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**Therapeutic Potential of (-)-Epigallocatechin 3-*O*-gallate on Renal Damage  
in Diabetic Nephropathy Model Rats**

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EGCg on Renal Damage in Diabetic Nephropathy

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**Abbreviations:**

STZ, streptozotocin; EGCg, (-)-epigallocatechin 3-*O*-gallate; TBA, thiobarbituric acid; BSA, bovine serum albumin; PMSF, phenylmethyl sulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; iNOS, inducible nitric oxide synthesis; COX-2, cyclooxygenase-2; NF- $\kappa$ B, nuclear factor- $\kappa$ B; I $\kappa$ B- $\alpha$ , inhibitor binding protein  $\kappa$ B- $\alpha$ ; RAGE, receptor for advanced glycation end-product; TGF- $\beta$ <sub>1</sub>, transforming growth factor- $\beta$ <sub>1</sub>; HRP, horseradish peroxidase; Cr, creatinine; C<sub>cr</sub>, creatinine clearance; AGE, advanced glycation end-product; PAS, periodic acid-Schiff's; MAPK, mitogen-activated protein kinase; ECM, extracellular matrix

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### Abstract

Previous investigations have demonstrated that green tea polyphenols and partially hydrolysed guar gum as dietary fibre play antioxidative and hypolipidemic activities, respectively, supporting their reduction of risk factors in the course of diabetic nephropathy via a hypoglycemic effect and ameliorating the decline of renal function through their combined administration to rats with subtotal nephrectomy plus streptozotocin (STZ) injection. As a further study, we examined whether (-)-epigallocatechin 3-*O*-gallate (EGCg), the main polyphenolic compound, could ameliorate the development of diabetic nephropathy. Rats with subtotal nephrectomy plus STZ injection were orally administered EGCg at doses of 25, 50 and 100 mg/kg body weight/day. After a 50-day administration period, EGCg treated groups showed suppressed hyperglycemia, proteinuria, and lipid peroxidation, though there were only weak effects on the levels of serum creatinine and glycosylated protein. Furthermore, EGCg reduced renal advanced glycation end-product accumulation and its related protein expression in the kidney cortex as well as associated pathological conditions. These results suggest that EGCg ameliorates glucose toxicity and renal injury, thus alleviating renal damage caused by abnormal glucose metabolism-associated oxidative stress involved in renal lesions of diabetic nephropathy.

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## Introduction

Diabetic nephropathy is one of the most serious complications in diabetes mellitus and has been the most common cause of end-stage renal failure among patients undergoing chronic hemodialysis therapy since 1998 (Nakai et al., 2005). At present, patients with diabetes mellitus in Japan are estimated to number above seven million, and the number is increasing; however, maintaining prolonged dialysis therapy is a great burden on patients both mentally and physically, and social problems including financial issues have also been raised. Therefore, the daily consumption of foods and drinks containing effective agents for the management of onset and/or progression of diabetic complications has been receiving attention to reduce the number of patients with end-stage renal failure.

In Japan, there is much debate over the health benefits of green tea extracts and it is common to drink tea with a meal. In the previous studies, we reported that green tea polyphenols have antioxidant properties, and green tea is a useful agent to protect against protein oxidation- and glycation-associated diseases (Yokozawa et al., 1996, 1997, 1998; Nakagawa and Yokozawa, 2002; Nakagawa et al., 2002). Green tea polyphenols were also indicated as beneficial agents to manage the development of diabetic nephropathy induced by subtotal nephrectomy plus streptozotocin (STZ) injection (Yokozawa et al., 2005). On the other hand, of the catechins, (-)-epigallocatechin 3-*O*-gallate (EGCg) is known to be the most abundant in green tea. Recently, we have reported that EGCg had an antioxidant effect on creatinine (Cr) oxidation in rats with chronic renal failure, and thus inhibited methylguanidine production in an adenine-induced renal failure model (Nakagawa et al., 2004). Furthermore, there are some experimental reports of EGCg on diabetes mellitus, e.g., administration of EGCg reduced the level of mRNAs for glyconeogenesis enzymes (Koyama et al., 2004), and EGCg caused many similar effects to insulin, including repression of glucose production and phosphoenolpyruvate carboxykinase and glucose-6-phosphatase gene expression in cells

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(Waltner-Law et al., 2002); however, the mechanisms of EGCg action in diabetic nephropathy remain unclear.

Therefore, to evaluate the effect of EGCg as a representative polyphenol on diabetic nephropathy, we investigated the effect of EGCg on glucose-derived metabolic disorders like glycation and oxidative stress in diabetic nephropathy induced by subtotal nephrectomy plus STZ injection.

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## Materials and Methods

**Materials.** The following reagents were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan: 4,6-dihydroxy-2-mercaptopyrimidine (2-thiobarbituric acid (TBA)), coomassie brilliant blue R-250 (CBB R-250), bovine serum albumin (BSA), 2-amino-2-hydroxymethyl-1,3-propadiol (tris (hydroxymethyl) aminomethane), Tween 20, glycerol, phenylmethyl sulfonyl fluoride (PMSF), protease inhibitor cocktail and skim milk powder. Prestained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) standards, low range, and the Bio-Rad protein assay kit were purchased from Bio-Rad Laboratories, Japan. Mouse anti-mouse NOS2 monoclonal antibody (primary antibody for inducible nitric oxide synthesis (iNOS)) and mouse anti-human cyclooxygenase-2 (COX-2) monoclonal antibody, rabbit anti-human nuclear factor- $\kappa$ B (NF- $\kappa$ B) p65 polyclonal antibody, rabbit anti-human inhibitor binding protein  $\kappa$ B- $\alpha$  (I $\kappa$ B- $\alpha$ ) polyclonal antibody, rabbit anti-human receptor for advanced glycation end-product (RAGE) polyclonal antibody, rabbit anti-human transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ) polyclonal antibody, goat anti-rabbit IgG horseradish peroxidase (HRP) conjugated secondary antibody and goat anti-mouse IgG HRP conjugated secondary antibody were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA. Rabbit anti-human fibronectin polyclonal antibody was purchased from DAKO Cytomation, Denmark. Anti-mouse  $\beta$ -actin antibody and STZ were purchased from Sigma-Aldrich, St. Louis, MO, USA. ECL Western blotting detection reagents were purchased from Amersham Bioscience, Piscataway, NJ, USA.

**EGCg.** One hundred g of commercial green tea was boiled gently in 1,000 ml of water for 1 h. The extract was then evaporated to dryness under reduced pressure. The yield of green tea was 14.7%, by weight, of the starting material. The green tea polyphenol was prepared from a hot-water extract of green tea, as reported previously (Sakanaka et al., 1989). For purification of EGCg, recycling high-performance liquid chromatography was performed

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on a JAI-LC-908 high-performance liquid chromatograph (Japan Analytical Industry Co., Tokyo, Japan) equipped with JAI RI and JAI UV detectors, operating at 280 nm, as described previously (Sakanaka et al., 1989). A prepacked PVA HP-GPC column (JAIGEL GS-320, 50 x 2 cm i.d.) was used. Methanol was used as the eluting solvent at a flow rate of 3 ml/min. The isolated EGCg was identified by analysis on a GC mass spectrograph (JMS-DX 303, JEOL, Tokyo, Japan) and an NMR apparatus (GSX-400, JEOL). The chemical structure of this compound is illustrated in Fig. 1.

