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Original Article

Combination therapy with artemether and enalapril improves type 1 diabetic nephropathy through enhancing antioxidant defense

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Abstract: Previous studies have demonstrated that both artemether and enalapril are effective in treating diabetic nephropathy (DN). However, the effects and underlying mechanisms of their combination in treating DN remain unknown. The experimental DN model was induced by injecting streptozotocin (STZ) into male C57BL/6J mice. Mice were randomly allocated to the Type 1 diabetes control (T1D-ctrl), STZ, STZ + artemether (STZ + Art), STZ + enalapril (STZ + ACEi), or STZ + artemether + enalapril (STZ + Art + ACEi) group. The interventions lasted for 8 weeks. At the end of the experiment, related urine and serum biochemical values, such as urinary albumin excretion (UAE) and fasting blood glucose (FBG), were measured. In addition, blood pressure (BP) and kidney morphologic changes were also evaluated. The expression of oxidative stress related molecules, such as catalase, acetylated SOD2 (k68) and acetylated SOD2 (k122) in the kidney were measured. Results: combination therapy showed more pronounced effects in reducing UAE, FBG, and BP than any single drug. Typical diabetic kidney injuries, such as heavier kidney weight, and glomerular and tubular hypertrophy, were also further alleviated by combination therapy. Combination therapy also up-regulated the expression of catalase and down-regulated the expression of acetylated SOD2 (k68) and acetylated SOD2 (k122). Combination therapy with artemether and enalapril exhibited renoprotective effects in STZ-induced T1D mice superior to a single drug. The mechanism might be associated with their synergistic effects in enhancing antioxidant defense.

Keywords: Diabetic nephropathy, artemether, enalapril, ACEi, combination

Introduction

About 700 million people are projected to suffer from diabetes mellitus (DM) by 2045 [1]. Approximately 40% of individuals with DM develop DN [2], which ultimately relentlessly progresses to renal failure. In addition, DN is the dominant cause that contributes to end stage renal disease (ESRD) which obliges patients to receive renal replacement therapy in developed countries [3]. A variety of mechanisms are implicated in the pathogenesis of DN. So far, commonly used approaches, such as blood glucose and BP control, have failed to completely retard the development of DN. Given the high incidence of DN and the consequent socioeconomic burden, it is urgent to develop more effective treatments.

The importance of oxidative stress in the pathogenesis and development of DN is increasingly realized. When the production of reactive oxygen species (ROS) overwhelms the antioxidant system, oxidative stress occurs [4]. Thus, either inhibiting the overproduction of ROS or enhancing the antioxidant defense may be an effective intervention in slowing the progress of DN. However, the efficacy of antioxidant in the treatment of DN remains controversial. A meta-analysis written by Kandhare et al. concluded that antioxidant treatment was effective in treating DN [5], while another meta-analysis concluded

that antioxidant therapy had no significant effect in retarding the development of ESRD in DN patients [6]. Given the importance and controversy over antioxidants, it is urgent to develop more targeted and effective antioxidant therapies.

Ample clinical trials have demonstrated that inhibition of the renin-angiotensin aldosterone system (RAAS) could decrease albuminuria and delay the progression to renal failure [7-10]. However, the simultaneous administration of angiotensin receptor blocker (ARB) and angiotensin-converting enzyme inhibitor (ACEi), could increase the risk of ESRD and hyperkalemia or doubled blood creatinine [11], which seems not recommended to be administrated in clinical practice. In fact, enalapril has been found to enhance the activity of antioxidant enzymes in renal of rats with diabetes [12]. Besides, Duarte and colleagues demonstrated that enalapril could not only reduce blood glucose levels, but also alleviate proteinuria and improve insulin resistance in obese rats [13]. These results evoked our interest to develop additional approaches using RAAS blockade.

Artemether is a derivative of artemisinin with a series of biologic effects such as anti-malaria [14] and anti-cancer [15]. A report by Guo et al. indicated that artemether had anti-diabetic and anti-obesity effects on db/db mice [16]. Consistently, our previous studies found that artemether could not only reduce the elevated blood glucose levels, but also alleviate kidney injuries in T1D mice [17] and db/db mice [18]. These studies suggested that artemether had abroad application prospects against DN. However, the anti-diabetic and renoprotective effects of artemether combined with enalapril on DN have not been reported yet. Thus, we designed this study to investigate the effects of artemether concomitant with enalapril treatment and to explore the mechanism.

