$) and decreased glomerular volume (MD: 2669.06; 95% CI: 2066.31, 3271.80; $P<0.001$). Similarly, treatment group 2 significantly inhibited mesangial expansion (MD: -19.21; 95% CI: -21.26, -17.16; $P<0.001$) and decreased glomerular volume (MD: 2488.04; 95% CI: 1885.29, 3090.78; $P<0.001$) compared to the positive control. While treatment group 1 exhibited better effects than treatment group 2, although the difference was not statistically significant.

Conclusion: In STZ-induced diabetic rats, combination therapy of RA with candesartan or losartan can inhibit mesangial expansion and decrease glomerular volume.

Implication for health policy/practice/research/medical education:
It is anticipated that the outcomes of this study will yield significant insights into the potential of combining the antioxidant rosmarinic acid with specific angiotensin receptor blockers to impede the progression of diabetic nephropathy. This combination could potentially be formulated as an innovative therapeutic approach for diabetic kidney disease, serving as both a prophylactic intervention and a treatment strategy to avert the progression to chronic kidney disease and end-stage renal disease.

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Introduction

Diabetes mellitus is still a significant health burden throughout the world, including Indonesia. In 2015, the number of people with diabetes exceeded 400 million, and projections suggest this figure is anticipated to surpass 600 million by the year 2040. One third of diabetes mellitus patients are estimated to experience diabetic nephropathy (DN), making it a primary contributor to kidney failure worldwide. Current therapeutic treatments for diabetic kidney disease still do not provide satisfactory results in slowing the progression of kidney damage and do not have the ability to prevent the occurrence of diabetic kidney disease (1). DN also leads changes in the histological structure of the glomerulus, namely the thickening of

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mesangial expansion and basement membrane with accumulation of extracellular matrix proteins accompanied by intraglomerular mesangial proliferation and interstitial fibrosis (2). DN induces alterations in the histological composition of the glomerulus, including the thickening of the basement membrane and expansion of the mesangium. These changes involve the accumulation of extracellular matrix proteins, concurrent with interstitial fibrosis and intraglomerular mesangial proliferation (3). The progression of diabetic kidney disease involves numerous intricate pathways in its pathophysiology. Activation system of the renin-angiotensin and oxidative stress are considered pivotal factors in this complex process (2). Therefore, the strategy for managing DN by inhibiting oxidative stress pathways and controlling renin-angiotensin-aldosterone system (RAAS) is expected to be one way to prevent further progression of diabetic kidney disease.

Rosmarinic acid (RA) is recognized for an antioxidant and anti-inflammatory. which is contained in several types of plants, especially the Boraginaceae family. RA has the effect of reducing nuclear factor-κB (NF-κB), increasing glutathione transferase, anti-Bcl-2 activity, consuming peroxynitrite, improving superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) in the kidneys and is able to inhibit connective tissue growth factor. The role of RA has potential as a therapy for DN (4). Likewise, interventional studies have shown that inhibition of the RAAS has proven beneficial in preventing and treating patients with diabetic kidney disease (2). Losartan and irbesartan are angiotensin II receptor blocker (ARB) drugs, which also have an effect on diabetic kidney disease and have been recommended for use in DN therapy in the United States and Europe. Likewise with other ARB groups, such as telmisartan, candesartan and valsartan (2,5,6). The Reduction of Endpoints in non-insulin-dependent diabetes with the Angiotensin II Antagonist Losartan (RENAAL) clinical research indicated a renoprotective effect of administering ARB (losartan) in patients with DN and type 2 diabetes mellitus, in addition to its ability to control blood pressure (7). The Candesartan and Lisinopril Microalbuminuria (CALM) clinical trial showed that the candesartan/lisinopril combination was more effective in reducing the albumin creatinine ratio by 50% compared to candesartan monotherapy (24%) or lisinopril (39%) (8). Based on the RENAAL and CALM studies which showed the success of losartan in the treatment of DN and the potential of candesartan in the treatment of DN (9), Hence, researchers are encouraged to undertake studies to investigate the synergistic effect of combining RA either losartan or candesartan, specifically in terms of the inhabitation mesangial expansion and the reduction of glomerular volume in DN.

