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ΕΡΕΥΝΗΤΙΚΗ ΕΡΓΑΣΙΑ

Modulation of asymmetric dimethylarginine (ADMA), nitric oxide, and urinary nephrin levels in diabetic nephropathy rats by rosmarinic acid with candesartan or losartan treatment

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ΑΡΧΕΙΑ ΕΛΛΗΝΙΚΗΣ ΙΑΤΡΙΚΗΣ 2026, 43(1):42–51

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OBJECTIVE **To assess asymmetric dimethylarginine (ADMA), nitric oxide, and urinary nephrin levels following treatment with rosmarinic acid combined with either candesartan or losartan in diabetic nephropathy (DN) rats.** METHOD **A post-test-only control group study was conducted between January and October 2021. The study groups included healthy rats (placebo), untreated DN rats (streptozotocin-induced), T1 (streptozotocin+ rosmarinic acid+candesartan), and T2 (streptozotocin+rosmarinic acid+losartan). After the treatments, ADMA, nitric oxide, and urinary nephrin levels were measured. Associations were determined using ANOVA and *post hoc* Tukey tests.** RESULTS **A total of 20 rats were analyzed. DN rats treated with the combination of rosmarinic acid and candesartan showed lower levels of urinary nephrin compared to untreated DN rats. However, the combination of rosmarinic acid and losartan did not significantly impact urinary nephrin levels. Additionally, we were unable to demonstrate a clear effect of the combination of rosmarinic acid with either candesartan or losartan on ADMA and nitric oxide levels.** CONCLUSIONS **Rosmarinic acid combined with candesartan has a beneficial effect on reducing urinary nephrin levels in DN rats.**

Ρύθμιση των επιπέδων της ασύμμετρης διμεθυλαργινίνης (ADMA), του νιτρικού οξειδίου και της νεφρίνης ούρων σε αρουραίους με διαβητική νεφροπάθεια μετά από θεραπεία με ροσμαρινικό οξύ σε συνδυασμό με καντεσαρτάνη ή λοσαρτάνη

Περίληψη στο τέλος του άρθρου

Key words

Angiotensin receptor blockers
Asymmetric dimethylarginine
Diabetic nephropathy
Nitric oxide
Rosmarinic acid
Urinary nephrin

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Diabetic nephropathy (DN) is a serious health issue and is considered the predominant cause of end-stage renal disease (ESRD).[1,2] The prevalence of ESRD is estimated to be between 10.6% and 13.4%,[3,4] with approximately 35% to 50% of cases attributed to DN.[1] The mortality rate for DN has been reported to range from 16.9% to 26.7%.[3,5] Proper management of DN remains challenging, as its pathogenesis is complex and involves various mechanisms, including oxidative stress,[6] endothelial dysfunction,[7] and podocyte detachment.[8] These processes can be detected through specific biomarkers: Asymmetric dimethylarginine (ADMA) and nitric oxide for endothelial dysfunction,[7] nitric oxide for oxidative stress,[6] and urinary nephrin for podocyte detachment.[8]

Over the past decade, research into potential therapeutic agents for managing DN has significantly advanced, leading to the identification of therapeutic targets such as oxidative stress,[9] and endothelial dysfunction.[10] Rosmarinic acid, a class of aqueous phenolic compounds, has been shown to possess antioxidant properties.[11] However, reports on the antioxidant activity of rosmarinic acid specifically in DN are limited. Nonetheless, its antioxidant effect has

been demonstrated in cases of lung and liver injury involving oxidative stress.[12] Therefore, rosmarinic acid may also have a beneficial impact on reducing oxidative stress in DN. Additionally, as angiotensin II is an essential factor in the development of endothelial dysfunction,[13–15] treatment with angiotensin receptor blockers (ARBs) may offer benefits for endothelial dysfunction in DN.[16–18] Previous studies have shown that ARBs can improve endothelial progenitor cell function in hypertension.[19] Of all ARBs, candesartan and losartan have been reported to exhibit the most significant angiotensin II receptor blocking, antioxidant, and anti-inflammatory effects.[20,21] Given their potential to improve oxidative stress and endothelial dysfunction, these treatments may also indirectly benefit podocyte detachment. We hypothesized that the combination of rosmarinic acid with either candesartan or losartan could improve ADMA, nitric oxide, and urinary nephrin levels in DN. Currently, no studies have explored these potential combination treatments in DN. Accordingly, our study aimed to investigate ADMA, nitric oxide, and urinary nephrin levels following treatment with rosmarinic acid and either candesartan or losartan in a DN rat model.

MATERIAL AND METHOD

Animal preparation and allocation

Male brown rats (Rattus norvegicus), aged seven to nine weeks, were sourced from the Central Animal House of Universitas Brawijaya. The Ethical Committee of Universitas Brawijaya, Malang, Indonesia (no 29/EC/KEPK/02/2021) approved all animal protocols, which were performed in strict adherence to Indonesian laws and experimental animal guidelines, in line with Institutional Animal Care and Use Committee protocols.[22] The study was conducted at the Biomedical Laboratory, Pharmacological Laboratory, and Pathological Anatomy Laboratory of Universitas Brawijaya. The animal housing area was maintained at a temperature of 23±1°C.