Materials and methods

Animal studies

Guangzhou University of Chinese Medicine Institutional Animal Care and Use Committee and experimental animal Ethics Committee approved all procedures administrated in this experiment (No. 20190301011). Specific pathogen free male C57BL/6J mice were obtained from Guangdong Medical Laboratory Animal Center and were maintained in the Central Animal Facility at Shenzhen Graduate School of Peking University. STZ diluted in citrate buffer, with a dose of 55 mg/kg body weight, was intraperitoneally injected into the mice to induce diabetes for five consecutive days. The mice receiving the same volume of citrate buffer by being intraperitoneally injected were served as T1D-ctrl group. In total, five groups were included in the study: the T1D-ctrl group, STZ group, STZ + Art group, STZ + ACEi group, and STZ + Art + ACEi group respectively. The T1D-ctrl group and STZ group were maintained with a normal diet. Mice in the STZ + Art group and STZ + ACEi group were fed with diet contained with 0.8 g/kg artemether (ChengDuConBon Biotech Co., LTD, China), 0.4 g/kg enalapril (MedChemExpress, N.J, USA) respectively for 8 weeks. Mice in the STZ + Art + ACEi group were fed with a normal diet which supplemented with 0.8 g/kg artemether and 0.4 g/kg enalapril.

Urinary albumin assay

Urine was collected with the aid of metabolic cages at the end of the study. The mice urinary albumin ELISA kit (Montgomery, TX, USA) was used to determine the amount of urine albumin under the guidance of the manufacturer's protocols.

Physiological and metabolic values

The concentration of FBG was measured by blood glucose meter (Roche, Basel, Switzerland) biweekly throughout the experiment. The concentration of HbA1c was detected by Ultra2 HbA1c Analyzer (Primus, Kansas City, MO, USA) at the end of this experiment. The serum biochemical and urine related indexes, such as serum creatinine, urea nitrogen and urinary N-acetyl-β-D glucosaminidase (NAG), were detected by automatic biochemical analyzer (Roche, Basel, Switzerland). BP was monitored at 8 weeks post treatment by the tail-cuff MRBP system (IITC Life Science Inc., CA, USA).

Light microscopy

Kidney tissues were sliced into 3 μm-thick sections, then subjected to staining with Periodic acid-Schiff (PAS) and scanned by a digital slide scanner (3DHistech Ltd., Budapest, Hungary)

to assess the diabetic kidney structural changes. Approximately 30-50 renal glomerular tuft areas (GTAs), 40-50 renal glomerular mesangial matrix areas and 80-100 proximal renal tubular areas (PAS staining containing brush border) were measured. The ways to calculate the renal glomerular tuft volume (GTV), tubular cross-section area, and tubular wall area were done as described previously [17, 19, 20].

Immunohistochemistry staining analysis

Immunohistochemistry (IHC) staining was done on 3 μm formalin-fixed, paraffin-embedded kidney slides using the rabbit antibodies against catalase (CST, Danvers, MA, USA), acetylated SOD2 (k122) (Abcam, Cambridge, UK) and acetylated SOD2 (k68) (Abcam). First, kidney sections were deparaffinized and rehydrated. Later, the slices were boiled in a citric acid buffer (pH=6) for 20 minutes for the purpose of antigen retrieval. The kidney sections were exposed to 3% H_2O_2 for 15 minutes to inactive intrinsic peroxidases. Then sections were incubated with the primary antibodies at 4°C overnight. After flushing with phosphate-buffered saline (PBS) three times, the sections were incubated with horseradish peroxidase-polymer conjugated secondary antibodies (MaixinBio, Fuzhou, China) for 15 minutes at room temperature. Then diaminobenzidine was used as chromogen and hematoxylin was applied as counterstain.

Immunoblotting

For immune blotting, the kidney cortex was lysed and prepared in sample loading buffer (Bio-Rad, Hercules, CA, USA). Then the proteins were loaded onto 10% SDS-PAGE gels and blotted onto polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Danvers, MA, USA). The membranes were blocked in 5% non-fat dry milk for 1 h at room temperature and then incubated with primary antibodies against β-actin (Sigma Aldrich, St. Louis, MO, USA), catalase (CST), acetylated SOD2 (k122) (Abcam) and acetylated SOD2 (k68) (Abcam) overnight at 4°C. After washing three times with Tris-buffered saline (TBS), the membranes were exposed to secondary antibodies for 1 h at room temperature. The ChemiDoc MP imaging system (Bio-Rad, Hercules, CA, USA) was employed to detect and analyze the bands. β-actin was probed for measuring of equal loading with protein samples.