**Experimental Design.** The Guidelines for Animal Experimentation approved by the University of Toyama were followed in all experimental studies. Five-week-old male Wistar rats, weighing 120-130 g, were obtained from Japan SLC, Inc. (Hamamatsu, Japan), kept in wire-bottomed cages and exposed to a 12-h/12-h light/dark cycle. The room temperature and humidity were maintained automatically at about 25°C and 60%, respectively. They were allowed free access to laboratory pellet chow (CLEA Japan Inc., Tokyo, Japan; comprising 24.0% protein, 3.5% lipid and 60.5% carbohydrate) and water. After several days of adaptation, according to the method reported previously (Yokozawa et al., 2001), the rats underwent resection of half of the left kidney and total excision of the right kidney 10 days later. Thereafter, they were injected intraperitoneally with STZ (25 mg/kg body weight) in 10 mM citrate buffer (pH 4.5). The blood glucose and urea nitrogen levels were determined after recovery from the injection, and the rats were divided into four groups (a control and three treatment groups), avoiding any inter-group differences in these blood indices. A normal group of rats which underwent sham operation was also included. Each experimental group contained 10 rats, and while the 50-day experiment was performed, the normal and control groups received water and the other three groups received EGCg at 25, 50 and 100 mg/kg body weight/day via oral gavage, respectively, and food intake (about 25 g/day) of the EGCg treated groups did not differ from that of the diabetic nephropathy control

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rats. At the end of this experiment, 24-h urine samples were collected using metabolic cages and blood samples were obtained via cardiac puncture. The serum was immediately separated from the blood samples by centrifugation. After renal perfusion through the renal artery with ice-cold physiological saline, the remaining kidney was removed from each rat and one part of the tissue was immersed in formalin for histological examination and the other part was frozen at  $-80^{\circ}\text{C}$  until analysis.

**Serum and Urine Constituent Levels.** Serum levels of glucose, total protein, albumin, total cholesterol, triglyceride, urea nitrogen and Cr were examined using commercial reagents (Glucose CII-Test Wako, A/G B-Test Wako, Cholesterol E-Test Wako and Triglyceride E-Test Wako obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan; BUN Kainos and CRE-EN Kainos obtained from Kainos Laboratories, Inc., Tokyo, Japan). Serum TBA-reactive substance level was determined using the methods of Naito and Yamanaka (1978). Urine component levels were determined as follows: protein by the sulfosalicylic acid method (Sakagishi, 1968) and Cr using a commercial reagent (CRE-EN Kainos). The Cr clearance ( $C_{\text{Cr}}$ ) was calculated on the basis of urinary Cr, serum Cr, urine volume and body weight using the following equation:  $C_{\text{Cr}}$  (ml/min/kg body weight)=[urinary Cr (mg/dl)  $\times$  urinary volume (ml)/serum Cr (mg/dl)]  $\times$  [1,000/body weight (g)]  $\times$  [1/1,440 (min)].

**Electrophoretic Pattern Analysis of Proteinuria.** Equal amounts (0.5  $\mu\text{g}$ ) of urinary protein were loaded onto a 10% acrylamide gel, subjected to SDS-PAGE and the gel was stained with CBB R-250 to visualize the protein bands and destained overnight in 7% acetic acid. The molecular masses of the urinary protein bands were estimated by comparison with those of the bands of standard proteins.

**Advanced Glycation End-Product (AGE) Contents in Kidney.** Renal AGE contents were determined by the method of Nakayama et al. (1993). Minced renal tissue was delipidated by shaking gently with chloroform and methanol (2:1, v/v) overnight. After



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washing, the tissue was homogenized in 0.1 N NaOH, followed by centrifugation at 8,000 g for 15 min at 4°C. The amounts of AGEs in these alkali-soluble samples were measured at an emission wavelength of 440 nm and excitation wavelength at 370 nm against a blank of 0.1 N NaOH solution using a spectrofluorometric detector (Shimadzu RF/550, Kyoto, Japan). A native BSA preparation (1 mg/ml in 0.1N NaOH) was used as a reference, and its fluorescent intensity was defined as one unit of fluorescence. The fluorescence intensities of the samples at a protein concentration of 1 mg/ml were measured and expressed in arbitrary units (AU) compared with a native BSA preparation.

**Mitochondrial TBA-Reactive Substance Levels in Kidney.** Mitochondria were prepared from kidney homogenates by differential centrifugation (800 g and 12,000 g, respectively) at 4°C, according to the methods of Johnson and Lardy (1967) and Jung and Pergande (1985), respectively, with slight modifications. The pellets were resuspended in preparation medium and the TBA-reactive substance levels were determined by the method of Uchiyama and Mihara (1978). Protein levels were determined by the method of Itzhaki & Gill (1964) with BSA as the standard.

**Protein Extraction and Western Blot Analyses.** Renal cortical sections were homogenized with ice-cold lysis buffer (pH 7.5) containing 137 mM NaCl, 20 mM Tris-HCl, 1% Tween 20, 10% glycerol, 1 mM PMSF and protease inhibitor cocktail. Samples were then centrifuged at 2,000 g for 10 min at 4°C. To ensure equal loading of the lanes, the protein concentration of each tissue was determined using a Bio-Rad protein assay kit with BSA as a standard, and then immunoblotting was carried out.

For determination of iNOS, COX-2, NF- $\kappa$ B p65, I $\kappa$ B- $\alpha$ , RAGE, TGF- $\beta$ <sub>1</sub> and fibronectin protein expression, each sample (30  $\mu$ g protein) was electrophoresed through 8, 12 and 15% SDS-PAGE. Separated proteins were electrophoretically transferred to a nitrocellulose membrane, blocked with 5% skim milk solution for 1 h, and then incubated with the

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corresponding primary anti-iNOS, COX-2, NF- $\kappa$ B p65, I $\kappa$ B- $\alpha$ , RAGE, TGF- $\beta$ <sub>1</sub>, fibronectin and  $\beta$ -actin antibody overnight at 4°C. After the blots were washed, they were incubated with goat anti-rabbit and/or goat anti-mouse IgG HRP-conjugated secondary antibodies for 90 min at room temperature. Each antigen-antibody complex was visualized using ECL Western blotting detection reagents and detected by chemiluminescence with LAS-1000 plus (FUJIFILM, Japan).

Band densities were determined by Scion image software (Scion Corporation, Frederick, MD, USA) and quantified as a ratio of the density of the  $\beta$ -actin band. The evaluation for these protein levels at mean values against normal rats is represented as 1 and the corresponding values for the diabetic nephropathy rats are expressed as the ratios of these values.

**Histopathological Evaluation.** Renal tissues were fixed in 10% neutral formalin solution, embedded in paraffin and cut into 4- $\mu$ m sections. The sections were stained with hematoxylin-eosin and periodic acid-Schiff's (PAS) reagent, and then examined by light microscopy. The glomerular area was determined as the average area of a total of 30 glomeruli, cut at their vascular poles, by tracing along the outline of the capillary loop using a VH analyzer VH-H1A5 (Keyence, Osaka, Japan). Diffuse and exudative lesions were also examined by one hundred or fewer glomeruli in each sample and scored histologically as 0=0%, 1=0-5%, 2=5-10%, and 3=>10% of each glomerulus.

**Statistical Analysis.** The results are presented as the mean  $\pm$  S.E.M. The effect of EGCg on each parameter was examined using one way analysis of variance. Individual differences among groups were analyzed by Dunnett's test and significance was accepted at  $p<0.05$ .