Objectives

The objective of this study is to prove the role of oxidative stress and angiotensin-II in the development and progression of DN, as well as to prove whether there is synergism in the combination of anti-oxidants with certain ARBs, in this case candesartan and losartan, and whether there are differences in the effects of the two types of ARBs against inhibiting the development of DN.

Materials and Methods

Study design

In this study, streptozotocin (STZ) was utilized, and procured from Bio World, which was identified by the catalog number 41910012-3 (714990). RA (weight of molecular: 360.31, formula of molecular: C18H16O8) 96% was procured from Sigma-Aldrich Co (St Louis, MO, USA), whereas the product number: 536954 and batch number: BCCF0185. Losartan potassium 50 mg is produced by Kalbe Farma Tbk with registration number GKL0808515209A1 and batch number KTLSTA92071 and Candesartan cilexetil 8 mg is produced by Dexa Medica with registration number GKL1105048016A1 and batch number 52G4137.

Animals

This study used Wistar strain rats (Rattus norvegicus) from animal research facility in Universitas Brawijaya Malang. Prior to commencing the experiment, the Wistar strain rats underwent a period of adaptation lasting 7 days, each housed in individual cages. The rats used were male, 10-12 weeks old, weighing 150-200 g, in good health, characterized by active movement, no defects, and no hair loss. Exclusion criteria were diabetic mice that did not achieve hyperglycemia and died during treatment. This research has received ethical approval from the Faculty of Medicine, Universitas Brawijaya Malang with number 29/EC/KEPK/02/2021.

Diabetes induction

Following a 7-day adjustment period, the rats had treated a high-fat diet for 21 days. Then, on the 22nd day the Wistar strain rats in diabetes group were given STZ to induce diabetes mellitus. Diabetes induction was carried out by injecting a single dose of 40 mg/kg STZ diluted in 0.1 M sodium citrate buffer (pH 4.5) to a concentration of 4 mg/ml, administered intraperitoneally (10). Rats in the age-matched control group were administered comparable volumes of sodium citrate buffer without STZ. Following the STZ injection, the rats had access to a regular diet and water. On the fifth day post-STZ injection, blood glucose levels were assessed using a glucose meter (Nesco). Rats

were considered to have developed diabetes if their blood glucose exceeded 270 mg/dL (15 mmol/L) and were subsequently included in the study (10).

Experimental design

The number of rat used was 16, with 4 rats taken randomly from each group, namely the healthy rats as negative control (NC) group, diabetic rats without therapy as the positive control (PC) group, the combination treatment group of AR 75 mg/kg/d with candesartan 1 mg/kg/d (therapy group 1) and the combination treatment group of AR 75 mg/kg/d with losartan 2.5 mg/kg/d (therapy group 2). Once the blood sugar target was achieved, rats in the respective treatment groups received therapy, whereas rats in the control group were administered only saline solution. After an eight-week period following the initiation of treatment, and collection of urine specimens. Anesthetized rats conducted for blood collection through the kidneys, and cardiac punctures were performed for subsequent analysis of biochemical. Any remaining biological material was disposed of in accordance with established standards of biosecurity.

Renal histological analysis

The analysis of renal histological was conducted following established procedures (11). The kidneys were subsequently embedded in paraffin, preserved in 10% buffered formalin, and sliced into 3-µm sections. These sections were then stained with eosin and hematoxylin for further examination. Observations were conducted with a light microscope (Olympus) at a magnification of 400×. Photographs were taken of forty randomly chosen glomeruli per sample section. The glomerular area (GA) was determined using ImageJ 1.48 software, and the glomerular volume (GV) was computed utilizing the formula of Rangan and Tesch; $GV = 1.2545 \times (GA)^{1.5}$ (12).