Statistical analysis

* symbol means compared with the T1D-ctrl group; # symbol means compared with the STZ group; Δ symbol means compared with the STZ + Art group; ♦ symbol means compared with the STZ + ACEi group. Data are expressed as mean ± SD. Data analysis was performed using SPSS statistics software (IBM, NY, USA). Comparisons between the T1D-ctrl and the STZ group were analyzed by unpaired Student's t test. Differences among multiple groups (STZ, STZ + Art, STZ + ACEi, STZ + Art + ACEi) were analyzed by using one-way analysis of variance (ANOVA) followed by Bonferroni or Dunnett T3 post hoc analysis.

Results

Combination therapy prevented hyperglycemia and improved diabetic symptoms

The level of FBG had substantially increased and had an incremental tendency in the STZ group vs. the T1D-ctrl group during the experiment. However, neither artemether nor enalapril could decrease the level of FBG within the first 6 weeks of treatment. Treatment with artemether ($P<0.01$) and combination therapy significantly ($P<0.001$) lowered the FBG level compared to the STZ group at 8 weeks post-treatment (**Figure 1A**). Similarly, both treatments of artemether ($P<0.001$) and combination therapy significantly ($P<0.001$) lowered the level of HbA1c compared with the STZ group (**Figure 1B**). The excretion of urinary glucose significantly increased in the STZ group in contrast to the T1D-ctrl group. Neither treatment with artemether nor enalapril could decrease the level of urine glucose, while the combination therapy was effective (**Figure 1C**). Metabolic symptoms such as polydipsia, polyuria, polyphagia and overproduction of feces were obvious in the STZ group. Both administration of artemether and combination therapy decreased the excretion of feces and urine, and combination therapy showed superior effects (**Figure 1D-G**).

Combination therapy improved BP, serum albumin, total protein, and lipid level

Mice in the STZ group vs. the T1D-ctrl group had no differences in BP during the experiment. Administration of artemether showed no effects on lowering BP in this study. However, the treatment with enalapril or combination therapy significantly lowered BP, and the combi-