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## Results

**Body and Kidney Weight Changes.** Table 1 shows the effects on the changes in body and kidney weights of rats with diabetic nephropathy after the oral administration of EGCg. At the beginning, the body weight was lower in diabetic nephropathy rats than normal rats, however, oral administration of 25, 50 and 100 mg/kg body weight/day for 50 days did not lead to any significant differences in body weight gain. On the other hand, the kidney weight in diabetic nephropathy control rats was 2.3-times larger than the normal value (from 0.31 g/100 g body weight to 0.70 g/100 g body weight), showing reduced enlargement via EGCg administration at 25, 50 and 100 mg dose-dependently (0.64, 0.62 and 0.59 g/100 g body weight, respectively).

**Serum Constituents.** The effects of EGCg on serum constituents of rats with diabetic nephropathy are shown in Table 2. The level of serum glucose was reduced by oral administration of 25, 50 and 100 mg of EGCg to 497, 487 and 460 mg/dl, respectively, while diabetic nephropathy control rats showed a value of 592 mg/dl, which was 3.1-fold higher than normal rats. The serum total protein and albumin levels of rats with diabetic nephropathy were significantly lower than those of normal rats, however, after EGCg administrations at 50 and 100 mg/kg body weight/day, these levels were significantly improved. We also examined the serum total cholesterol, triglyceride and TBA-reactive substance levels (index of lipid peroxidation); as shown in Table 2, the condition of diabetic nephropathy led to 2.4-, 2.2- and 2.4-fold increases in these parameters, respectively, compared with normal values, while EGCg decreased the levels of total cholesterol and TBA-reactive substance significantly, but only showed a slight tendency to decrease the triglyceride level.

**Renal Functional Parameters.** Table 3 shows that the effects of EGCg on serum and urinary parameters of renal function. The serum urea nitrogen and Cr levels and urinary

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protein excretion rate were increased significantly (2.6-, 2.5- and 4.3-fold, respectively) by diabetic nephropathy. After oral administration of EGCg, serum urea nitrogen declined significantly at all doses; however, there was only a significant decrease in serum Cr at a dose of 100 mg. On the contrary, 50 and 100 mg of EGCg markedly decreased urinary protein excretion from 82.3 mg/day to 47.9 mg/day (42% decrease,  $p < 0.001$ ) and 40.6 mg/day (51% decrease,  $p < 0.001$ ), respectively. In addition, the decrease in  $C_{Cr}$  from 7.20 to 3.35 ml/kg body weight/min was ameliorated at 100 mg of EGCg (4.09 ml/kg body weight/min) as well as the serum Cr level.

**Electrophoretic Patterns of Proteinuria.** The patterns of urinary protein in five groups, i.e., normal, untreated and EGCg 25, 50 and 100 mg-treated diabetic nephropathy groups, are presented in Fig. 2. Electrophoretic separation of urinary protein showed a large albumin fraction ( $M_r$ : 67 kDa) and low- and high-molecular-weight proteins divided by albumin band in the control rats with diabetic nephropathy, but all of these proteins were absent in normal rats. On the other hand, as the dose of EGCg increased the albumin and low-molecular-weight of proteins was effectively decreased compared with the control group.

**Renal AGEs and TBA-Reactive Substance Levels.** The levels of AGEs and mitochondrial TBA-reactive substance in the kidney were both increased approximately 1.7-fold in diabetic nephropathy untreated rats compared to normal values (Table 4). Significant changes were observed from the lowest dose of 25 mg, and there was a dose-dependent decrease in AGE accumulation from 2.53 AU (control) to 2.35, 2.20 and 2.04 AU (EGCg 25, 50 and 100 mg), respectively. The rats given EGCg at 50 and 100 mg showed a decrease to the same degree in the TBA-reactive substance level ( $p < 0.001$ ), while in rats treated with 25 mg, there was a slight but significant decrease compared with these two administration groups ( $p < 0.05$ ).

**Expression of iNOS, COX-2, NF- $\kappa$ B p65 and I $\kappa$ B- $\alpha$  Proteins.** To elucidate the

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expression levels of inflammatory enzymes, iNOS and COX-2, as well as the up-stream regulators, NF- $\kappa$ B p65 and I $\kappa$ B- $\alpha$ , we performed Western blot analyses of the renal cortex and presented these results in Fig. 3. Diabetic nephropathy rats showed increases in the levels of iNOS and COX-2 protein expression to 1.60- and 4.65-times the normal value, respectively. On the contrary, down-regulation of COX-2 protein expression was observed dose-dependently and the rats given 50 and 100 mg of EGCg showed values of 69% and 62% of the control value, respectively; however, there was no effect observed on the level of iNOS protein expression except in the 100 mg treated group. The levels of NF- $\kappa$ B p65 and phosphorylated I $\kappa$ B- $\alpha$  in the kidney cortex of diabetic nephropathy control rats likewise were elevated 1.48- and 1.31-fold above those in the kidney cortex of normal animals ( $p < 0.001$  and  $p < 0.01$ , respectively), and treatment with EGCg at 50 and 100 mg doses significantly inhibited the diabetic nephropathy-induced increases ( $p < 0.01$  and  $p < 0.001$ , respectively).

**Expression of RAGE, TGF- $\beta_1$  and Fibronectin Proteins.** We examined the expression of RAGE, TGF- $\beta_1$  and fibronectin proteins in the kidney cortex. As shown in Fig. 4, diabetic nephropathy rats showed a slight increase in RAGE ( $1.13 \pm 0.07$  vs.  $1.00 \pm 0.09$ ) (not significant), but showed increased expressions of TGF- $\beta_1$  and fibronectin in the kidneys of  $3.20 \pm 0.62$  vs.  $1.00 \pm 0.12$  ( $p < 0.001$ ) and  $3.50 \pm 0.42$  vs.  $1.00 \pm 0.28$  ( $p < 0.001$ ), respectively. The 50 and 100 mg administrations of EGCg intensely inhibited the induction of fibronectin protein relative to that of TGF- $\beta_1$  protein, that is, the fibronectin protein levels were reduced to  $1.98 \pm 0.25$  and  $1.97 \pm 0.33$  ( $p < 0.001$ , respectively), and the TGF- $\beta_1$  protein levels were reduced to  $2.33 \pm 0.27$  ( $p < 0.05$ ) and  $2.09 \pm 0.13$  ( $p < 0.01$ ), though there was no effect observed in diabetic nephropathy rats given 25 mg of EGCg. In contrast, only a slight tendency to decrease RAGE protein was noted in the EGCg treated groups.

**Histopathological Changes.** PAS-stained specimens from the experimental rats revealed exudative lesions and a mild increase of mesangial matrix in the diabetic

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nephropathy control group (Fig. 5 (B,C)). The EGCg 50 and 100 mg-administered diabetic nephropathy groups showed minimal lesions (Fig. 5 (E,F)) as compared with the control group, while no changes were observed in the 25 mg treated group (Fig. 5 (D)). Table 5 summarizes the histopathological changes in the kidneys of the experimental groups. The total glomerular area, obtained by tracing along the outline of the capillary loop, showed a 2.2-fold increase in diabetic nephropathy in the control group compared with that in the normal group, but the EGCg 50 and 100 mg-treated groups showed significant ameliorations of this increase. There were significant increases in diffuse and exudative lesions between the diabetic nephropathy control rats and normal rats, from none to  $2.60 \pm 0.16$  and none to  $1.60 \pm 0.27$ , however, the EGCg 50 and 100 mg-administered diabetic nephropathy rats showed significantly lower levels.

