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Research Article

Aqueous chicory seed extract ameliorates diabetic kidney damage via alteration of renal renin-angiotensin system (RAS) balance

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ABSTRACT

Objectives: This study investigated the effects of aqueous chicory seed extract (CSE), metformin (Met), and aspirin (Asp) on the Renin-Angiotensin System (RAS) in healthy Wistar rats, as well as in early (NIA/STZ) and late-stage diabetes (STZ).

Methods: Rats were divided into Control, NIA/STZ, and STZ groups. NIA/STZ rats received niacinamide/streptozotocin, while STZ rats received STZ to induce early and late stages of diabetes. Subgroups received CSE (125 mg/kg), metformin (100 mg/kg), or aspirin (120 mg/kg). Measurements included mRNA levels of AGT, ACE, and ACE2; activities of ACE and ACE2; levels of Ang II and Ang-(1-7); protein carbonyl content (PCC); nitric oxide (NO); and kidney collagen.

Results: Late-stage diabetes (STZ) decreased AGT, ACE, and ACE2 mRNA, but increased ACE activity, Ang II, Ang-(1-7), the ACE/ACE2 ratio, PCC, and collagen. CSE increased AGT and ACE2 mRNA, and decreased ACE activity, Ang II, the ACE/ACE2 ratio, and PCC. Metformin boosted AGT mRNA and reduced PCC and collagen. Aspirin lowered collagen. Early diabetes (NIA/STZ) decreased AGT, ACE2, and Ang-(1-7), while increasing ACE activity and Ang II levels. CSE increased AGT and Ang-(1-7), and reduced Ang II and the Ang II/Ang-(1-7) ratio. Metformin reduced ACE mRNA and increased Ang-(1-7). CSE decreased reactive oxygen species (ROS) and improved Ang-(1-7) levels, especially in early stages. Both CSE and metformin helped reduce fibrosis.

Conclusion: Our findings suggest that CSE supports renal tissue repair in both early and late stages of T2D by increasing levels of the protective peptide Ang-(1-7).

Keywords: Diabetic nephropathy; Renin-Angiotensin System; Angiotensinogen; ACE; ACE2; chicory seed extract

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Introduction

Diabetic nephropathy (DN), which signifies severe kidney damage associated with diabetes, results from the prolonged accumulation of minor structural abnormalities in various components, including the glomerulus, tubular epithelial cells, interstitial fibroblasts, and vascular endothelial cells (1). Recent studies suggest that the onset of renal complications in diabetes is primarily linked to the heightened activity of the renin-angiotensin-aldosterone system (RAAS or RAS) (2, 3). RAS is a crucial vasoactive system responsible for regulating blood pressure, as well as cardiovascular and renal functions. An overactive RAS can lead to a range of cardiovascular and kidney disorders (4). According to the canonical pathway, the systemic RAS begins with the action of renin, which cleaves angiotensinogen (AGT) to yield the inactive decapeptide angiotensin I. The endothelial enzyme angiotensin-converting enzyme (ACE) then converts angiotensin I into angiotensin II (Ang II). This process triggers the secretion of aldosterone from the adrenal glands, leading to vasoconstriction, increased sodium and water retention, and a rise in blood pressure (5, 6).

Numerous RAS components are found within various tissues, including the brain, kidneys, vascular endothelium, and heart. RAS consists of two opposing pathways. The pressor arm features the ACE–Angiotensin II–AT1 receptor axis, with the octapeptide Angiotensin II being the most extensively studied element. The second arm, which serves a counter-regulatory function, is characterized by the ACE2–Angiotensin (1-7)–Mas axis, where the heptapeptide angiotensin (1-7) [Ang-(1-7)] exhibits multiple biological effects (7). Ang II activates multiple signaling pathways, leading to the generation of reactive oxygen species (ROS) and promoting inflammation, apoptosis, and fibrosis in cellular structures. Ang-(1-7), synthesized by ACE2, binds to the Mas receptor to elicit advantageous effects, including vasodilation, anti-inflammatory responses, inhibition of cell proliferation, reduction in ROS production, prevention of apoptosis, and mitigation of fibrosis (8). Ang-(1-7) is widely distributed throughout the body, circulating in various tissues, including the heart, brain, liver, spleen, testes, lungs, and kidneys (9). It is recognized that the dysregulation between the two components of the RAS could be a significant factor contributing to renal damage in diabetes. Conditions associated with oxidative stress, ROS, and metabolic syndrome—such as elevated blood glucose levels, increased lipid levels, and inflammation—may provoke the pathological aspect of the RAS, overshadowing its protective function (10).

The management of diabetes and its associated complications has increasingly focused on downstream intracellular signaling pathways and their modulators as potential therapeutic targets (11). Currently, antihypertensive medications that disrupt the RAS—such as ACE inhibitors, angiotensin II receptor blockers (ARBs), aldosterone antagonists, and renin inhibitors—are considered first-line treatments to prevent or delay the onset of nephropathy in patients with microalbuminuria or proteinuria associated with Type 1 and Type 2 diabetes (12). Additionally, the use of antioxidants and anti-inflammatory agents may also prove beneficial. For instance, the anti-inflammatory properties found in various natural products and extracts from medicinal plants may help restore balance to the RAS and potentially slow the progression of diabetic kidney disorders (13, 14). Our earlier studies indicated that the aqueous extract of *Cichorium intybus* L. (commonly known as chicory), referred to as CSE, exerts beneficial effects on the kidneys of rats with diabetes induced by streptozotocin (STZ) and a combination of niacinamide and STZ (NIA/STZ) (15). The objective of the present study was to examine the mRNA expression levels of AGT, ACE, and ACE2; assess ACE and ACE2 enzyme activity; measure the concentrations of Ang II, Ang-(1-7), PCC, and NO; and evaluate collagen fibrosis in the renal tissues of rats.