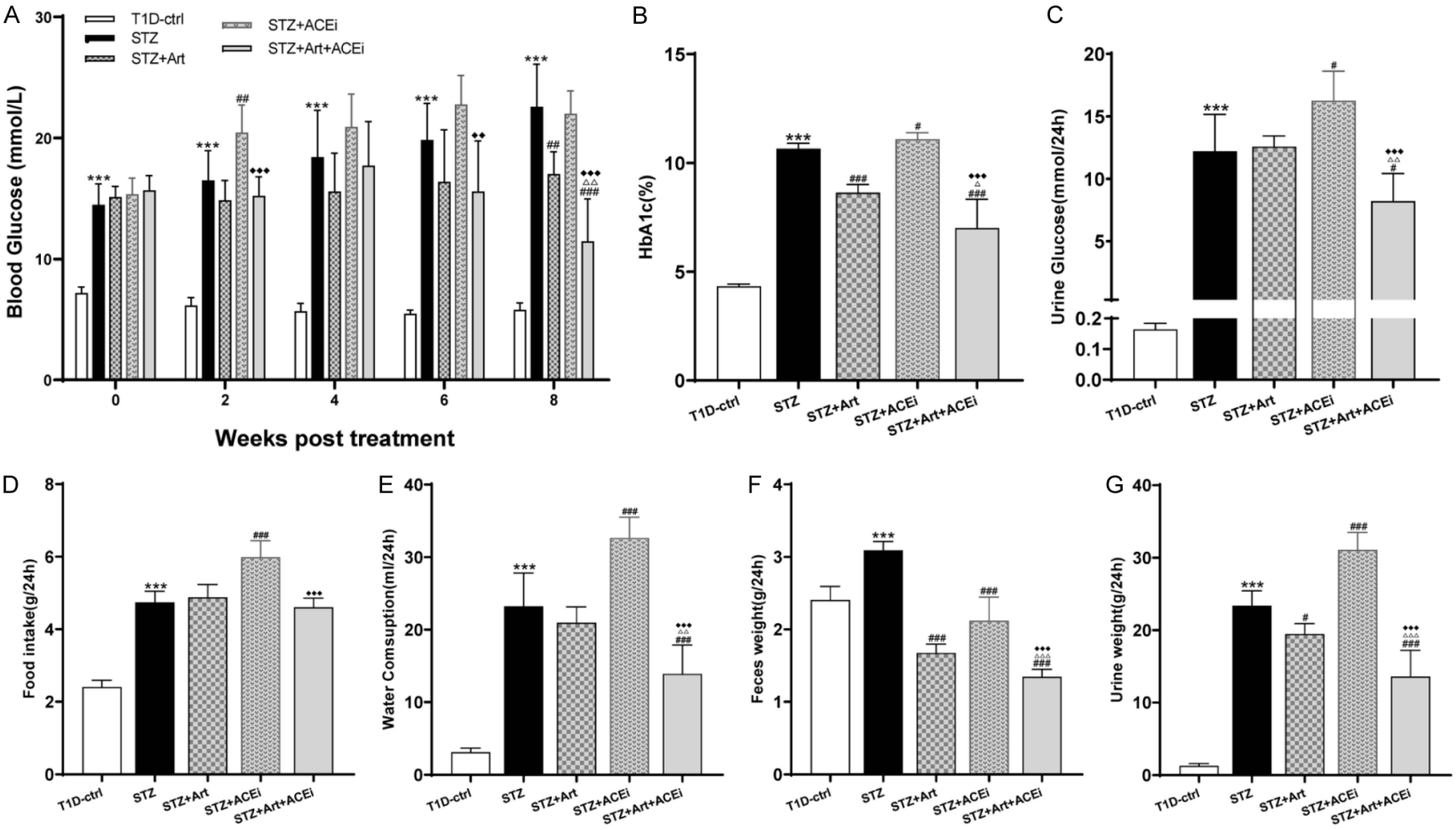

Figure 1. Effects of artemether, enalapril alone, or their combination on blood glucose, HbA1c, urine glucose and diabetic symptoms. A: Bar graphs representing the concentration of fasting blood glucose at 0, 2, 4, 6, 8 weeks post-treatment. B, C: Bar graphs representing the levels of HbA1c and urinary glucose at the end of the experiment. D-G: Bar graphs representing the quantification of food intake, water consumption, feces, and urine weight in all groups at the end of the experiment. n=7-8 per group. $^{***}P<0.001$ vs. the T1D-ctrl group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, and $^{\#\#\#}P<0.001$ vs. the STZ group; $^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.01$, and $^{\Delta\Delta\Delta}P<0.001$ vs. STZ + Art group; $^{\blacklozenge\blacklozenge}P<0.01$, and $^{\blacklozenge\blacklozenge\blacklozenge}P<0.001$ vs. STZ + ACEi group.

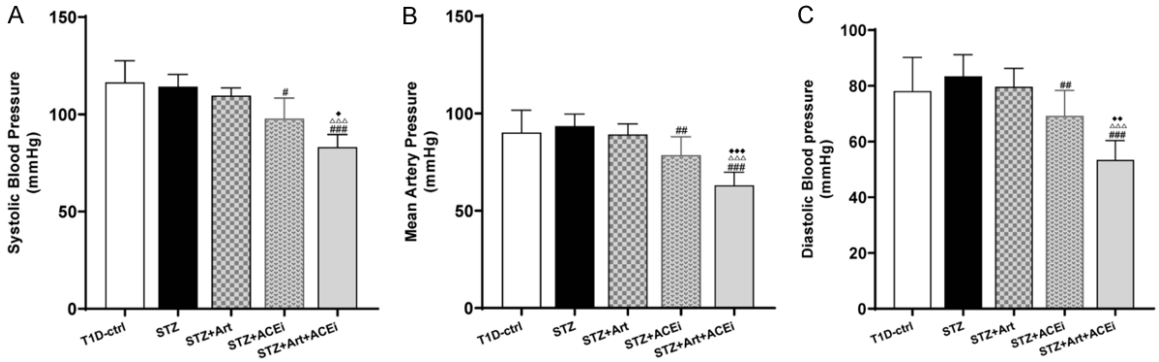

Figure 2. Combination therapy with artemether and enalapril decreased blood pressure. A-C: Bar graphs denoting the quantification of systolic blood pressure, mean artery pressure, and diastolic blood pressure in different groups. n=8 per group. $^{\#}P<0.05$, $^{\#\#}P<0.01$, and $^{\#\#\#}P<0.001$ vs. the STZ group; $^{\Delta\Delta\Delta}P<0.001$ vs. STZ + Art group; $^{\blacklozenge}P<0.05$, $^{\blacklozenge\blacklozenge}P<0.01$ and $^{\blacklozenge\blacklozenge\blacklozenge}P<0.001$ vs. STZ + ACEi group.