The rats were housed individually and fed an adaptation diet consisting of 60% fats for one week to induce insulin resistance and diabetes. Twenty rats were randomly distributed among four different study groups: Healthy rats (placebo), untreated DN rats (streptozotocin-induced), T1 (streptozotocin+rosmarinic acid 40 mg/kg+candesartan 1 mg/kg), and T2 (streptozotocin+rosmarinic acid 40 mg/kg+losartan 2.5 mg/kg). The doses of rosmarinic acid and ARBs used in this study were based on previous recommendations.[23,24]

Rosmarinic acid and ARBs were administered starting on the first day after the target blood glucose levels were reached. The healthy and the untreated DN rats were administered a saline solution. Rosmarinic acid, freshly dissolved in 25% ethanol at a concentration of up to 0.1 mL per 100 g, was administered via gavage once a day for eight weeks in the morning.

Induction of diabetic rats

After the adaptation period, the rats were given a high-fat diet for a duration of three weeks. They were then fasted for six-eight hours before receiving streptozotocin. The streptozotocin utilized in this study was obtained from Bio World (catalog number 41910012-3, 714990; Bio World, Dublin, OH, US). To induce diabetes mellitus, the study groups (untreated DN rats, T1, and T2) were administered streptozotocin, while the healthy rats received a citrate buffer. Streptozotocin was administered intraperitoneally at a dose of 40 mg/kg, diluted in 0.1 M sodium-citrate buffer (pH 4.5). Afterward, food and water were made available to the rats ad libitum.

Blood glucose levels were assessed on the fifth day after streptozotocin administration using blood samples collected from the tail and assessed with a portable glucometer (Nesco, Fitech UK Ltd, Southampton, UK). We included only rats with fasting blood glucose levels exceeding 280 mg/dL in the study. The protocol for diabetes induction was adapted from a previous study.[25] Blood glucose level data are provided in the supplementary files.

Measurement of albuminuria and urinary nephrin

The protocols for measuring albuminuria and urinary nephrin were adapted from a previous study.[26] Briefly, at the end of the study period, we collected 24-hour urine samples using metabolic cages. Measurements were performed using a rat NPHS2 ELISA kit (96T), catalog no E1595Ra (Bio-Assay Technology Laboratory, Shanghai Korain Biotech Co Ltd, Shanghai, China). We centrifuged the urine samples at 3000–6000×g and used the supernatant to evaluate albuminuria and urinary nephrin levels.

Measurement of creatinine, nitric oxide, and asymmetric dimethylarginine levels

We collected blood samples from the abdominal vena cava with a 19- to 23-gauge needle. For the measurement of creatinine levels, as adapted from a previous study,[26] the blood samples were subjected to centrifugation at a force of approximately 15,000×g, and the supernatant was used to assess creatinine levels. For nitric oxide measurement, blood samples were collected in heparinized tubes, homogenized, and centrifuged at 10,000 r/min for five minutes at 4 °C. The supernatant was used to measure nitric oxide levels. The Modified Griess Method was employed, using the Cobas Mira Automated Analyzer (Block Scientific Inc, Bellport NY, US), as described in a previous study.[27] For ADMA measurement, serum was collected in a tube containing 20 mg of 5-sulfosalicylic acid and stored at 4 °C for 10 minutes. We then centrifuged the samples at 2500×g for 15 minutes at 4 °C, and the supernatant was used to assess ADMA levels, following previously established methods.[28]

Statistical analysis

Data were shown as the mean with standard deviation (SD).

The levels of albuminuria, urinary nephrin, creatinine, nitric oxide, and ADMA between groups were analyzed using an ANOVA test. Pairwise comparisons between groups were conducted using *post hoc* Tukey tests and to determine effect estimates. A p-value below 0.05 indicates statistical significance. Prior to performing the ANOVA, data from each group were assessed for normality. We conducted statistical analysis with Statistical Package for Social Sciences (SPSS), version 18.0 (SPSS Inc, Chicago, IL, USA). A scatter plot was used to visualize comparisons between groups.

RESULTS

Initial data assessment

We assessed the levels of albuminuria, creatinine, urinary nephrin, nitric oxide, ADMA, nephrin-to-creatinine ratio, and albumin-to-creatinine ratio among the different groups: Untreated DN rats, T1, T2, and healthy rats. Before evaluating the final associations, we tested the normality of the data. Our normality test showed that all data within each group conformed to a normal distribution ($p>0.05$). A summary of the normality test results is provided in the supplementary file.