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## Discussion

Diabetic nephropathy results from an interaction between metabolic and hemodynamic factors. Glucose dependent pathways are activated within the diabetic kidney, such as increasing oxidative stress, polyol formation and AGE accumulation. Hemodynamic factors also participate in systemic and intraglomerular pressure and in the activation of various vasoactive hormone pathways. These hemodynamic pathways, alone and in conjunction with metabolic pathways, stimulate intracellular secondary messengers such as protein kinase C and mitogen-activated protein kinase (MAPK), nuclear transcription factors such as NF- $\kappa$ B, and various growth factors such as the pro-sclerotic cytokine, TGF- $\beta$ , and the angiogenic, permeability enhancing growth factor, vascular endothelial growth factor. These pathways eventually result in increased renal albumin permeability and extracellular matrix (ECM) accumulation which causes increasing proteinuria, glomerular sclerosis and tubulointerstitial fibrosis. Therefore, therapeutic strategies targeting the management and prevention of diabetic nephropathy include agents for controlling hyperglycemia, glucose-derived oxidative stress and renal damage.

Hyperglycemia is the principle factor responsible for structural alterations at the renal level, and the Diabetes Control and Complications Trial Research Group (1993) has elucidated that hyperglycemia is directly linked to diabetic microvascular complications, particularly in the kidney; therefore, glycemic control remains the main target of therapy. In this study, the glucose level of diabetic nephropathy rats showed about a three-fold, significant increase; however, EGCg inhibited this increase dose-dependently. In addition, the typical pattern of serum constituents, that is, a decrease in total protein and albumin due to their excessive excretion via urine, and also increase in lipids, e.g., total cholesterol and triglyceride, whose abnormal metabolisms have been proven to play roles in the pathogenesis of diabetic nephropathy (Sun et al., 2002) and enhance lipid peroxidation, were all improved

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by administration of the EGCg. Therefore, we believe that EGCg had a positive effect on serum glucose and lipid metabolic abnormalities.

A progressive decline in the glomerular filtration rate, reflecting serum Cr and  $C_{Cr}$  levels, is the most common characteristic in the development of diabetic nephropathy, which causes proteinuria leading to histological damage in the kidney. This decline is thought to be due to changes in renal hemodynamic initiated by the loss of functioning nephrons, which leads to an increased urinary flow rate, ammonia production and oxygen consumption (Brenner et al., 1982). The results of the study presented here demonstrate that diabetic nephropathy rats showed significant increases in the serum urea nitrogen, Cr and urinary protein excretion rate, while the  $C_{Cr}$  level showed a significant decrease compared with normal rats, representing a decline in renal function. However, the EGCg treatment positively affected these parameters, especially in the group given 100 mg. For further investigation, we performed pattern analysis of proteinuria using SDS-PAGE, and the EGCg treatment showed obvious decrease at all parts of the molecule. These data suggest that not only improvement of proteinuria but also its individual fractions may, at least in part, ameliorate the development of glomerular and tubulointerstitial injury.

As mentioned above, EGCg may improve the typical parameters under the development of diabetic nephropathy; therefore, in this study, we also determined whether EGCg could affect glucose-dependent renal injury. In the state of diabetic nephropathy, there is increased glomerular basement membrane thickening and mesangial ECM deposition, followed by mesangial hypertrophy and diffuse and nodular glomerular sclerosis, and these structural changes may be directly influenced by AGEs through excessive crosslinking of matrix molecules in a receptor-independent way (Vlassara et al., 1992, 1994). Moreover, another pathway exists in the action of AGEs, i.e., receptor-dependent processes, whereby AGEs bind to their cognate cell-surface receptor, RAGE, resulting in the activation of post-receptor



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signaling, generation of intracellular oxygen free radicals and activation of gene expression. Particularly, the RAGE promoter contains NF- $\kappa$ B binding sites, through which AGEs are involved in the activation of reactive oxygen species and NF- $\kappa$ B via AGE-RAGE interaction (Yan et al., 1994; Li and Schmidt, 1997; Wang et al., 1999), and in turn, upregulation of RAGE ensures that sustained NF- $\kappa$ B activation is not only maintained but also amplified (Bierhaus et al., 2001). Furthermore, AGE-RAGE and signaling pathways including NF- $\kappa$ B and MAPK modulate the activation of TGF- $\beta$  with subsequent effects in inducing the accumulation of matrix in synergistic ways, i.e., TGF- $\beta$  causes renal cell hypertrophy and promotes the production of ECM molecules including type I and IV collagen, fibronectin and laminin while inhibiting their decomposition, and induces the expression of receptors for the matrix protein, integrin, resulting in renal sclerosis and fibrosis (Schiffer et al., 2000; Fukami et al., 2004). On the contrary, activated NF- $\kappa$ B is known not only to play a critical role mediating immune and inflammatory responses involving transcription of target genes like iNOS, COX-2, TNF- $\alpha$ , IL-1 and chemokines (Karin and Ben-Neriah, 2000; Nishikawa et al., 2000), but also to have a significant correlation with the degree of severity in albuminuria in diabetic patients with renal complications (Hofmann et al., 1999). Furthermore, COX-2 up-regulation was reported in a model of STZ-induced diabetes and 5/6 nephrectomy to participate in renal damage (Komers et al., 2001; Fujihara et al., 2003). In this study, we demonstrated that renal AGE accumulation observed in diabetic nephropathy rats was decreased by EGCg administration, though EGCg showed only a slight tendency to reduce renal RAGE expression in diabetic nephropathy rats. However, remarkable antioxidative activity of renal tissue was shown in the level of lipid peroxidation at 50 and 100 mg doses of EGCg, resembling the results of iNOS, COX-2, NF- $\kappa$ B and phosphorylated I $\kappa$ B- $\alpha$ , and the fibrogenic cytokines, TGF- $\beta$ <sub>1</sub> and fibronectin protein expression in the renal cortex.

Lin and Lin (1997) have reported that EGCg decreases the activity and protein levels of

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iNOS by reducing the expression of iNOS mRNA, and this reduction could occur through the blocking of NF- $\kappa$ B binding to the iNOS promoter, thereby inhibiting the induction of iNOS transcription in mouse macrophages stimulated with lipopolysaccharide, and Ahmed et al. (2002) also demonstrated the effect of EGCg in human chondriocytes in an interleukin-1 $\beta$ -induced inflammatory study, in which inhibition of NO and prostaglandin E<sub>2</sub> production correlated with iNOS and COX-2 activity. Additionally, EGCg was revealed to prevent the progression of insulin-dependent diabetes mellitus according to research on IL-1 $\beta$  and IFN- $\gamma$ -induced  $\beta$ -cell destruction via reducing NO production and iNOS mRNA and protein levels including the inhibition of NF- $\kappa$ B activation (Han, 2003); however, no investigation has been performed with EGCg treatment against the induction and/or regulation of iNOS and COX-2 expression using the diabetic nephropathy rat model. Hence, this study may provide new evidence of the effect of EGCg on the NF- $\kappa$ B regulated transcriptional target genes, iNOS and COX-2, and protein expression in the process of developing diabetic nephropathy.