Statistical analysis

Data analysis used the one-way ANOVA comparative hypothesis test and post-hoc Tukey HSD (Honestly Significant Difference test) which met the requirements of the data normality test and variance homogeneity test with a confidence level of 95% ($\alpha = 0.05$), and a significance level of 0.05 ($P = 0.05$). Data analysis using IBM SPSS version 25 software.

Results

The results of blood glucose evaluation from not only the treatment group, but also the positive control group showed that all rat had fasting blood glucose of more than 270 mg/dL, thus meeting the criteria for diabetes model rat. The results of the average blood glucose levels

in each study group are presented in Figure 1, while the glomerular histology is presented in Figure 2.

From mesangial matrix calculations, the treatment group significantly showed an increase in mesangial expansion compared to healthy mice (NC group) (MD: 22.66; 95% CI: 20.62, 24.72; $P < 0.001$). Therapy group 1 significantly inhibited mesangial expansion compared with PC control (MD: -19.92; 95% CI: -21.97, -17.87; $P < 0.001$). Likewise, the therapy group 2 significantly inhibited mesangial expansion compared with the PC group (MD: -19.21; 95% CI: -21.26, -17.16; $P < 0.001$). When compared between therapy group 1 and 2, therapy group 1 compared to the therapy group 2 indicated a better effect in inhibiting mesangial expansion, but it was
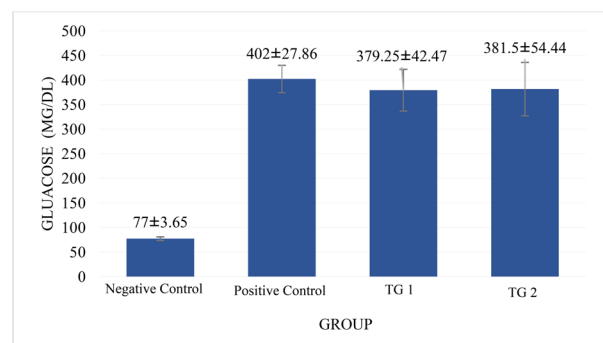

Figure 1. Histogram of blood glucose levels. The findings are expressed in the form of mean ± SD. Abbreviations: TG 1; Therapy group 1, TG 2; Therapy group 2.
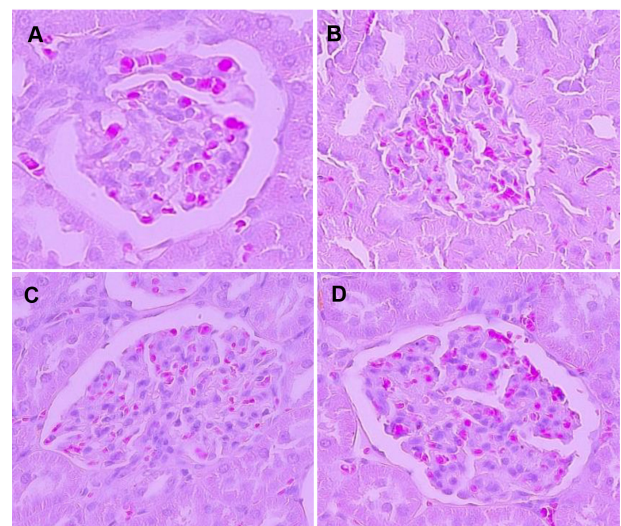

Figure 2. Representative photomicrographs of all groups, captured at an original magnification of 400×, are displayed for the HE-stained kidney sections among the various groups. Notes: A. negative control; B. positive control; C. therapy group 1; D. Therapy group 2. In image B, there is periglomerular fibrosis, capsule thickening, wrinkled tuft, narrowing of Bowman space, small glomerular volume and denser mesangial matrix compared to images A, C, and D.

not significant statistically (MD: -0.71; 95% CI: -2.76, 1.34; $P = 0.735$) (Figure 3).