The differences in data between groups were analyzed using ANOVA. Notable differences between groups were observed in the levels of albuminuria, creatinine, urinary nephrin, nitric oxide, and ADMA. However, no significant differences were found in the nephrin-to-creatinine and albumin-to-creatinine ratios between groups. Consequently, *post hoc* Tukey tests were performed to further evaluate the differences in albuminuria, creatinine, urinary nephrin, nitric oxide, and ADMA levels. A summary of the ANOVA test results is presented in table 1.

Albuminuria levels between groups

The average albuminuria levels were 2.50 (±0.54) ng/µL, 1.83 (±0.30) ng/µL, 2.12 (±0.25) ng/µL, and 1.59 (±0.25) ng/µL for the untreated DN rats, T1, T2, and healthy rats, respectively (tab. 1). Our analysis revealed that treatment with the combination of rosmarinic acid and candesartan (T1) (tab. 2) or the combination of rosmarinic acid and losartan (T2) (tab. 3) had no significant impact on albuminuria levels ($p>0.05$). No notable differences were observed when comparing T1 versus T2, healthy rats versus T1, and healthy

Table 1. Summary of the combination of rosmarinic acid and ARB in treating diabetic nephropathy.

Parameters	Untreated DN rats	T1	T2	Healthy rats	p
Albuminuria (ng/µL)	2.50±0.54	1.83±0.30	2.12±0.25	1.59±0.25	0.0190
Creatinine (mg/dL)	0.50±0.26	0.21±0.12	0.75±0.37	0.14±0.08	0.0130
Urinary nephrin ($\times10^3$ ng/µL)	0.09±0.02	0.05±0.01	0.06±0.02	0.05±0.00	0.0020
NO (ng/µL)	69.64±3.85	73.24±5.12	62.01±4.85	93.19±16.15	0.0020
ADMA (ng/µL)	62.60±6.24	48.67±6.10	53.36±11.81	44.18±7.06	0.0420
Nep/Cr ratio	0.23±0.17	0.29±0.17	0.10±0.05	0.50±0.32	0.0920
Alb/Cr ratio	6.99±5.88	10.86±5.04	3.88±3.08	16.63±13.75	0.1890

DN: Diabetic nephropathy, NO: Nitric oxide, ADMA: Asymmetric dimethylarginine

T1: Treatment 1 (rosmarinic acid 40 mg/kg body weight [BW]+candesartan 1 mg/kg BW); T2: Treatment 2 (rosmarinic acid 40 mg/kg BW+losartan 2.5 mg/kg BW)

Table 2. Efficacy of the rosmarinic acid and candesartan combination in treating diabetic nephropathy.

Parameters	Rosmarinic acid and candesartan	Untreated DN rats	MD	95% CI	p
Albuminuria (ng/µL)	1.83±0.30	2.50±0.54	(-0.67)	(-1.42) – (0.07)	0.0830
Creatinine (mg/dL)	0.21±0.12	0.50±0.26	(-0.29)	(-0.79) – (0.20)	0.3420
Urinary nephrin ($\times10^3$ ng/µL)	0.05±0.01	0.09±0.02	(-0.04)	(-0.07) – (-0.02)	0.0020
NO (ng/µL)	73.24±5.12	69.64±3.85	3.60	(15.34) – (22.52)	0.9410
ADMA (ng/µL)	48.67±6.10	62.60±6.24	(-13.93)	(-31.04) – (3.17)	0.1260
Nep/Cr ratio	0.29±0.17	0.23±0.17	0.06	(-0.36) – (0.49)	0.9640
Alb/Cr ratio	10.86±5.04	6.99±5.88	3.87	(-13.00) – (20.74)	0.9020

NO: Nitric oxide, ADMA: Asymmetric dimethylarginine, MD: Mean difference, CI: Confidence interval

Table 3. Efficacy of the rosmarinic acid and losartan combination in treating diabetic nephropathy.

Parameters	Rosmarinic acid+losartan	Untreated DN rats	MD	95% CI	p
Albuminuria (ng/μL)	2.12±0.25	2.50±0.54	(-0.38)	(-1.13) – (0.37)	0.4700
Creatinine (mg/dL)	0.75±0.37	0.50±0.26	0.25	(-0.25) – (0.74)	0.4860
Urinary nephrin ($\times 10^3$ ng/μL)	0.06±0.02	0.09±0.02	(-0.03)	(0.05) – (0.00)	0.0500
NO (ng/μL)	62.01±4.85	69.64±3.85	(-7.63)	(-26.56) – (11.30)	0.6400
ADMA (ng/μL)	53.36±11.81	62.60±6.24	(-9.24)	(-26.34) – (7.86)	0.4120
Nep/Cr ratio	0.10±0.05	0.23±0.17	(-0.13)	(-0.55) – (0.30)	0.8120
Alb/Cr ratio	3.88±3.08	6.99±5.88	(-3.11)	(-19.98) – (13.77)	0.9460

NO: Nitric oxide, ADMA: Asymmetric dimethylarginine, DN: Diabetic nephropathy, MD: Mean difference, CI: Confidence interval

rats versus T2 (p>0.05). However, a significant difference in albuminuria levels was observed between untreated DN rats and healthy rats, with a mean difference (MD) of -0.92 ng/μL (p<0.05) (fig. 1).