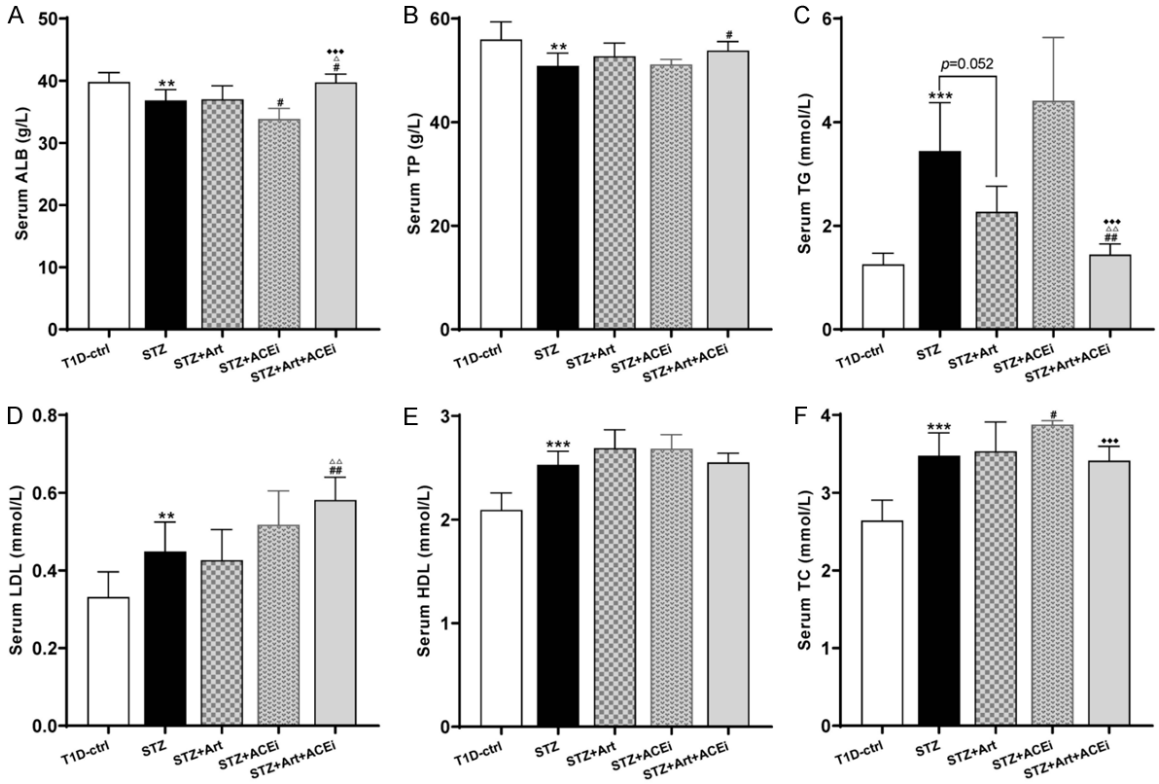

Figure 3. Effects of artemether, enalapril alone, or their combination on biochemical measures. A, B: Bar graphs representing the levels of serum albumin and total protein. C-F: Bar graphs representing the levels of TG, LDL, HDL, and TC in different groups. n=8 per group. $^{**}P<0.01$ and $^{***}P<0.001$ vs. the T1D-ctrl group; $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs. the STZ group; $^{\Delta}P<0.05$ and $^{\Delta\Delta}P<0.01$ vs. STZ + Art group; $^{\blacklozenge\blacklozenge\blacklozenge}P<0.001$ vs. STZ + ACEi group.

nation therapy showed superior effects (**Figure 2A-C**). As shown in **Figure 3A**, **3B**, combination therapy could undo the fall in serum albumin and total protein in the STZ group. To confirm the effects of all treatments on lipid metabolism, the levels of serum triglycerides (TG), low density lipoprotein (LDL), high density lipoprotein (HDL), and total cholesterol (TC) were measured. As shown in **Figure 3C-F**, the levels of serum TG, LDL, HDL, and TC were elevated in the STZ group, while combination therapy decreased the serum TG level.