Moreover, diabetic nephropathy rats used in the present study showed significant glomerular hypertrophy and diffuse and exudative lesions. Longitudinal hyperfiltration is associated with renal enlargement such as the increase of glomerular size, and diffuse lesion development is dependent on increased mesangial matrix and glomerular basement membrane thickening as both are composed of ECM molecules, as in the case of the TGF- $\beta$  system, and they also correlate with proteinuria, and the other phenomenon, the exudative lesion called capsular drop and fibrin cap, is suggested to consist of plasma components, such as IgM, fibrinogen, and AGEs. According to the results of histopathological evaluation, while diabetic nephropathy rats showed a 2.2-fold increase in glomerular area, mild but significant increases in diffuse and exudative lesions and also a slight increase in the mesangial matrix, EGCg could affect glomerular hypertrophy and these lesions at 50 and 100 mg doses,

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reflecting the effects of AGEs, TGF- $\beta_1$  and fibronectin levels. Hence, we may hypothesize that EGCg could be advantageous against diabetic kidney damage, which correlates with AGEs with or without a receptor-dependent pathway and their relating inflammatory responses, and then EGCg subsequently suppresses the induction of mesangial hypertrophy and fibronectin synthesis in diabetic nephropathy.

In conclusion, our observations presented here suggest that EGCg has a beneficial effect on diabetic nephropathy via suppressing hyperglycemia, AGEs, their related oxidative stress and cytokine activations, and also pathological states due to its synergistic effect. This study may provide original and strong supporting evidence for the efficacy of EGCg in the early stage of diabetic nephropathy, suggesting that it would be a superior aid for the management of patients with diabetic nephropathy.

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### Footnotes

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### Legends for Figures

**Fig. 1.** Chemical structure of EGCg.

**Fig. 2.** SDS-PAGE pattern of proteinuria in normal rats (N) and diabetic nephrectomized rats treated with EGCg 25 mg/kg body weight/day (E25), 50 mg/kg body weight/day (E50), 100 mg/kg body weight/day (E100) or water (control, C) for 50 days. Lane M shows the molecular weight marker.

**Fig. 3.** Western blot analyses of iNOS (A), COX-2 (B), NF- $\kappa$ B p65 (C) and I $\kappa$ B- $\alpha$  (phosphorylated and non-phosphorylated) (D) protein expression in the renal cortex of normal rats (N) and in diabetic nephrectomized rats treated with EGCg 25 mg/kg body weight/day (E25), 50 mg/kg body weight/day (E50), 100 mg/kg body weight/day (E100) or water (control, C) for 50 days. Values represent the means  $\pm$  S.E.M. (\* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001 vs. normal values; # $p$ <0.05, ## $p$ <0.01, ### $p$ <0.001 vs. diabetic nephropathy control values).

**Fig. 4.** Western blot analyses of RAGE (A), TGF- $\beta$ <sub>1</sub> (B) and fibronectin (C) protein expression in the renal cortex of normal rats (N) and in diabetic nephrectomized rats treated with EGCg 25 mg/kg body weight/day (E25), 50 mg/kg body weight/day (E50), 100 mg/kg body weight/day (E100) or water (control, C) for 50 days. Values represent the means  $\pm$  S.E.M. (\* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001 vs. normal values; # $p$ <0.05, ## $p$ <0.01, ### $p$ <0.001 vs. diabetic nephropathy control values).

**Fig. 5.** Photomicrographs of the glomeruli in normal rats (A) and diabetic nephrectomized

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rats treated with EGCg 25 mg/kg body weight/day (D), 50 mg/kg body weight/day (E), 100 mg/kg body weight/day (F) or water (control, B and C) for 50 days. Scale bar = 100  $\mu$ m.

TABLE 1

Body and kidney weight changes

Data are means  $\pm$  S.E.M.

Groups	Body weight			Kidney weight (g/100 g body weight)
	Initial (g)	Final (g)	Gain (50 days)	
Normal	266.3 $\pm$ 5.2	356.5 $\pm$ 15.2	90.3 $\pm$ 7.3	0.31 $\pm$ 0.02
Diabetic nephropathy				
Control	245.3 $\pm$ 5.5**	269.5 $\pm$ 11.7**	24.2 $\pm$ 8.0**	0.70 $\pm$ 0.05**
EGCg (25 mg/kg body weight/day)	250.1 $\pm$ 6.9*	280.0 $\pm$ 11.6**	29.9 $\pm$ 8.2**	0.64 $\pm$ 0.02**,#
EGCg (50 mg/kg body weight/day)	242.5 $\pm$ 5.4**	268.0 $\pm$ 6.7**	25.5 $\pm$ 6.5**	0.62 $\pm$ 0.03**##
EGCg (100 mg/kg body weight/day)	245.2 $\pm$ 7.2**	271.2 $\pm$ 6.6**	26.1 $\pm$ 6.3**	0.59 $\pm$ 0.03**###

\* $p < 0.05$ , \*\* $p < 0.001$  vs. normal values; # $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.001$  vs. diabetic nephropathy control values.

TABLE 2

Serum constituents at 50 days of administration

Data are means  $\pm$  S.E.M.

Items	Normal	Control	EGCg (mg/kg body weight/day)		
			25	50	100
Glucose (mg/dl)	193 $\pm$ 9	592 $\pm$ 38 <sup>***</sup>	497 $\pm$ 22 <sup>***,##</sup>	487 $\pm$ 22 <sup>***,##</sup>	460 $\pm$ 19 <sup>***,##</sup>
Total protein (g/dl)	4.75 $\pm$ 0.11	4.21 $\pm$ 0.08 <sup>***</sup>	4.20 $\pm$ 0.10 <sup>***</sup>	4.37 $\pm$ 0.07 <sup>***,#</sup>	4.44 $\pm$ 0.06 <sup>***,##</sup>
Albumin (g/dl)	2.88 $\pm$ 0.04	2.38 $\pm$ 0.08 <sup>***</sup>	2.43 $\pm$ 0.06 <sup>***</sup>	2.56 $\pm$ 0.06 <sup>***,##</sup>	2.62 $\pm$ 0.05 <sup>***,##</sup>
Total cholesterol (mg/dl)	46.4 $\pm$ 2.4	113.6 $\pm$ 12.7 <sup>***</sup>	102.3 $\pm$ 6.0 <sup>***</sup>	83.3 $\pm$ 6.4 <sup>***,##</sup>	77.7 $\pm$ 6.8 <sup>***,##</sup>
Triglyceride (mg/dl)	63.7 $\pm$ 6.3	143.1 $\pm$ 31.4 <sup>***</sup>	126.6 $\pm$ 15.7 <sup>*</sup>	120.9 $\pm$ 27.3 <sup>*</sup>	116.6 $\pm$ 26.3 <sup>*</sup>
TBA-reactive substance (nmol/ml)	1.56 $\pm$ 0.08	3.70 $\pm$ 0.39 <sup>***</sup>	2.48 $\pm$ 0.18 <sup>**,#</sup>	2.50 $\pm$ 0.34 <sup>**,#</sup>	2.16 $\pm$ 0.24 <sup>##</sup>

\* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001 vs. normal values; # $p$ <0.05, ## $p$ <0.001 vs. diabetic nephropathy control values.

TABLE 3

Renal functional parameters at 50 days of administration

Data are means  $\pm$  S.E.M.