Creatinine levels between groups

The mean creatinine levels for untreated DN rats, T1, T2, and healthy rats were 0.50 (±0.26) mg/dL, 0.21 (±0.12) mg/dL, 0.75 (±0.37) mg/dL, and 0.14 (±0.08) mg/dL, respectively (tab. 1). Our statistical analysis did not reveal a significant impact of treatment with either the combination of rosmarinic acid and candesartan (T1) (tab. 2) or the combination of rosmarinic acid and losartan (T2) (tab. 3) on creatinine levels compared to untreated DN rats (p>0.05). We also did not detect any notable differences in creatinine levels between untreated DN rats and healthy rats or between healthy rats and T1 (p>0.05). However, lower creatinine levels were observed in the T1 and healthy rat groups compared to T2, with MDs of -0.54 mg/dL and -0.61 mg/dL, respectively (p<0.05) (fig. 2).

Urinary nephrin levels between groups

The average levels of urinary nephrin were 0.09 (±0.02) ng/μL, 0.05 (±0.01) ng/μL, 0.06 (±0.02) ng/μL, and 0.05 (±0.00)$\times 10^3$ ng/μL for untreated DN rats, T1, T2, and healthy rats, respectively (tab. 1). Our analysis found that urinary nephrin levels were significantly lower in healthy rats compared to untreated DN rats (MD: -0.04; 95% confidence interval [CI]: -0.06 to -0.01; p=0.0060). Comparable results were noted in the group treated with the combination of rosmarinic acid and candesartan (T1) compared to untreated DN rats (MD: -0.04; 95% CI: -0.07 to -0.02; p=0.0020) (tab. 2). However, no significant differences were found in urinary nephrin levels between T2 and untreated DN

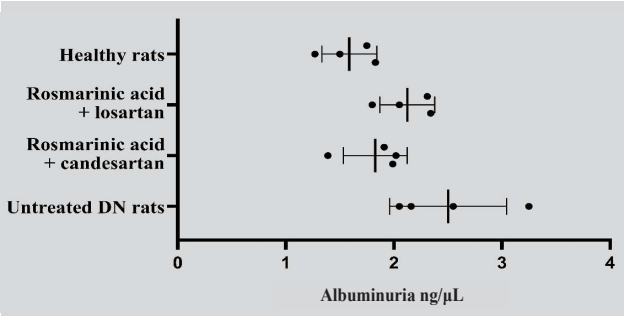

Figure 1. Comparison of albuminuria levels among groups: Healthy rats versus untreated DN rats (MD: [-0.92]; 95% CI: [-1.66, -0.17]; p=0.0160); T1 versus untreated DN rats (MD: [-0.68]; 95% CI: [-1.42, 0.07]; p=0.0830); T2 versus untreated DN rats (MD: [-0.38]; 95% CI: [-1.13, 0.37]; p=0.4700); T1 versus T2 (MD: [-0.30]; 95% CI: [-1.05, 0.45]; p=0.6510); healthy rats versus T1 (MD: [-0.24]; 95% CI: [-0.99, 0.51]; p=0.7790); healthy rats versus T2 (MD: [-0.54]; 95% CI: [-1.29, 0.21]; p=0.1990). DN: Diabetic nephropathy, T1: Treatment 1 (rosmarinic acid 40 mg/kg body weight [BW]+candesartan 1 mg/kg BW); T2: Treatment 2 (rosmarinic acid 40 mg/kg BW+losartan 2.5 mg/kg BW), MD: Mean difference, CI: Confidence interval.

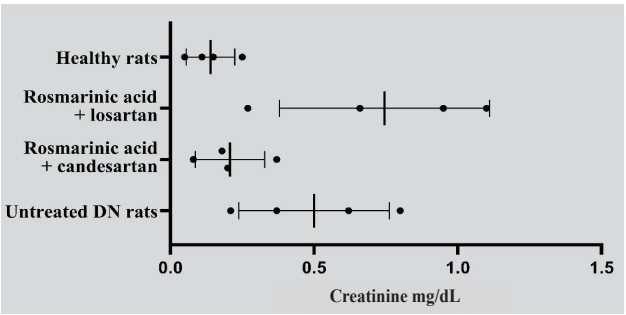

Figure 2. Comparison of creatinine levels among groups: Healthy rats versus untreated DN rats (MD: [-0.36]; 95% CI: [-0.86, 0.14]; p=0.1920); T1 versus untreated DN rats (MD: [-0.29]; 95% CI: [-0.79, 0.20]; p=0.3420); T2 versus untreated DN rats (MD: [0.25]; 95% CI: [-0.25, 0.74]; p=0.4860); T1 versus T2 (MD: [-0.54]; 95% CI: [-1.04, -0.04]; p=0.0330); healthy rats versus T1 (MD: [-0.07]; 95% CI: [-0.56, 0.43]; p=0.9770); healthy rats versus T2 (MD: [-0.61]; 95% CI: [-1.10, -0.11]; p=0.0160). DN: Diabetic nephropathy, T1: Treatment 1 (rosmarinic acid 40 mg/kg body weight [BW]+candesartan 1 mg/kg BW), T2: Treatment 2 (rosmarinic acid 40 mg/kg BW+losartan 2.5 mg/kg BW), MD: Mean difference, CI: Confidence interval.

rats, T1 and T2, healthy rats and T1, or healthy rats and T2 (p>0.05) (fig. 3).