Materials and methods

All experiments were conducted using kidney tissues collected from rats in our earlier studies (15, 16).

Preparation of plant extract, metformin, and aspirin

Powdered chicory seeds were immersed in water at a concentration of 20% (w/v), heated in a boiling water bath, and then subjected to lyophilization. The resulting extract was stored at −20 °C, following the method described by Ghamarian et al. (17). Metformin and aspirin tablets were obtained from Chemidaru Industrial Company (Tehran, Iran) and manually crushed prior to use.

Animal care

Eight-week-old healthy adult male Wistar albino rats, weighing between 190 and 260 grams, were obtained from the Institute of Biochemistry and Biophysics at the University of Tehran. The animals were housed in sanitized standard cages (two rats per cage) within a controlled facility maintained at 22 ± 2 °C, under a 12-hour light/dark cycle. Rats had ad libitum access to standard chow and water throughout the study.

Induction of experimental diabetes

Diabetes was induced by administering streptozotocin (STZ) at a dose of 55 mg/kg. For the early-stage diabetes (ET2D) model, NIA was injected at 200 mg/kg, 15 minutes after STZ administration. No additional injections were given for the late-stage diabetes (LT2D) model. Both STZ and NIA were freshly prepared in chilled citrate buffer (0.3 ml) immediately

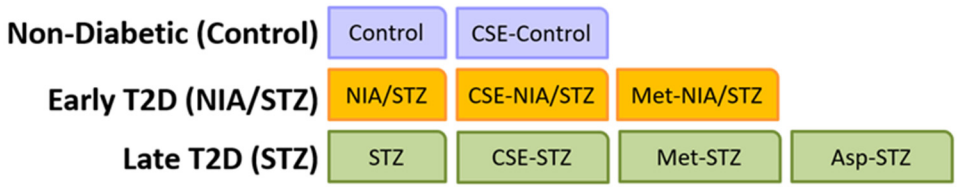

Figure 1. Designations of Groups. Treatment duration: 21 days. Treatments consisted of CSE, 125 mg; metformin, 100 mg; and aspirin, 120 mg, administered daily on a per-kilogram basis of weekly body weight. Citrate buffer was used as vehicle; CSE: chicory seed extract; Met: metformin; Asp: aspirin).

before injection (17). Hyperglycemia was confirmed by measuring peripheral blood glucose from the tail vein on days 4 and 10 post-injection using a GlucoSure STAR device (ApexBio, Taiwan). Rats receiving STZ and exhibiting fasting blood sugar (FBS) levels above 300 mg/dl on both days were classified as LT2D. Those treated with NIA/STZ and maintaining FBS levels between 140 and 220 mg/dl were categorized as ET2D (16). Control rats received vehicle injections, excluding STZ and NIA. STZ and NIA were purchased from Sigma-Aldrich Co. (St. Louis, MO).

Experimental design, group designation, and treatment

Following the induction of diabetes, the rats were categorized into nine distinct groups, each consisting of six individuals, as shown in Figure 1. The treatment regimen commenced on the tenth-day post-induction. The vehicle was administered to Groups 1 (Control, non-diabetic), 3 (NIA/STZ or ET2D), and 6 (STZ or LT2D) at a volume of 0.3 ml. CSE was administered to Groups 2 (CSE-control), 4 (CSE-NIA/STZ), and 7 (CSE-STZ), while metformin was injected into Groups 5 (Met-NIA/STZ) and 8 (Met-STZ). For 21 consecutive days, daily intraperitoneal injections of CSE and metformin were administered. Furthermore, Group 9 (Asp-STZ) received oral aspirin via gavage daily. CSE, metformin, and aspirin were accurately measured into individual microtubes and stored in refrigeration following weekly body weight evaluations. Each substance was mixed with 0.3 mL of freshly prepared, autoclaved, and cooled citrate buffer (20 mM, pH 4.5) immediately before administration (16).

Sample collection

At the conclusion of the treatment period on day 31, rats were anesthetized using diethyl ether and subsequently euthanized. Kidneys were extracted, immediately immersed in liquid nitrogen, and stored at −80 °C for further analysis.

Real-time PCR

A transverse section of each kidney tissue sample (weighing <200 mg/ml) was homogenized on ice in 1 ml of cold TRIzol. Total RNA was extracted following the manufacturer's protocol (GeneAll Hybrid-R Blood RNA, Seoul, Korea) and purified using GeneALL RNA Rioboclear (Seoul, South Korea). Complementary DNA (cDNA) was synthesized via reverse transcription using the First Strand cDNA Synthesis Kit (Takara PrimeScript 1st strand cDNA Synthesis Kit, Kusatsu, Shiga, Japan). Primer sequences used for PCR are available upon request.