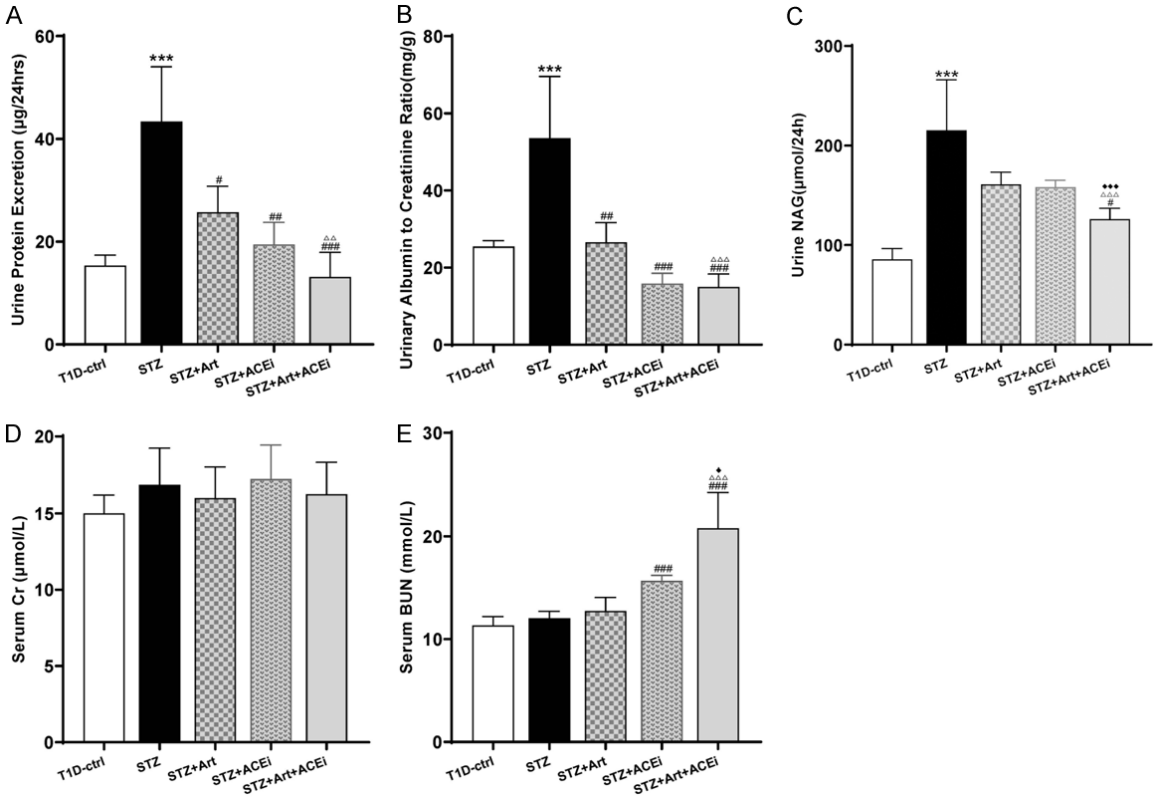

Figure 4. Effects of artemether, enalapril alone, or their combination on urine and serum measures related to kidney function. A-C: Urinary albumin excretion, urinary albumin to creatinine ratio, and urinary excretion of NAG at the end of the study in various groups. D, E: Blood creatinine and urea nitrogen concentration at the end of the study in each group. n=6-8 per group. $^{***}P<0.001$ vs. the T1D-ctrl group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, and $^{\#\#\#}P<0.001$ vs. the STZ group; $^{\Delta\Delta}P<0.01$, and $^{\Delta\Delta\Delta}P<0.001$ vs. STZ + Art group; $^{\bullet}P<0.05$ and $^{\bullet\bullet\bullet}P<0.001$ vs. STZ + ACEi group.

Combination therapy further decreased albuminuria and urinary NAG

Figure 4A-C revealed that the mice in the STZ group had significantly ($P<0.001$) higher levels of UAE, urinary albumin to creatinine ratio (UACR) and NAG, compared with which in the T1D-ctrl group. Treatment with artemether or enalapril single significantly reduced the levels of UAE and UACR versus to that in STZ group. Furthermore, combination therapy provided greater protection on UAE, UACR, and NAG. However, none of the treatments showed effects on decreasing serum creatinine and urea nitrogen (**Figure 4D**, **4E**).

Combination therapy prevented diabetic kidney hypertrophy and alleviated renal pathologic injuries

As shown in **Figure 5A**, the kidney weight was very increased in the STZ group versus the T1D-ctrl group. Typical diabetic renal pathologic injuries, such as glomerular and tubular hypertrophy and expansion of extracellular matrix, were all observed in the STZ group (**Figure 5B-I**). The intervention with either artemether or combination therapy could alleviate enlargement of the kidney and attenuate glomerular hypertrophy. Combination therapy could also attenuate expansion of extracellular matrix and tubular hypertrophy, and exhibited greater effects.

Combination therapy enhanced renal antioxidant defense

As shown in **Figure 6A-D**, increased expression of acetylated SOD2 (k68) and acetylated SOD2 (k122), although not significant at k122, were observed by western blot analysis in the STZ group compared with the T1D-ctrl group, and administration of both artemether and enalapril could decrease the expression of these two indexes. Furthermore, a more noticeable decrease was observed in the combination