Nitric oxide levels between groups

The mean levels of nitric oxide were 69.64 (±3.85) ng/µL for untreated DN rats, 73.24 (±5.12) ng/µL for T1, 62.01 (±4.85) ng/µL for T2, and 93.19 (±16.15) ng/µL for healthy rats (tab. 1). Our data indicated that treatment with either the combination of rosmarinic acid and candesartan (tab. 2) or the combination of rosmarinic acid and losartan (tab. 3) had no significant impact on nitric oxide levels compared to untreated DN rats (p>0.05). Additionally, nitric oxide levels were similar between T1 and T2 (p>0.05). However, elevated levels of nitric oxide were observed in healthy rats compared to untreated DN rats, T1, and T2, with MDs of 23.55 ng/µL, 19.95 ng/µL, and 31.17 ng/µL, respectively (p<0.05) (fig. 4).

Asymmetric dimethylarginine levels between groups

The mean levels of ADMA were 62.60 (±6.24) ng/µL for untreated DN rats, 48.67 (±6.10) ng/µL for T1, 53.36 (±11.81) ng/µL for T2, and 44.18 (±7.06) ng/µL for healthy rats (tab. 1). Our analysis revealed that ADMA levels were significantly lower in healthy rats compared to untreated DN rats, with a MD of -18.42 ng/µL (p<0.05). However, we did not observe any significant differences in ADMA levels between T1 and untreated DN rats, T2 and untreated DN rats, T1 and T2, healthy rats and T1, or healthy rats and T2 (p>0.05) (fig. 5).

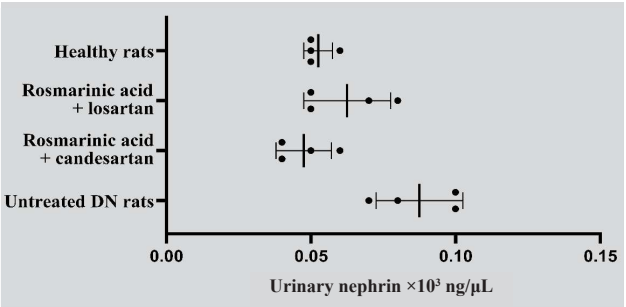

Figure 3. Comparison of urinary nephrin levels among groups: Healthy rats versus untreated DN rats (MD: [-0.04]; 95% CI: [-0.06, -0.01]; p=0.0060); T1 versus untreated DN rats (MD: [-0.04]; 95% CI: [-0.07, -0.02]; p=0.0020); T2 versus untreated DN rats (MD: [-0.03]; 95% CI: [-0.05, 0.00]; p=0.0500); T1 versus T2 (MD: [-0.02]; 95% CI: [-0.04, 0.01]; p=0.3280); healthy rats versus T1 (MD: 0.01; 95% CI: [-0.02, 0.03]; p=0.9320); healthy rats versus T2 (MD: [-0.01]; 95% CI: [-0.04, 0.02]; p=0.6450). DN: Diabetic nephropathy, T1: Treatment 1 (rosmarinic acid 40 mg/kg body weight [BW]+candesartan 1 mg/kg BW), T2: Treatment 2 (rosmarinic acid 40 mg/kg BW+losartan 2.5 mg/kg BW), MD: Mean difference, CI: Confidence interval.

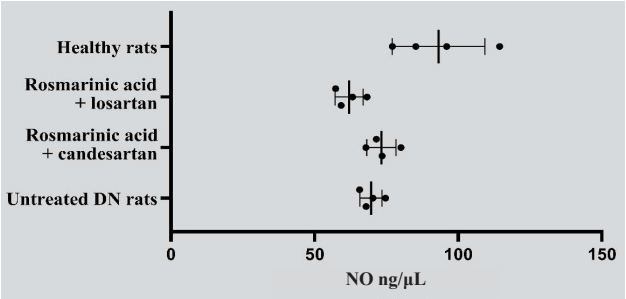

Figure 4. Comparison of nitric oxide levels among groups: Healthy rats versus untreated DN rats (MD: 23.55; 95% CI: [4.62, 42.47]; p=0.0140); T1 versus untreated DN rats (MD: 3.59; 95% CI: [-15.34, 22.52]; p=0.9410); T2 versus untreated DN rats (MD: [-7.63]; 95% CI: [-26.55, 11.30]; p=0.6400); T1 versus T2 (MD: 11.22; 95% CI: [-7.71, 30.15]; p=0.3380); healthy rats versus T1 (MD: 19.95; 95% CI: [1.02, 38.88]; p=0.0380); healthy rats versus T2 (MD: 31.17; 95% CI: [12.24, 50.10]; p=0.0020). DN: Diabetic nephropathy, NO: Nitric oxide, T1: Treatment 1 (rosmarinic acid 40 mg/kg body weight [BW]+candesartan 1 mg/kg BW), T2: Treatment 2 (rosmarinic acid 40 mg/kg BW+losartan 2.5 mg/kg BW), MD: Mean difference, CI: Confidence interval.