Items	Normal	Control	EGCg (mg/kg body weight/day)		
			25	50	100
s-Urea nitrogen (mg/dl)	16.8 $\pm$ 0.5	44.5 $\pm$ 3.1**	37.9 $\pm$ 1.8**,#	38.0 $\pm$ 2.6**,#	28.8 $\pm$ 1.4**,#
s-Cr (mg/dl)	0.38 $\pm$ 0.01	0.94 $\pm$ 0.09**	0.90 $\pm$ 0.08**	0.82 $\pm$ 0.06**	0.66 $\pm$ 0.05**,#
CCr (ml/kg body weight/min)	7.20 $\pm$ 0.26	3.35 $\pm$ 0.43**	3.41 $\pm$ 0.32**	3.65 $\pm$ 0.37**	4.09 $\pm$ 0.35**,#
u-Protein (mg/day)	19.1 $\pm$ 0.7	82.3 $\pm$ 13.3**	64.0 $\pm$ 11.9**	47.9 $\pm$ 14.6*,#	40.6 $\pm$ 6.4##

\* $p < 0.05$ , \*\* $p < 0.001$  vs. normal values; # $p < 0.05$ , ## $p < 0.001$  vs. diabetic nephropathy control values.

TABLE 4

Renal AGEs and mitochondrial TBA-reactive substance levels

Data are means  $\pm$  S.E.M.

Groups	AGEs (AU)	Mitochondrial TBA-reactive substance (nmol/mg protein)
Normal	1.52 $\pm$ 0.04	5.97 $\pm$ 0.84
Diabetic nephropathy		
Control	2.53 $\pm$ 0.10*	9.88 $\pm$ 0.39*
EGCg (25 mg/kg body weight/day)	2.35 $\pm$ 0.09* <sup>##</sup>	9.05 $\pm$ 0.35* <sup>#</sup>
EGCg (50 mg/kg body weight/day)	2.20 $\pm$ 0.04* <sup>###</sup>	8.14 $\pm$ 0.43* <sup>###</sup>
EGCg (100 mg/kg body weight/day)	2.04 $\pm$ 0.05* <sup>###</sup>	8.11 $\pm$ 0.37* <sup>###</sup>

\* $p$ <0.001 vs. normal values; # $p$ <0.05, ## $p$ <0.01, ### $p$ <0.001 vs. diabetic nephropathy control values.

TABLE 5

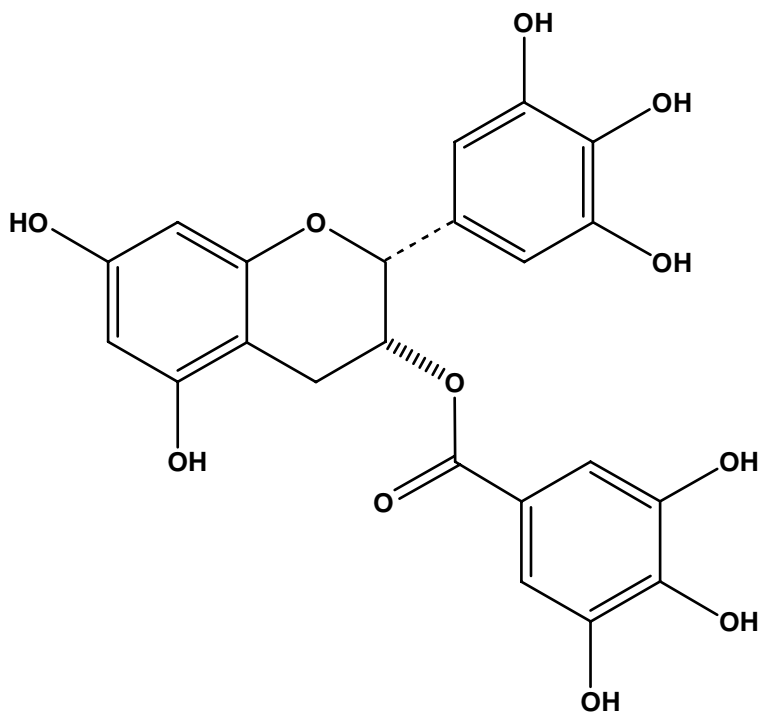
Renal histopathological evaluation

Data are means  $\pm$  S.E.M.

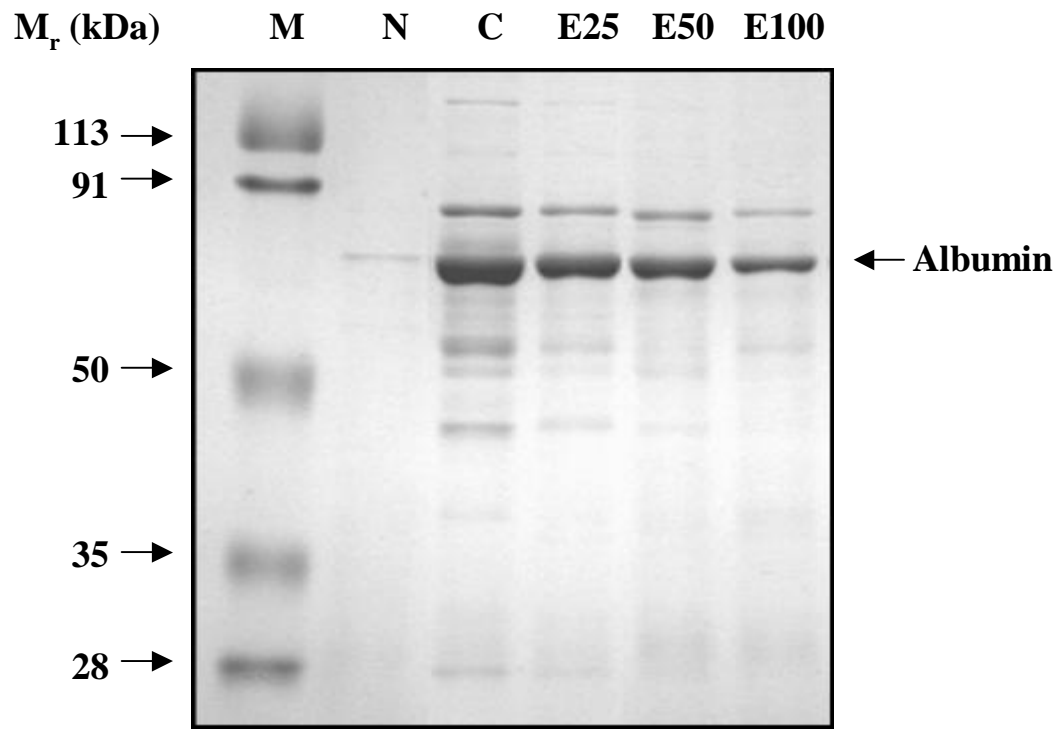
Groups	Glomerular size ( $\mu\text{m}^2 \times 10^3$ )	Diffuse lesion (score)	Exudative lesion (score)
Normal	11.0 $\pm$ 0.1	0	0
Diabetic nephropathy			
Control	23.8 $\pm$ 0.7*	2.60 $\pm$ 0.16*	1.60 $\pm$ 0.27*
EGCg (25 mg/kg body weight/day)	22.1 $\pm$ 0.4*,##	2.45 $\pm$ 0.21*	1.45 $\pm$ 0.16*
EGCg (50 mg/kg body weight/day)	20.7 $\pm$ 0.7*,##	2.27 $\pm$ 0.14*.,#	1.00 $\pm$ 0.19*,##
EGCg (100 mg/kg body weight/day)	19.8 $\pm$ 0.6*,##	1.90 $\pm$ 0.18*,##	0.90 $\pm$ 0.10*,##

\* $p < 0.001$  vs. normal values; # $p < 0.05$ , ## $p < 0.001$  vs. diabetic nephropathy control values.