Real-time PCR was performed on the cDNA samples using the SYBR Premix Ex Taq II kit (Takara, Kusatsu, Shiga, Japan), with fluorescence monitored via the Rotor-Gene Q system (Qiagen, Germany). Target mRNA levels were normalized to β-actin and quantified using the $2^{-\Delta\Delta Ct}$ method.

Enzyme-linked immunosorbent assay of kidney tissue AngII and Ang-(1-7)

Kidney tissue sections were homogenized using a glass homogenizer in 1 ml of ice-cold PBS buffer containing PMSF and an antiprotease cocktail (Sigma Chemical). The homogenates were centrifuged at 10,000×g for 15 minutes at 4 °C, and the supernatants were stored at −80 °C for subsequent analysis. Angiotensin II and Angiotensin-(1-7) concentrations were quantified using the Rat Angiotensin II ELISA kit (Cloud-Clone Corp., Houston, USA) and the Rat Angiotensin-(1-7) ELISA kit (MyBioSource, San Diego, USA), respectively. ACE activity was assessed using the spectrophotometric method described by Cushman and Cheung, which measures the production of hippuric acid from the substrate Hippuryl-L-Histidyl-L-Leucine (HHL) (18). The assay protocol followed the product datasheets provided by Sigma-Aldrich (St. Louis, USA). ACE activity was expressed in units per milliliter, and specific activity was calculated in units per milligram of protein.

ACE2's specific activity assay

The catalytic activity of ACE2 was assessed fluorometrically utilizing Abz-Ser-Pro-Tyr(NO2)-OH (Abz-SPY(NO2)) as the substrate (Peptides International, USA). Each sample's tissue homogenate supernatant was incubated in 1 mL of 100 mM Tris–HCl buffer at pH 7.5, containing 10 µM ZnCl2, 0.5 µM captopril (an ACE inhibitor), 50 nM thiorphan (a neprilysin inhibitor), and 10 µM of the fluorogenic substrate, at 37 °C for 4 hours.

The fluorescence was quantified using a spectrometer, set to an excitation wavelength of 320 nm and an emission wavelength of 420 nm (19). After determining the ACE2 activity for each sample (expressed in RFU/min), the specific activity of the enzyme was calculated (in RFU/min per mg of protein).

Determination of protein carbonyl content (PCC)

PCC was determined using the method developed by Reznick and Packer (1994) (20). Both sample and control tubes contained 200 µl of tissue homogenate. In the experimental tube, 800 µl of 2,4-dinitrophenylhydrazine (DNPH) at a concentration of 10 mM in 2.5 mM HCl was added, while the control tube received 800 µl of 2.5 mM HCl alone. Tubes were incubated at room temperature for 1 hour, with vortex mixing every 15 minutes. After incubation, 1 ml of 20% (w/v) trichloroacetic acid (TCA) was added, and the mixture was centrifuged at 10,000 rpm for 5 minutes at 4 °C to collect protein precipitates. A final precipitation step was performed using 10% TCA. The pellet was washed three times with 1 ml of a 1:1 (v/v) ethanol–ethyl acetate solution. The final precipitate was dissolved in 500 mg of 6 M guanidine hydrochloride and centrifuged for 10 minutes to remove residual debris. Subsequently, 220 µl of the supernatant from both sample and control tubes was transferred to a 96-well plate, and absorbance was measured at 370 nm using an Eon Microplate Spectrophotometer. Protein carbonyl content was calculated using the equation specified in the original protocol.

Protein Carbonyl (nmol/ml) = [(ODsample-ODcontrol) / ($0.01 \mu M^{-1}$)] ($500\mu l_{GdnHCl}/200\mu L_{Sample}$). The actual extinction coefficient for DNPH at 370 nm is 22000 $M^{-1}cm^{-1}$ (0.022 $\mu M^{-1}cm^{-1}$). This value has been adjusted for the solution's path length in the well. Protein carbonyl content was quantified by calculating the ratio of protein carbonyl levels (in nmol/ml) to total protein concentration (in mg/ml), determined using the Bicinchoninic acid (BCA) Protein Quantification Kit (Parstous, Mashhad, Iran).

Carbonyl Content (nmol/mg) = (Protein Carbonyl nmol/ml)/ (protein mg/ml).

Measurement of NO by Griess assay

Nitric oxide production was assessed in tissue homogenates through the measurement of nitrite (NO2) concentration. For protein precipitation, 200µl of the tissue homogenate was incubated with NaOH and ZnSO4 at room temperature. The supernatant and Griess reagent were subsequently added to each well of a 96-well plate. Absorbance was measured at a wavelength of 570 nm utilizing an Eon Microplate Spectrophotometer. The concentration of nitric oxide (NO) was determined using a standard curve derived from sodium nitrite (NaNO2) solutions with concentrations ranging from 1.5 to 100 mM.