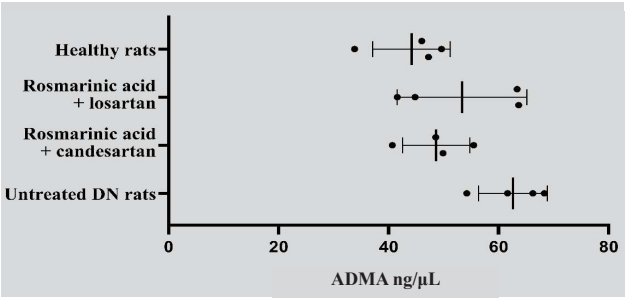

Figure 5. Comparison of asymmetric dimethylarginine (ADMA) levels among groups: Healthy rats versus untreated DN rats (placebo) (MD: [-18.42]; 95% CI: [-35.52, -1.32]; p=0.0340); T1 versus untreated DN rats (MD: [13.94]; 95% CI: [-31.04, 3.17]; p=0.1260); T2 versus untreated DN rats (MD: [-9.24]; 95% CI: [-26.34, 7.86]; p=0.4120); T1 versus T2 (MD: [-4.69]; 95% CI: [-21.79, 12.41]; p=0.8460); healthy rats versus T1 (MD: [-4.49]; 95% CI: [-21.59, 12.62]; p=0.8630); healthy rats versus T2 (MD: [-9.18]; 95% CI: [-26.28, 7.92]; p=0.4180). DN: Diabetic nephropathy, T1: Treatment 1 (rosmarinic acid 40 mg/kg body weight [BW]+candesartan 1 mg/kg BW), T2: Treatment 2 (rosmarinic acid 40 mg/kg BW+losartan 2.5 mg/kg BW), MD: Mean difference, CI: Confidence interval.

DISCUSSION

Our study found that urinary nephrin levels were lower in DN rats treated with rosmarinic acid and candesartan compared to untreated DN rats. Additionally, lower urinary nephrin levels were observed in healthy rats compared to untreated DN rats. Notably, the MD in urinary nephrin levels between healthy rats and DN rats treated with rosmarinic acid and candesartan was similar to that observed between untreated DN rats and healthy rats, suggesting that rosmarinic acid and candesartan treatment may improve urinary

nephrin levels to a degree similar to that of healthy rats. This study is the first to report on the effects of rosmarinic acid and candesartan on urinary nephrin levels in a DN rat model, so direct comparisons with other studies were not possible. However, similar studies evaluating urinary nephrin levels in DN treatment have been conducted. Samsu et al found that DN rats treated with rosmarinic acid had lower urinary nephrin levels compared to a positive control group.[29] Additionally, other research has shown that DN rats treated with ARBs had reduced urinary nephrin levels. They also noted that untreated DN rats had decreased nephrin expression, suggesting that altered nephrin expression may occur. ARBs were associated with inhibited nephrin expression, which may contribute to lower nephrin levels.[30] Furthermore, a study using anti-inflammatory drugs found that the administration of the calcineurin inhibitor FK506 was associated with lower nephrin levels compared to placebo in DN rats.[31] Given that rosmarinic acid is known for its anti-inflammatory properties,[11] it is plausible that the combination of rosmarinic acid and candesartan in our study led to lower urinary nephrin levels compared to placebo in DN rats.

The underlying mechanism of how rosmarinic acid and candesartan affect urinary nephrin levels in DN rats is not fully defined. However, some hypotheses can be proposed. The pathogenesis of DN is complex and involves a range of biomarkers.[32] Regarding nephrin, its expression is transiently increased in the first eight weeks after diabetes onset, followed by a long-term decrease.[33] Nephrin is localized at the slit membrane between adjacent podocytes in the glomerulus, and its primary function is to prevent protein passage through the glomerular barrier. Disruption of nephrin can lead to increased urinary nephrin and proteinuria, contributing to diabetic kidney disease.[34] Additionally, angiotensin II has been reported to cause podocyte injury.[35] This injury and inflammation may downregulate nephrin expression, resulting in increased urinary nephrin.[26] Targeting inflammation in podocytes and inhibiting angiotensin II might offer promising therapeutic outcomes. In our study, we administered candesartan to inhibit angiotensin II activity and mitigate podocyte injury. Previous research has shown that ARBs can dilate efferent arterioles, reduce glomerular capillary pressure, and protect the glomerulus.[36] Studies have also indicated that ARB treatment can ameliorate high glucose-induced hypertrophy[37] and mitigate podocyte injury.[38] Additionally, podocytes are key responders to serum amyloid A, an important mediator in DN.[39] Rosmarinic acid has been documented to suppress the aggregation and activity of serum amyloid A.[40] Therefore, it is plausible that the combination of rosmarinic acid and candesartan in our study contributed to the improvement of urinary nephrin levels.