From the calculation of glomerular volume, in the positive control group showed a significant reduction in glomerular volume compared to healthy mice/NC group (MD: -2683.68; 95% CI: -3286.42, -2080.94; $P < 0.001$). The therapy group 1 significantly inhibited the reduction in glomerular volume compared with the positive control (MD: 2669.06; 95% CI: 2066.31, 3271.80; $P < 0.001$). Likewise, the therapy group 2 significantly inhibited the reduction in glomerular volume compared with the positive control (MD: 2488.04; 95% CI: 1885.29, 3090.78; $P < 0.001$). When comparing between therapy group 1 and 2, the therapy group 1 showed a better effect in inhibiting glomerular volume reduction than the therapy group 2 but it was not significant statistically (MD: 181.01; 95% CI: -421.72, 783.76; $P = 0.809$) (Figure 4).

Discussion

Chronic hyperglycemia conditions result in the production of reactive oxygen species (ROS) and the development of advanced glycation end-products (AGEs). Furthermore, these aberrant metabolic products can activate intercellular signaling for the expression of proinflammatory and profibrotic genes with the production of a number of mediators for cellular injury. One of the complications that arises from chronic hyperglycemia is the development of DN (13). Histopathological changes in the early stages of DN are an increase in the thickness of the glomerular basal membrane and extracellular matrix proteins accumulation in the mesangial which causes mesangial expansion (14). In the present study, findings revealed a noteworthy rise in mesangial expansion and a substantial reduction in glomerular volume of the positive control group which compared to the negative control group.
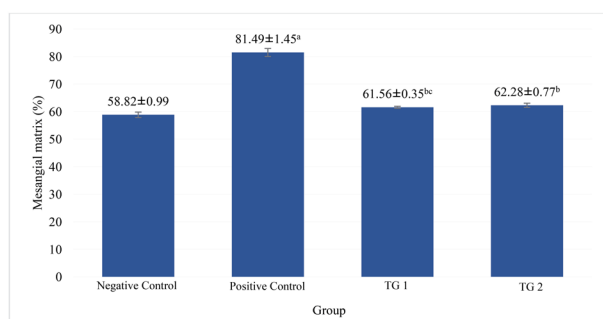
Accumulation of mesangial matrix proteins is characteristic of mesangial expansion. Immunohistochemical studies show that in DN, there is an accumulation of type III, IV, V, and VI collagen, fibronectin and laminin in the mesangial (1). Mesangial cell hypertrophy and accumulation of extracellular matrix that occurs is mediated by transforming growth factor-β (TGF-β). TGF-β production by mesangial cells is activated by hyperglycemia and angiotensin II, which then triggers the production of glomerular mesangial extracellular matrix and also reduces the production of matrix metalloproteinases which are responsible for keeping extracellular matrix degradation under control. The main mediator of TGF-β in mesangial expansion is connective tissue growth factor (CTGF). CTGF can also be stimulated directly by hyperglycemia and AGEs (13,14). In advanced stages, interstitial fibrosis is observed due to the continuous action of TGF-β stimulating the production of collagen and fibronectin (15).

Research by Denic et al has shown the presence of interstitial fibrosis in kidney biopsies with comorbid diabetes (16). Glomerular interstitial fibrosis of more than 25% correlates with smaller glomerular volumes at all depths of the renal cortex. A decrease in glomerular volume in the renal cortex region is associated with increased glomerulosclerosis (%GSG/globally-sclerotic glomeruli) in that area. Glomerulosclerosis (%GSG) is strongly associated with diabetes in deep regions of the renal cortex (17).