Collagen staining by Masson's trichrome method

The Trichrome Masson's method was used to identify collagen fibers in kidney tissues. Rat kidney specimens were fixed in 10% buffered formalin, embedded in paraffin, and sectioned at a thickness of 5 µm. Slides were stained using Masson's trichrome and analyzed under a light microscope. Images of the stained specimens were acquired, and ImageJ software was used to quantify the collagen area in each image, measured in μm^2.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 10. Comparisons among groups were conducted using ordinary one-way analysis of variance (ANOVA). Data are expressed as mean ± standard deviation. A significance level of $p < 0.05$ was established.

Results

Angiotensinogen mRNA expression

Renal AGT mRNA levels showed a significant decrease in both the LT2D (STZ group) and ET2D (NIA/STZ group) compared to the control groups (Control and CSE-Control) (Fig. 2). An increase in AGT mRNA expression was observed in the CSE-NIA/STZ group compared to the NIA/STZ group ($p < 0.05$). Administration of metformin (Met-NIA/STZ group) did not affect AGT mRNA levels. In the LT2D group, rats treated with CSE and metformin exhibited a significant increase in AGT gene expression compared to the STZ group, with a notably greater rise in the CSE-STZ group ($p < 0.001$) than in the Met-STZ group ($p < 0.05$). Aspirin treatment (Asp-STZ group) did not affect AGT gene expression in the LT2D group.

Ang II and Ang-(1-7) concentration

Diabetes development was associated with elevated Ang II levels in both the NIA/STZ and STZ groups. Administration of CSE and metformin significantly reduced Ang II concentrations in both ET2D and LT2D groups, as shown in Fig. 3. Aspirin treatment also led to a decrease in Ang II levels in the LT2D group.

The NIA/STZ group exhibited a slight decrease in Ang-(1-7) peptide levels, whereas the STZ group showed a significant increase compared to the control group ($p < 0.05$). In the ET2D group, administration of both CSE and metformin led to a significant elevation in Ang-(1-7) concentrations relative to the NIA/STZ group ($p < 0.05$). Within the LT2D group, no significant differences in Ang-(1-7) peptide levels were observed among treatments with CSE, metformin, or aspirin when compared to the STZ group, as depicted in Fig. 3.

ACE mRNA expression and ACE activity

The STZ group exhibited a significant decrease in ACE mRNA expression compared to the control groups

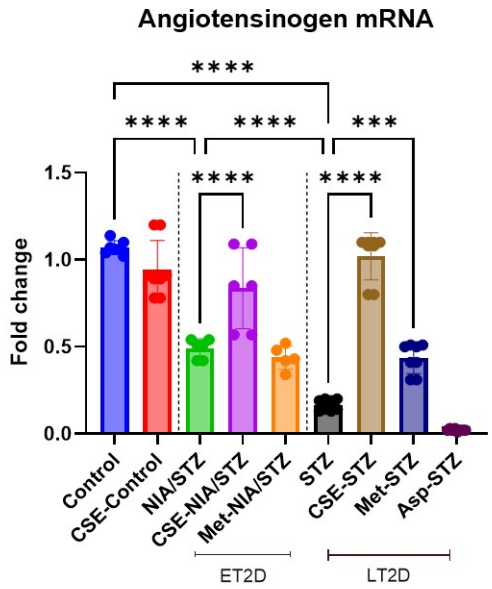

Figure 2. Angiotensinogen mRNA expression. Every histogram displays the mean ± standard deviation from three distinct trials. Ordinary one-way ANOVA test was used to identify which differences between each group were ****P<0.0001 from the control. Citrate buffer was used as vehicle; CSE: Chicory seed extract; Met: Metformin; Asp: Aspirin.

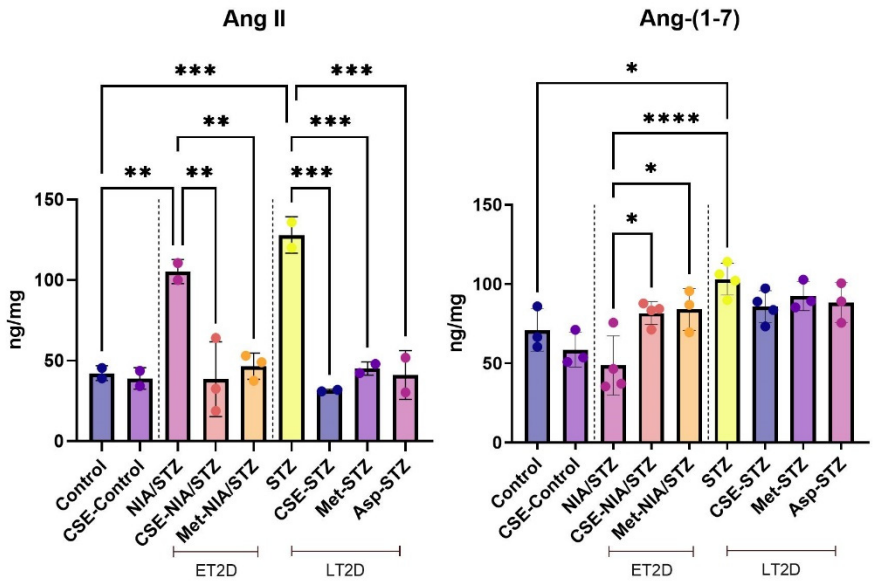

Figure 3. Ang II mRNA expression and Ang (1-7) concentration in the studied groups. (*p<0.05;**p<0.001; ***p<0.001).