Our current findings revealed that healthy rats had lower levels of albuminuria compared to untreated DN rats. However, we were unable to determine the impact of treatment with either the combination of rosmarinic acid and candesartan or the combination of rosmarinic acid and losartan on albuminuria levels in DN rats. Our study also indicated that healthy rats and DN rats treated with rosmarinic acid and candesartan had lower levels of creatinine compared to DN rats treated with rosmarinic acid and losartan. Previous studies have explored similar contexts. They found that rosmarinic acid administration was associated with a reduced risk of podocyte detachment and lower levels of albuminuria compared to control.[29] Another study reported that ARBs could prevent podocyte loss and subsequently reduce urinary albumin excretion.[30] Additionally, research indicated that elevated intrarenal angiotensin II levels were associated with the onset and advancement of albuminuria and podocyte dysfunction in type 2 diabetic rats.[41] Albuminuria is a key indicator of DN progression and reflects the severity of glomerular damage.[42] It also correlates with the degree of podocyte detachment, which is crucial for preventing protein loss in urine.[43] Inflammation of podocytes in DN may lead to excessive albuminuria.[26] These findings suggest that podocyte disruption might underlie the higher levels of albuminuria in DN rats. However, the effectiveness of the combination treatment with ARBs and rosmarinic acid remains uncertain.

Our findings showed that nitric oxide levels were lower in untreated DN rats and in DN rats treated with either the combination of rosmarinic acid and candesartan or the combination of rosmarinic acid and losartan compared to healthy rats. This suggests that treatment with either combination had insufficient power to improve nitric oxide levels in rats with DN. Evidence and mechanisms for DN treatment with rosmarinic acid and ARBs in relation to nitric oxide levels are limited. However, several proposed mechanisms might explain our findings. The pathogenesis of diabetic kidney disease involves a range of biomarkers, including oxidant markers.[44] Oxidative stress is known to affect the development of DN, which may explain why nitric oxide levels were lower in untreated DN rats compared to healthy rats. Key oxidative biomarkers implicated in DN include nuclear factor-E2-related factor 2 (Nrf2), silent information regulator factor 2-related enzyme 1 (Sirt1), nuclear factor kappaB (NFκB), and antioxidant response element (ARE).[45] Treatments targeting the balance between oxidants and antioxidants might offer beneficial outcomes. Rosmarinic acid, a phenolic compound with proven antioxidant proper-

ties[46] has been reported to reduce oxidative stress in DN.[47,48] Additionally, study indicated that rosmarinic acid is capable of suppressing connective tissue growth factor (CTGF) in treating DN.[49] In our study, the dosage of rosmarinic acid was 40 mg/kg, which is lower than the 50 mg/kg used in previous studies.[47,48] This lower dosage might account for the unexpected results. Furthermore, renal oxidative stress is also influenced by the renal expression of the p47phox component of NAD(P)H oxidase and endothelial nitric oxide synthase (eNOS).[50] Treatment with candesartan has been shown to improve renal oxidative stress, although this was typically administered via continuous infusion in other studies.[51] This difference in administration method might explain the discrepancy in outcomes.

Our study did not clarify the role of treatment with either the combination of rosmarinic acid and candesartan or the combination of rosmarinic acid and losartan in improving ADMA levels in rats with DN. This study is the first to assess the impact of combining rosmarinic acid and ARBs on ADMA levels in DN rats, and therefore, direct comparisons could not be made. The role of ADMA in DN remains unclear. It is hypothesized that ADMA, an endogenous inhibitor of NOS, could be essential in the development of endothelial dysfunction.[52,53] Elevated ADMA levels have been linked to a greater risk of tubulointerstitial ischemia in the initial phases of DN[54] and are linked to low nephron numbers and renal dysfunction.[55] Additionally, suppression of ADMA by dimethylarginine dimethylaminohydrolase (DDAH) has been shown to prevent progressive loss of peritubular capillaries (PTCs).[56] Endothelial dysfunction is a critical element in the advancement of kidney disease.[57] This theory may explain why ADMA levels were lower in healthy rats compared to untreated DN rats in our study. In endothelial dysfunction, biomarkers such as vascular cell adhesion molecule (VCAM) and reactive oxygen species (ROS) may be elevated.[57] Therefore, treatments aimed at improving endothelial dysfunction might offer beneficial outcomes. Studies have shown that ARB treatment is associated with reduced VCAM expression and increased NO levels.[58] Moreover, rosmarinic acid, with its antioxidant properties, may also positively impact endothelial dysfunction.[46] However, studies in rat models are limited and specific interventions, including drug dosage, gene-gene interactions, and pharmacogenetic aspects, might also influence the final findings.