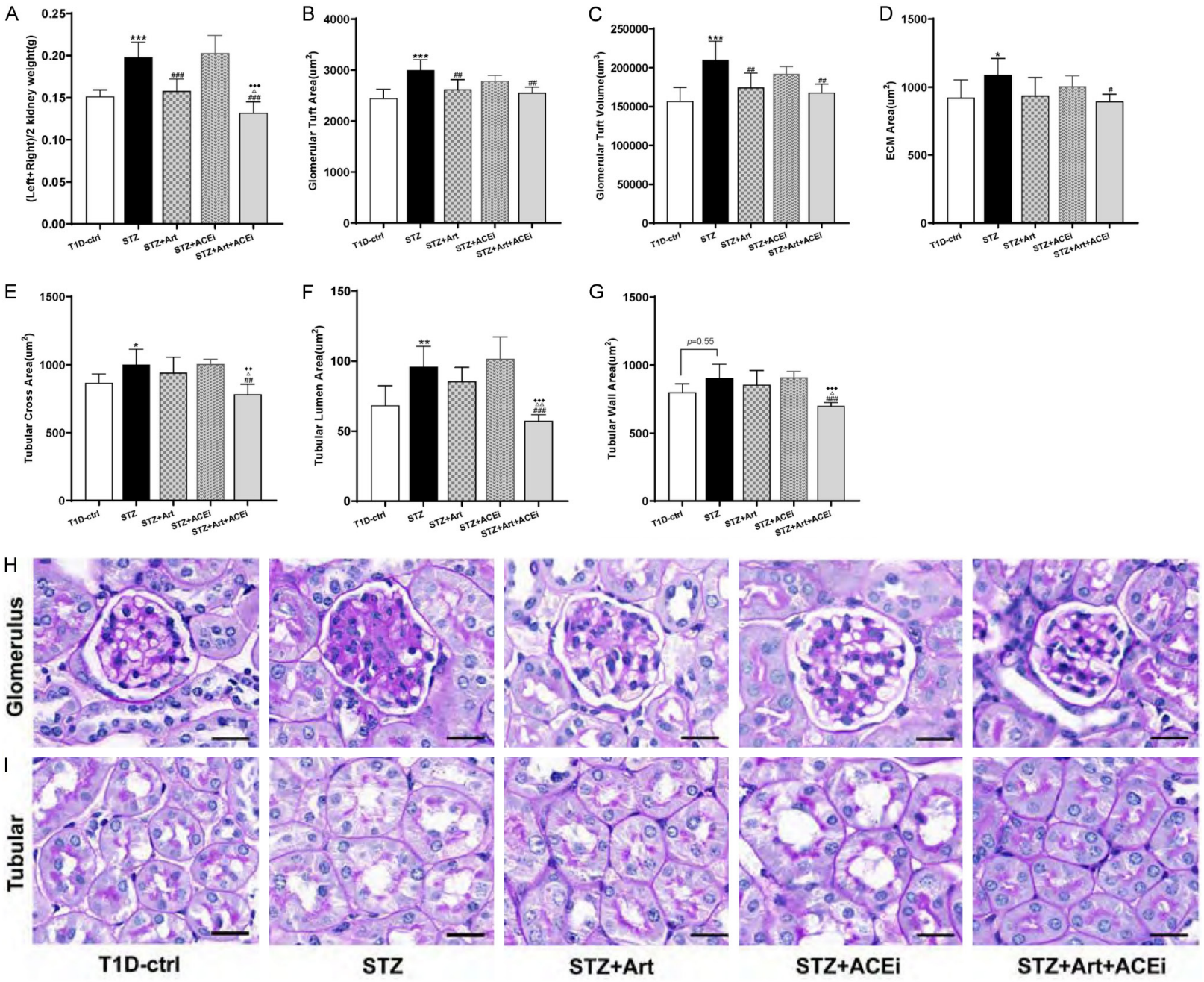
A
(Left+Right)/2 kidney weight(g)
B
Glomerular Tuft Area(um2)
C
Glomerular Tuft Volume(um3)
D
ECM Area(um2)
E
Tubular Cross Area(um2)
F
Tubular Lumen Area(um2)
G
Tubular Wall Area(um2)
p=0.55
T1D-ctrl
STZ
STZ+Art
STZ+ACEi
STZ+Art+ACEi
H
Glomerulus
I
Tubular

Figure 5. Combination therapy with artemether and enalapril prevented diabetic kidney enlargement and attenuated glomerular and tubular injuries. A: Kidney weights in indicated groups at the end of the experiment. B-D: Bar graphs representing the GTA, GTV, and mesangial matrix area in each group. E-G: Bar graphs indicating the tubular cross-section area, tubular lumen area, and wall area in various groups. H: Representative PAS staining images for the glomeruli. Scale bars, 20 μm. I: Representative PAS stained images of the tubules. Scale bars, 20 μm. n=7-8 per group. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs. the T1D-ctrl group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, and $^{\#\#\#}P<0.001$ vs. the STZ group; $^{\Delta}P<0.05$ and $^{\Delta\Delta}P<0.01$ vs. STZ + Art group; $^{\blacklozenge\blacklozenge}P<0.01$ and $^{\blacklozenge\blacklozenge\blacklozenge}P<0.001$ vs. STZ + ACEi group.