(p < 0.001). Treatments with CSE, metformin, and aspirin did not significantly alter ACE mRNA levels relative to the STZ group. However, metformin significantly increased ACE mRNA expression in the ET2D group compared to the NIA/STZ group (p < 0.001), whereas CSE treatment showed no significant effect (Fig. 4). ACE enzymatic activity was elevated in both the NIA/STZ and STZ groups. In the LT2D groups, treatments with CSE, metformin, and aspirin significantly inhibited ACE activity (Fig. 4).

ACE2 mRNA expression and ACE2 activity

As shown in Fig. 5, ACE2 mRNA expression was significantly reduced in both the STZ and NIA/STZ groups compared to the control groups. The reduction was more pronounced in the STZ group (p < 0.001) than in the NIA/STZ group (p < 0.05). In ET2D rats, administration of CSE or metformin did not result in a statistically significant change in ACE2 mRNA expression compared to the NIA/STZ group. In contrast, within the LT2D group, CSE treatment significantly

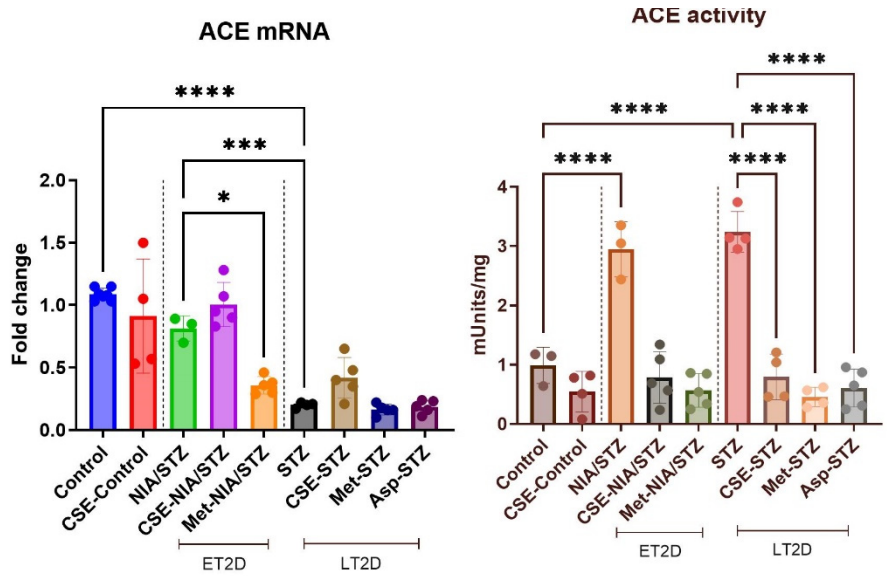

Figure 4. ACE mRNA Expression and activity in the analyzed groups. (*p < 0.05; ***p<0.001; ****p<0.0001).

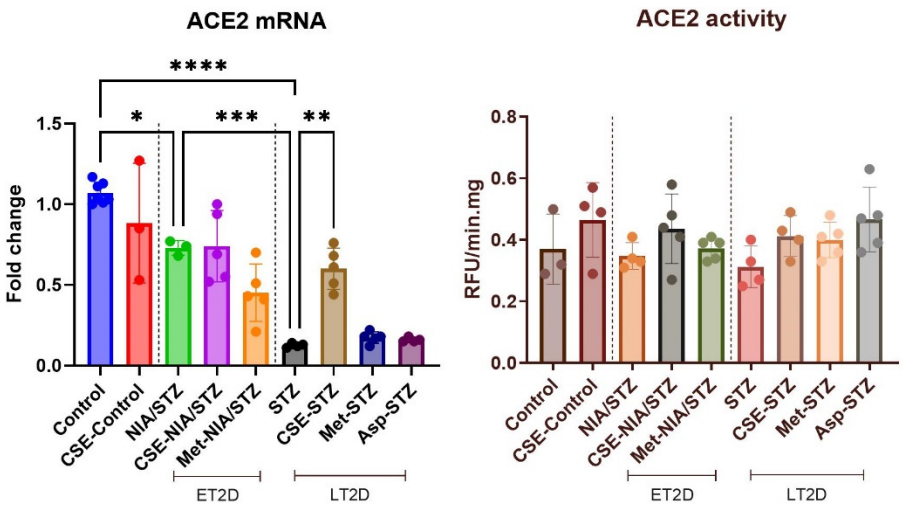

Figure 5. ACE2 mRNA expression and activity in the studied groups. (*p<0.05; **p<0.001; ***p<0.001; ****p<0.0001).

increased ACE2 mRNA expression relative to the STZ group ($p < 0.01$). Neither metformin nor aspirin produced significant changes in ACE2 mRNA expression in the LT2D group. ACE2 enzymatic activity remained unchanged following diabetes induction and subsequent treatments (Fig. 5).

Comparison of ACE: ACE2 mRNA and Ang II: Ang-(1-7) concentration ratios

Figure 6 illustrates that ACE and ACE2 mRNA expression levels followed a similar pattern across the experimental groups. In the STZ group, ACE expression was significantly higher than ACE2 ($p < 0.01$). Chicory extract (CSE) treatment significantly altered this ratio by enhancing ACE2 gene expression. Additionally, the Ang II/Ang-(1-7) concentration ratio was markedly elevated in the NIA/STZ group, a change that was significantly reduced following treatment with CSE and metformin (Fig. 6).