Our current study was the first to document the role of rosmarinic acid and ARBs in the management of DN by assessing the levels of urinary nephrin, nitric oxide, and ADMA. Of these markers, only urinary nephrin levels were significantly affected by the treatment with rosmarinic acid and ARBs. This study might provide additional insights into the roles of nephrin, nitric oxide, and ADMA in the pathological process of DN. Furthermore, it offers an initial investigation into the efficacy of rosmarinic acid and ARBs in managing DN. However, our study focused only on candesartan and losartan. Other ARBs should also be investigated to fully understand their potential effects.

In our present study, several potential limitations were discussed. First, we did not assess other potential biomarkers that might also influence the development of DN, such as markers of glomerular dysfunction, tubular dysfunction, and renal inflammation.[32] Second, our study utilized a post-test only control group design. A study employing a pre-test post-test control group design may provide more robust evidence. Third, we only evaluated candesartan and losartan as ARBs in our study. Further research involving additional ARBs may be necessary to obtain more comprehensive findings.

In conclusion, our study revealed that treatment with the combination of rosmarinic acid and candesartan has a beneficial impact on DN rats by ameliorating urinary nephrin levels. This study provided additional insights into the role of rosmarinic acid and ARBs in the treatment of DN.

ΠΕΡΙΛΗΨΗ

Ρύθμιση των επιπέδων της ασύμμετρης διμεθυλαργινίνης (ADMA), του νιτρικού οξειδίου και της νεφρίνης ούρων σε αρουραίους με διαβητική νεφροπάθεια μετά από θεραπεία με ροσμαρινικό οξύ σε συνδυασμό με καντεσαρτάνη ή λοσαρτάνη

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ΣΚΟΠΟΣ Η αξιολόγηση των επιπέδων της ασύμμετρης διμεθυλαργινίνης (ADMA), του μονοξειδίου του αζώτου και της νεφρίνης ούρων μετά από θεραπεία με ροσμαρινικό οξύ σε συνδυασμό είτε με καντεσαρτάνη είτε με λοσαρτάνη σε αρουραίους με διαβητική νεφροπάθεια (DN). **ΥΛΙΚΟ-ΜΕΘΟΔΟΣ** Η μελέτη διεξήχθη κατά το χρονικό διάστημα Ιανουαρίου– Οκτωβρίου 2021. Οι ομάδες μελέτης περιλάμβαναν υγιείς αρουραίους (εικονικό φάρμακο), αρουραίους DN που δεν έλαβαν θεραπεία (επαγόμενοι από στρεπτοζοτοκίνη), T1 (στρεπτοζοτοκίνη+ροσμαρινικό οξύ+καντεσαρτάνη) και T2 (στρεπτοζοτοκίνη+ροσμαρινικό οξύ+λοσαρτάνη). Μετά τις θεραπείες, μετρήθηκαν τα επίπεδα ADMA, μονοξειδίου του αζώτου και νεφρίνης ούρων. Οι συσχετίσεις προσδιορίστηκαν εφαρμόζοντας δοκιμασίες ANOVA και *post hoc* Tukey. **ΑΠΟΤΕΛΕΣΜΑΤΑ** Αναλύθηκαν συνολικά 20 αρουραίοι. Οι αρουραίοι DN που έλαβαν θεραπεία με τον συνδυασμό ροσμαρινικού οξέος και καντεσαρτάνης εμφάνισαν χαμηλότερα επίπεδα νεφρίνης ούρων σε σύγκριση με αρουραίους DN που δεν έλαβαν θεραπεία. Ωστόσο, ο συνδυασμός ροσμαρινικού οξέος και λοσαρτάνης δεν επηρέασε σημαντικά τα επίπεδα νεφρίνης στα ούρα. Επί πλέον, δεν κατέστη δυνατή η ανάδειξη μιας σαφούς επίδρασης του συνδυασμού ροσμαρινικού οξέος είτε με καντεσαρτάνη είτε με λοσαρτάνη στα επίπεδα ADMA και νιτρικού οξειδίου. **ΣΥΜΠΕΡΑΣΜΑΤΑ** Το ροσμαρινικό οξύ σε συνδυασμό με καντεσαρτάνη έχει ευεργετική επίδραση στη μείωση των επιπέδων νεφρίνης στα ούρα σε αρουραίους DN.

Λέξεις ευρετηρίου: Αναστολείς των υποδοχέων της αγγειοτασίνης, Ασύμμετρη διμεθυλαργινίνη, Διαβητική νεφροπάθεια, Μονοξείδιο του αζώτου, Νεφρίνη ούρων, Ροσμαρινικό οξύ

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