Hyperglycemia conditions activate the RAAS and many other mediators, which initially trigger an increase in kidney size, an increase in renal plasma flow (RPF), and an increase in filtration fraction (FF), which together produce abnormal glomerular increases. Glomerular filtration rate (GFR) in the early stages of diabetes is referred to as glomerular hyperfiltration. The increase in RPF and FF is largely the result of a disproportionate decrease in
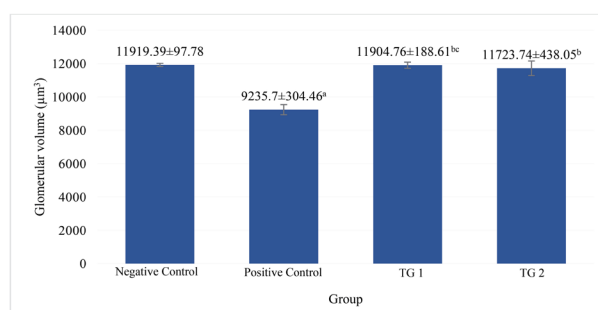

Figure 3. Diagram of Mesangial Matrix Percentage. The findings are expressed in the form of mean ± SD. Notes: a) significantly different compared to the negative control ($P<0.05$); b) significantly different compared to the positive control ($P<0.05$); c) not significantly different compared to therapy group 1 ($P>0.05$). Abbreviations: TG 1; Therapy group 1, TG 2; Therapy group 2.

Figure 4. Diagram of Average Glomerular Volume. The findings are expressed in the form of mean ± SD. Notes: a) significantly different compared to the negative control ($P<0.05$); b) significantly different compared to the positive control ($P<0.05$); c) not significantly different compared to therapy group 2 ($P>0.05$); Abbreviations: TG 1; Therapy group 1, TG 2; Therapy group 2.

afferent versus efferent arteriolar resistance. Increased vasoconstrictors, such as angiotensin II, thromboxane, and endothelin 1, have a stronger effect on increasing efferent arteriolar resistance. An imbalance between afferent and efferent arterioles will increase intraglomerular pressure and cause physical stress on the capillary, podocyte and mesangial walls over time, which will ultimately trigger a profibrotic response in DN characterized by a decrease in glomerular volume (13,18).

In this study, the results showed that diabetic mice that received candesartan and RA candesartan or losartan and RA significantly inhibited mesangial expansion and decreased glomerular volume compared to positive control mice. This condition may occur due to the synergy of the anti-inflammatory, anti-oxidant, and anti-fibrosis effects in RA with the angiotensin II inhibitory effects of candesartan and losartan. Not only in vivo but also in vitro studies have demonstrated the antioxidant effects of RA against peroxidative damage to biological membranes. Compared with caffeic acid and other derivatives, RA is one of the compounds that has the potential to inhibit the highly reactive 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (20). In kidney tissue, RA can improve CAT, SOD and GPX activity, and can reduce NF-κβ activity and increase peroxynitrite consumption, anti-Bcl-2 and glutathione transferase activity (9). In vivo studies show that RA is able to block TNF-α expressed by the NF-κB activation in diabetic mice (4). RA significantly decreased p65 NF-κB expression in diabetic mouse models compared with vitamin E (21).

Administration of RA 50 mg/kg significantly prevented mesangial expansion in the kidneys of rats that received nephrotoxic induction with CdCl2 (cadmium chloride) by eliminating oxidative free radicals, increasing cellular redox defense, accelerating cadmium clearance and preventing inflammatory signal transduction mediated by NF-κB, TNFRII, MAPK and protein kinase C delta (PKC-δ). RA also significantly prevented collagen deposition mediated by TGF-β1, alpha-smooth muscle actin (α-SMA), and SMAD3 in the kidneys of rat that received nephrotoxic induction with CdCl2 (cadmium chloride) which can induce renal fibrosis (22). Administration of RA can reduce the expression levels of TGF-β1, type I collagen, vimentin, fibronectin, phosphorylated AKT (p-AKT), and α-SMA. This study implies that RA may offer a viable therapeutic agent in effectively managing renal interstitial fibrosis (23).