Protein carbonyl content and NO concentration

The STZ group exhibited a significant increase in the protein carbonyl content (PCC) of kidney tissue compared to the control group ($p < 0.05$). No significant difference in PCC was observed between the NIA/STZ and control groups. In the ET2D model, treatment with CSE and metformin did not significantly alter PCC levels relative to the NIA/STZ group. However, in the LT2D model, administration of CSE and metformin led to a significant reduction in PCC levels compared to the STZ group ($p < 0.01$ and $p < 0.05$, respectively). Aspirin treatment did not produce a significant effect on PCC levels in the LT2D group (Fig. 7). Nitric oxide (NO) concentrations remained consistent across all experimental groups (Fig. 7).

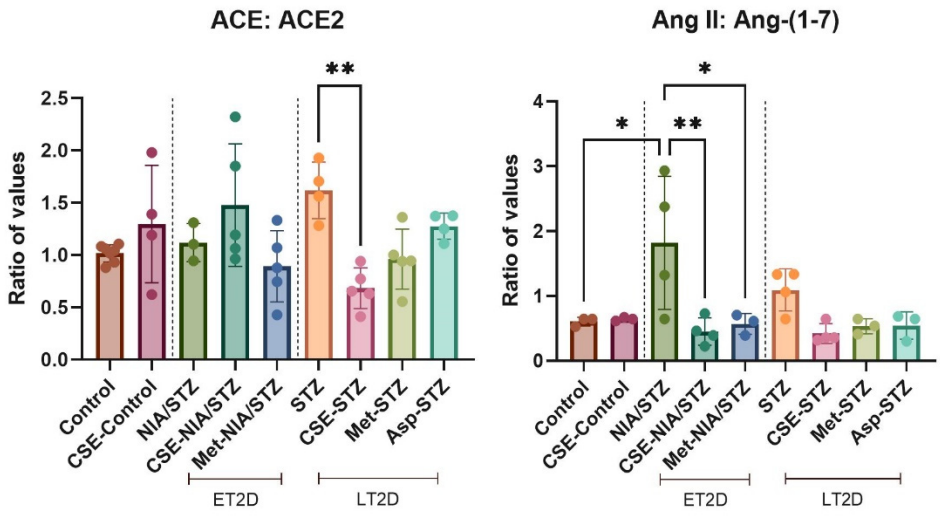

Figure 6. Comparison of ACE and ACE2 gene expression alongside Ang II and Ang (1-7) concentrations in the examined groups. ((*p < 0.05; ** p < 0.01).

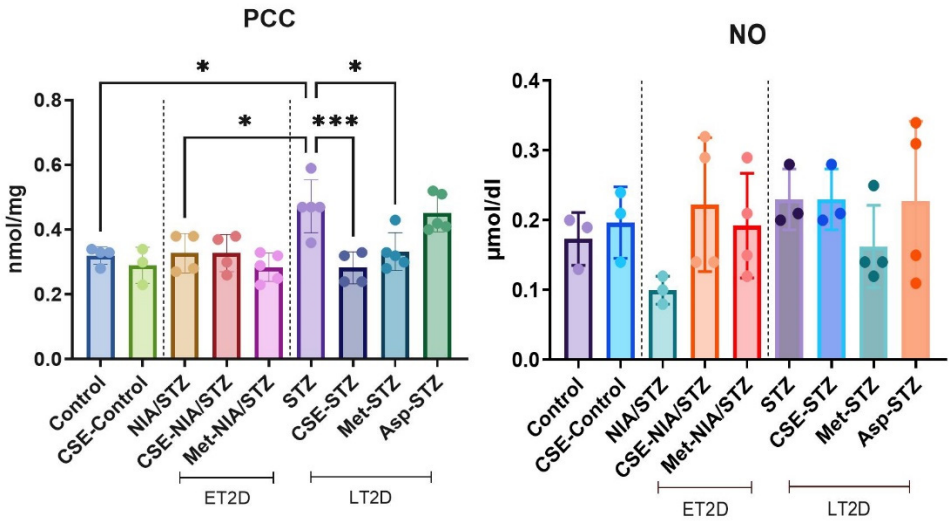

Figure 7. PCC levels and NO concentrations in the examined groups. (*p < 0.05; ***p < 0.001).

Collagen area measurement

LT2D rats in the STZ group showed a significant increase in renal collagen area compared to the control group ($p < 0.01$). Treatment with metformin and aspirin reduced collagen deposition, with a significantly greater effect observed in the Met-STZ group ($p < 0.01$) than in the Asp-STZ group ($p < 0.05$). In contrast, administration of chicory extract (CSE-STZ group) did not significantly affect collagen area compared to the STZ group (Fig. 8A and B).

Discussion

The present study aimed to investigate alterations in the expression of RAS genes in kidney tissue in response to diabetes and treatment with chicory seed extract (CSE). Both early and late stages of diabetes were examined to account for disease progression, and half of each rat's kidney was analyzed to address potential regional variability in gene expression.