**Fig. 1.**



**Fig. 2.**

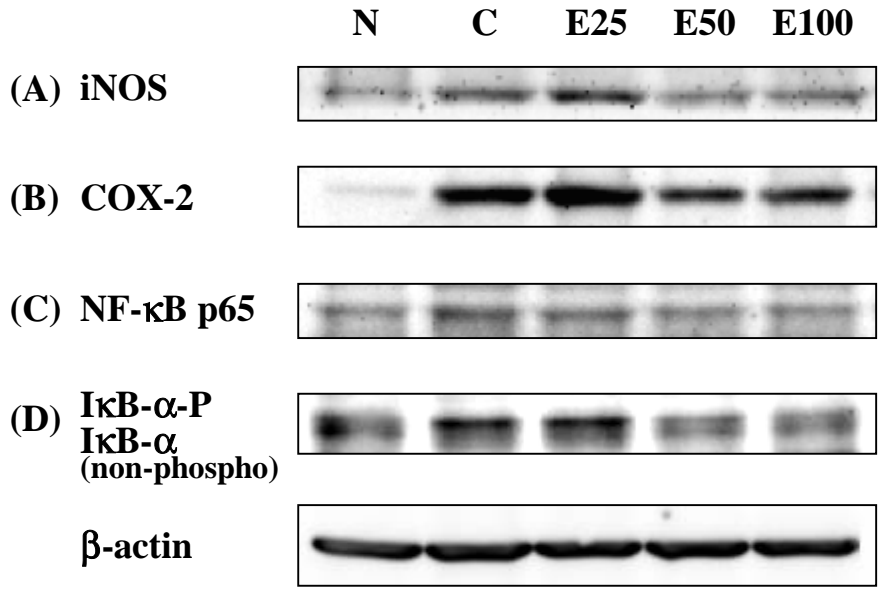
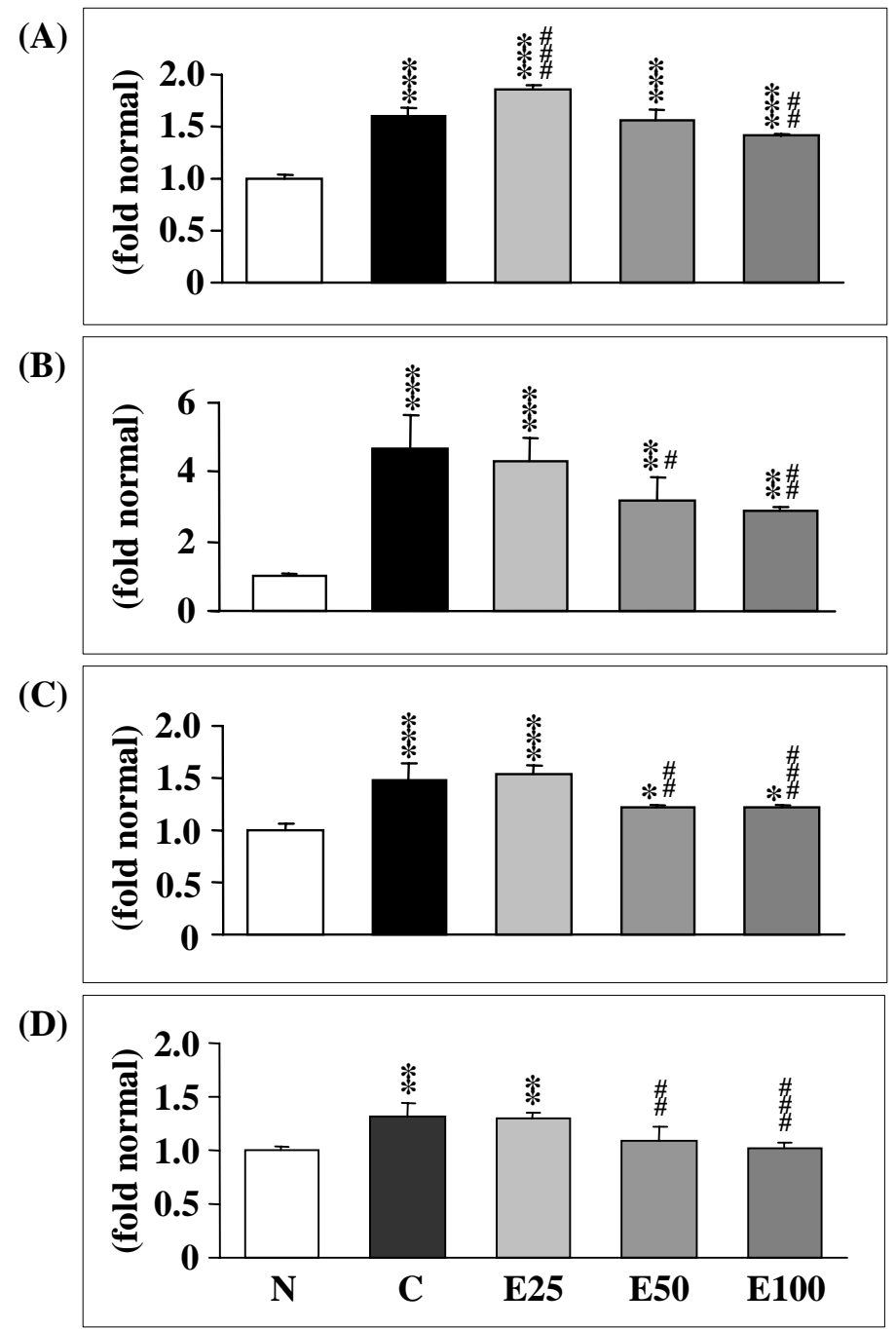


Fig. 3.