Candesartan is an ARB that highly selectively inhibits angiotensin II type I receptor (AT1R) in oxidative stress and kidney inflammation. Candesartan suppresses chemokine induced expression of NF-κB and TNF-α activation of AT1R blockade in cultured renal tubular epithelial cells. Inhibition of this receptor causes a direct antioxidant effect by reducing ROS production caused by TNF-α or restoring redox homeostasis and pro-oxidant hydrogen peroxide. A study evaluated whether candesartan could regulate angiotensin-converting enzyme 2 (ACE2)/ Mas receptor (protective axis of the renin-angiotensin system)/ Ang II type 2 receptor (AT2R) in diabetic rat. In diabetic rat that received a medium dose of candesartan 1 mg/kg/d for 4 weeks, there was improvement in renal tubular damage and albuminuria, increased expression of the ACE2/AT2R/Mas receptor axis, the effect of reducing ERK1/2 phosphorylation and reducing fibrosis (24).

Weil et al conducted a study on subjects receiving losartan 100 mg per day and placebo, finding lower volume of mesangial fractional in subjects treated with losartan (18.8 vs 25.6%; $P = 0.02$) (25). Losartan (20 μM), significantly decreased the proliferation of rat mesangial cells induced by Ang-II. All increases in the production of collagen type I, fibronectin, collagen type III, mRNA protein, and also collagen type IV expression induced by Ang II can be attenuated by administration of losartan (26). Angiotensin II can increase the expression of TRPC6 (transient receptor potential channel 6) and increase the concentration of intracellular calcium ions, both of which are associated with the proliferation of mesangial cells. Studies show that losartan can reverse the effects of Ang-II resulting in inhibition of mesangial cell proliferation (27).

Another study has shown that administration of losartan to DN model rat resulted in improvements in systolic blood pressure, serum glucose, urea, GFR, and a decrease in serum Ang-II levels. Histopathological results showed clear improvement in glomerulosclerosis, vascular and tubular injury parameters in the losartan group (28).

Based on the results of these studies, it has proven the important role of antioxidant-based therapy and RAAS blockade in inhibiting the progression of DN which aligns with the outcomes observed of the study. However, our study has limitations, especially the absence of a single treatment group, as well as using only one dose of therapy, either RA, candesartan or losartan.

Conclusion
Our research shows that the combination of RA either losartan or candesartan demonstrates an inhibitory effect on mesangial expansion and a reduction in glomerular volume in diabetic mouse models. Although not significant, the results of this study also showed a better effect of candesartan compared with losartan.

Limitations of the study
This study had several limitations, including no separate groups for RA or ARBs, the observation period was only 8 weeks, and only used one dose of the drug.

Authors' contribution

Conceptualization: Nur Samsu.
Data curation: All Authors.
Formal analysis: Nur Samsu.
Funding acquisition: Pandu Tridana Sakti, Achmad Rifai.
Investigation: Pandu Tridana Sakti.
Methodology: Nur Samsu.
Project administration: Pandu Tridana Sakti, Achmad Rifai.
Resources: All Authors.
Software: Pandu Tridana Sakti
Supervision: Nur Samsu.
Validation: Nur Samsu, Achmad Rifai.
Visualization: Nur Samsu, Pandu Tridana Sakti
Writing–original draft: Nur Samsu, Achmad Rifai.
Writing–review & editing: Nur Samsu, Achmad Rifai.

Conflicts of interest

The authors declare that they have no competing interests.

Ethical issues

This study adhered to the guidelines for animal studies and received approval from the Ethics Committee of the Medical Faculty of Universitas Brawijaya Malang under reference number 29/EC/KEPK/02/2021. We strived to adhere to the guidelines concerning animal experiments as stipulated by the United States National Institutes of Health (NIH, 1978). Ethical issues (including plagiarism, data fabrication, double publication) have been completely observed by the authors.

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