Various studies have reported differing impacts of STZ on the mRNA expression of three critical RAS components: AGT, ACE, and ACE2. Some research indicates that diabetes induced by STZ does not affect AGT expression (21), whereas other studies have noted an increase in AGT expression levels after STZ administration or in patients suffering from diabetes (22, 23). Furthermore, numerous studies have highlighted that ACE2 expression is notably diminished in both animal models of diabetes and diabetic patients (24, 25), with a significant number of investigations documenting decreased ACE2 levels among individuals with diabetes. However, higher baseline plasma ACE2 levels

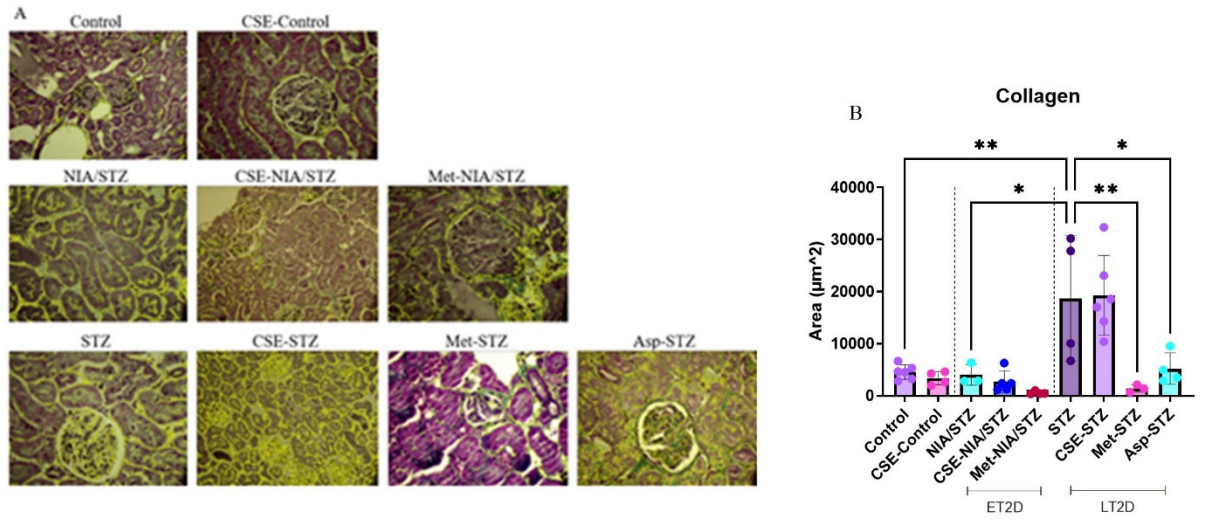

Figure 8. A) Images depicting collagen staining via the Masson trichrome method in the examined groups; B) The collagen area (µm²) in the kidney tissue across the studied groups after analysis with ImageJ software (*p < 0.05; **p < 0.01). Note: While the average collagen area appeared to be reduced by aspirin treatment in the Asp-STZ group, some individual samples showed high collagen accumulation (e.g., the image shown above for Asp-STZ). This highlights the variability in fibrosis response to aspirin.

in diabetic kidney disease (DKD) have been associated with protection against the development and progression of albuminuria and are linked to fewer renal endpoints (26). Hyperglycemia and diabetes typically lead to enhanced AGT mRNA expression through a combination of direct effects of high glucose levels, activation of the RAS, inflammatory responses, hormonal changes, and alterations in renal hemodynamics. However, specific conditions such as significant hormonal changes, local inflammatory responses, metabolic shifts, and severe renal injury, due to fibrosis for instance, could result in decreased levels of AGT mRNA in kidney tissue. In the present study, four weeks after STZ administration, a decline in AGT, ACE, and ACE2 mRNA levels was observed, consistent with the findings of Soler et al. (27). The observed decrease in the expression of these three genes can be linked to a negative feedback mechanism triggered by the initial increase in RAS products, including Ang II and Ang-(1-7), resulting from hyperglycemia and elevated ROS levels, which subsequently inhibit the expression of RAS enzymes (28). The observed decrease in ACE2 mRNA and ACE mRNA levels was such that a significant elevation of the ACE/ACE2 mRNA ratio was observed in STZ-injected rats. Consistent with prior research, our study demonstrated an increase in Ang II levels in the NIA/STZ and STZ experimental groups (24, 28). The increase in Ang II production is linked to oxidative stress related to diabetic nephropathy. Chen et al. proposed that Ang II can translocate to the nucleus, leading to genomic effects and influencing gene expression within the RAS (29). In kidney cells, Ang II binding sites were identified in the nucleus, suggesting that AT1 receptor subunits, traditionally localized on the cell membrane, can also be found intracellularly, including within the nucleus (29).

Oxidative stress plays a crucial role in the progression of diabetic nephropathy by activating detrimental pathways, particularly through elevated levels of Ang II. In this study, protein carbonyl content (PCC) was evaluated as a marker of oxidative stress in the renal tissue of rats with mild and severe diabetes. The results revealed a significant increase in PCC levels in the STZ group, supporting the findings of Comaoglu et al. (30). Ozsoy et al. also assessed multiple oxidative stress markers in the renal tissue of STZ-induced diabetic rats—including catalase, glutathione peroxidase, superoxide dismutase, and PCC with most showing elevated concentrations (31).