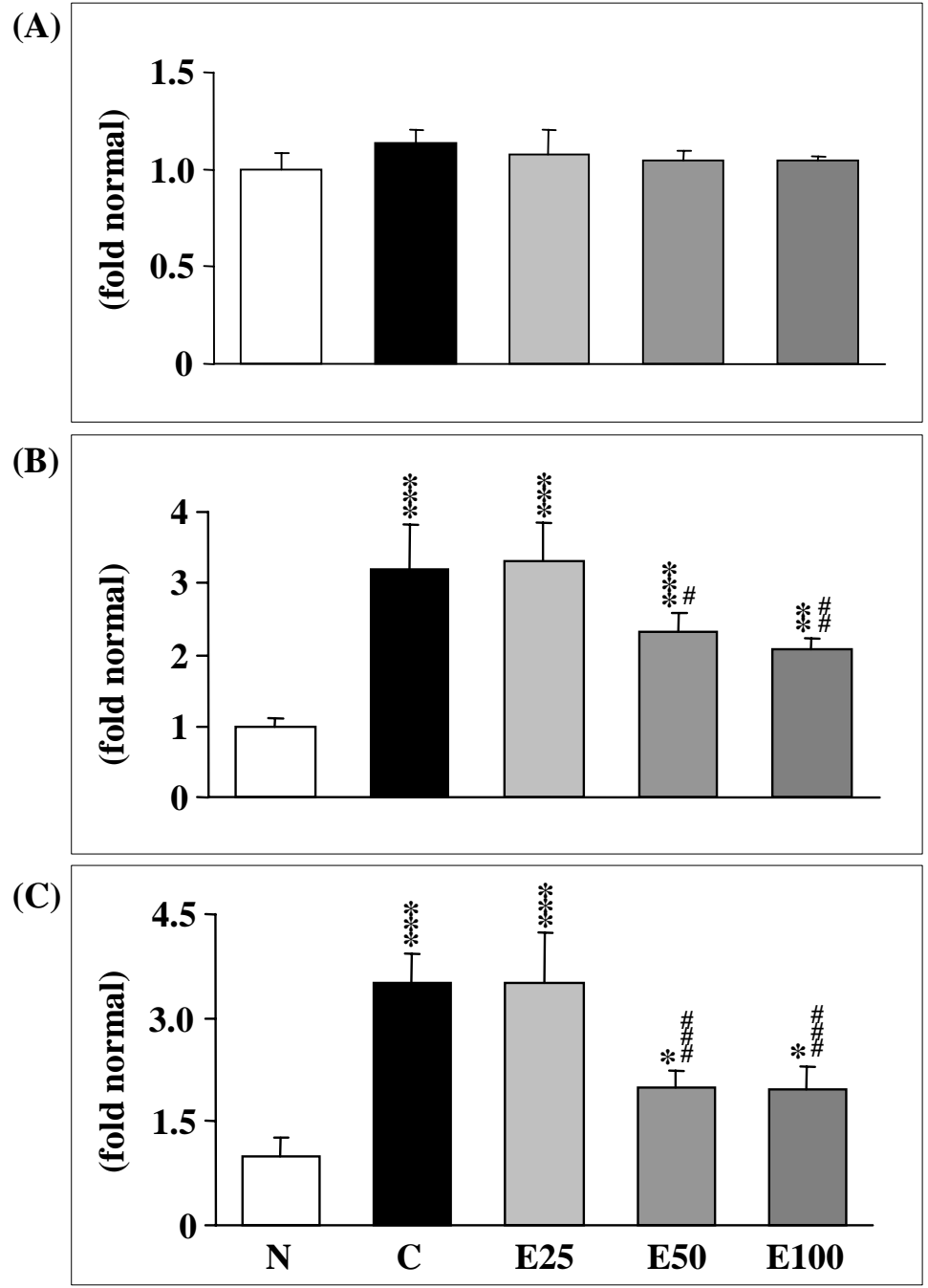
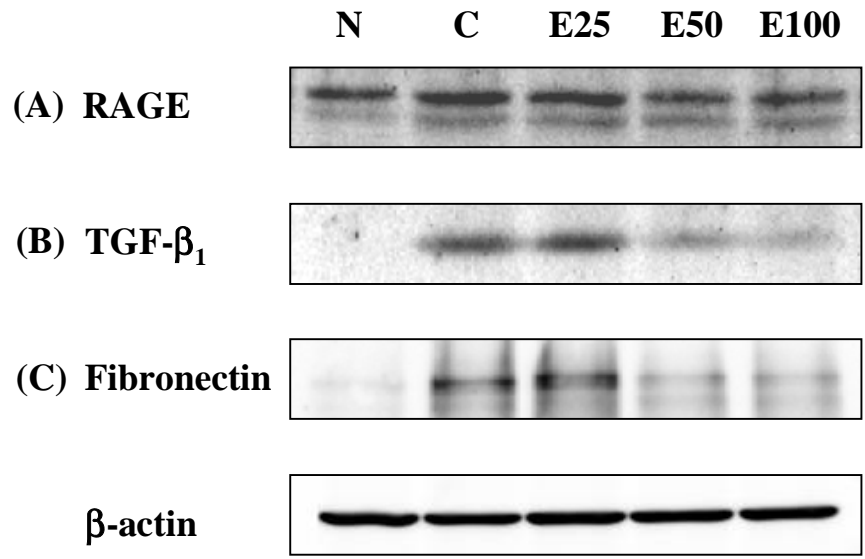
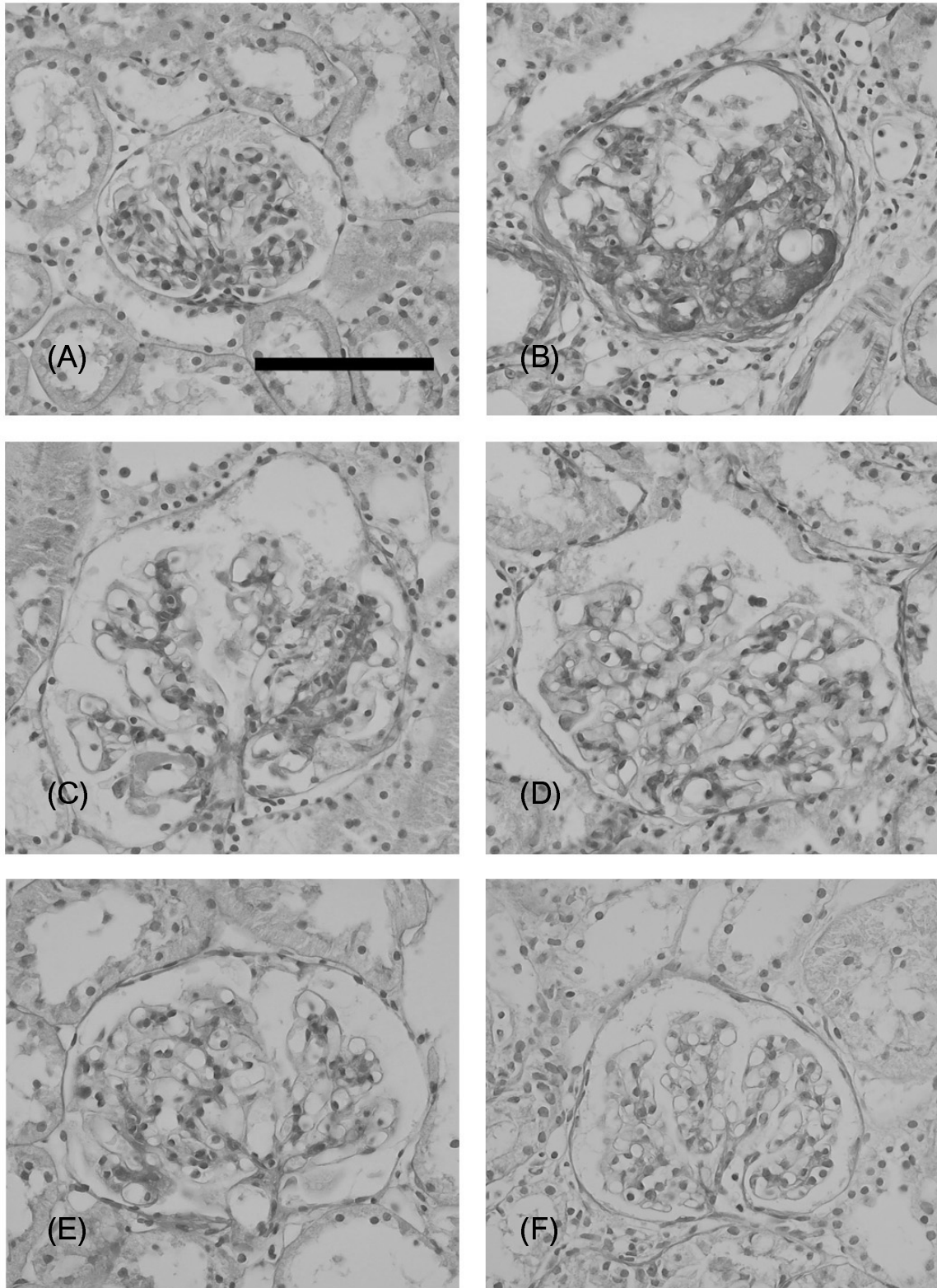


Fig. 4.



**Fig. 5.**