The accumulation of extracellular matrix and the onset of fibrosis are attributed to hyperglycemia and increased ROS production. Ang II promotes fibrosis under hyperglycemic conditions by activating the MAPK pathway. Previous research has shown that collagen levels rise in the renal tissue of STZ-induced diabetic rats (32), and our findings similarly indicate enhanced collagen synthesis and fibrosis in the STZ groups.

Prior studies have demonstrated that CSE significantly reduces blood glucose levels (15, 17, 33). While some diabetes-related changes in kidney tissue appear in both early-stage and late-stage diabetes, the severity of these changes differs (Fig. 1, Supplementary File). For instance, AGT mRNA expression decreases and PCC increases in both ET2D and LT2D groups; however, the increase in PCC is statistically significant only in the STZ group, with more pronounced alterations observed in advanced disease. Conversely, certain variables—such as Ang-(1-7)—exhibit stage-specific responses. It is therefore expected that more severe disease conditions

will result in greater deviations from the normal physiological state. Similarly, the efficacy of therapeutic interventions depends on disease severity. Although CSE treatment increases AGT mRNA expression and reduces Ang II levels in both diabetic groups, its effects do not follow a uniform pattern across stages. The further increase in AGT mRNA expression induced by CSE may represent a compensatory mechanism aimed at elevating Ang-(1-7) relative to Ang II. While CSE reduces Ang II in both ET2D and LT2D models, the goal of increasing Ang-(1-7) appears to be achieved only in milder cases of diabetes. In such cases, CSE may exert renoprotective effects against diabetic nephropathy by mitigating oxidative stress, inflammation, fibrosis, and lipotoxicity (34).

Studies suggest that in the circulating RAS, renin primarily regulates the production rate of plasma Ang II, whereas in tissues, ACE plays a dominant role in controlling local Ang II synthesis (35). Although ACE activity was elevated in both STZ and NIA/STZ rats paralleling the rise in Ang II levels—the reductions in renal Ang II concentrations induced by CSE and metformin occurred without significant changes in ACE mRNA expression. This highlights the complex regulation of ACE within the local RAS, distinguishing it from other genes in the pathway. Interestingly, in the CSE-STZ group, ACE activity was reduced despite unchanged ACE mRNA expression. In contrast, the CSE-NIA/STZ group showed no significant changes in either ACE mRNA or ACE activity following treatment. In the CSE-STZ group, reduced ACE activity coincided with elevated oxidative stress, as indicated by increased PCC levels. Meanwhile, in the CSE-NIA/STZ group, the primary effect of CSE appeared to be the enhancement of Ang-(1-7) levels. By decreasing the ACE/ACE2 ratio in the CSE-STZ group and reducing the Ang II/Ang-(1-7) ratio in the CSE-NIA/STZ group, CSE demonstrated its ability to act through distinct mechanisms depending on disease severity. This suggests that CSE can adapt its therapeutic effects to restore specific imbalances within the RAS.

Metformin exhibited similar effects to CSE in both LT2D and ET2D models. In LT2D, metformin increased AGT mRNA expression and reduced Ang II levels, ACE activity, and PCC. However, its efficacy was limited by its inability to elevate ACE2 mRNA expression. In ET2D, metformin was more effective than CSE in lowering ACE mRNA levels, indicating a stage-dependent therapeutic profile. The effects of aspirin were evaluated exclusively in the LT2D groups, and its route of administration differed from that of CSE and metformin. This variation, along with the limited scope of aspirin evaluation, restricts direct comparisons. Nonetheless, aspirin demonstrated a similar capacity to reduce Ang II concentrations and ACE activity, comparable to CSE and metformin. Additionally, aspirin reduced collagen deposition in the LT2D kidney, suggesting a potential antifibrotic effect. Although the average collagen area (μm^2) was significantly reduced in the Asp-STZ group, this conclusion is based on approximately five images. Notably, some individual samples—such as the one shown in Figure 8A—exhibited high collagen deposition. This variability suggests that aspirin's effect on kidney fibrosis in diabetic rats may be inconsistent, and under certain conditions, aspirin could potentially exacerbate fibrosis. Unlike CSE and metformin, aspirin did not significantly reduce PCC levels, indicating that its impact on oxidative stress may be limited in this context.

In summary, prior studies have demonstrated that chicory seed extract (CSE) significantly attenuates renal injury induced by STZ (LT2D) and STZ+NIA (ET2D) administration (Fig. 2 Supplementary file). Our findings suggest that CSE supports renal tissue repair in both early and late stages of T2D by reducing ROS levels and increasing the levels of the protective peptide Ang-(1-7), respectively. Additionally, metformin's effects on renal tissue closely resemble those of CSE, as it significantly decreases collagen accumulation and inhibits fibrosis in severely affected diabetic kidneys.

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Author contributions

Conceptualization and formal analysis: AN, SA; Funding acquisition: AN; Investigation and Methodology: SA, MBK; Supervision: AN, MBK; Writing original draft: SA; Writing review and editing: AN The first author's MSc thesis in Clinical Biochemistry led to the production of this article. All authors reviewed the manuscript and approved the final form for submission.

Conflict of interests

The authors declare no conflict of interest.

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