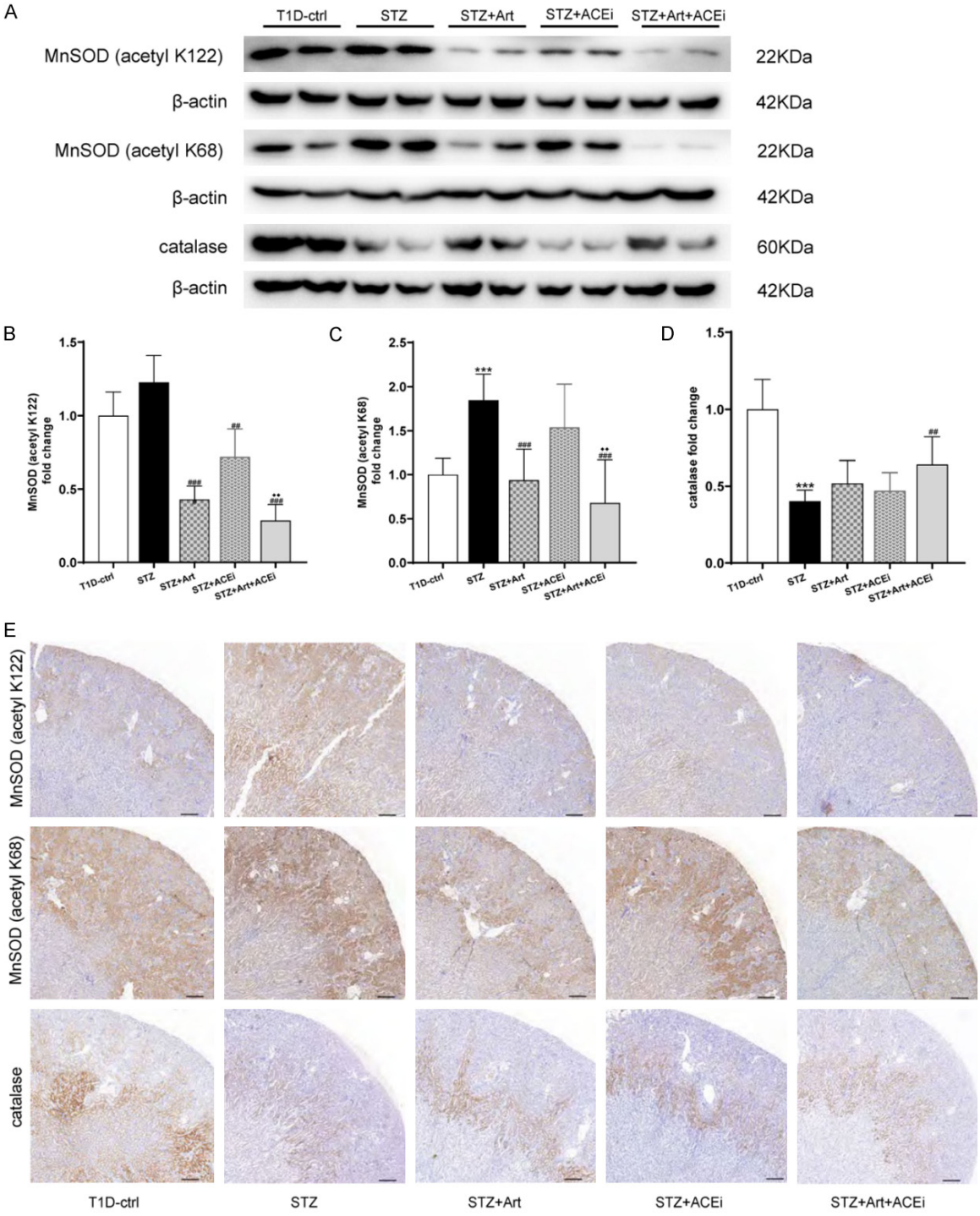

Figure 6. Combination therapy with artemether and enalapril regulated renal endogenous antioxidant protein expression. A: Western blot images of acetylated SOD2 (k122), acetylated SOD2 (k68), and catalase at 8 weeks

post-treatment. B-D: Bar graphs indicating the fold change in the aforementioned protein expression after normalization to β-actin. E: Immunohistochemical staining images of acetylated SOD2 (k122), acetylated SOD2 (k68), and catalase in the kidney of different groups. Scale bars: 200 μm. n=4-8 per group. $^{***}P<0.001$ vs. the T1D-ctrl group; $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ vs. the STZ group; $^{\blacklozenge\blacklozenge}P<0.01$ vs. STZ + ACEi group.

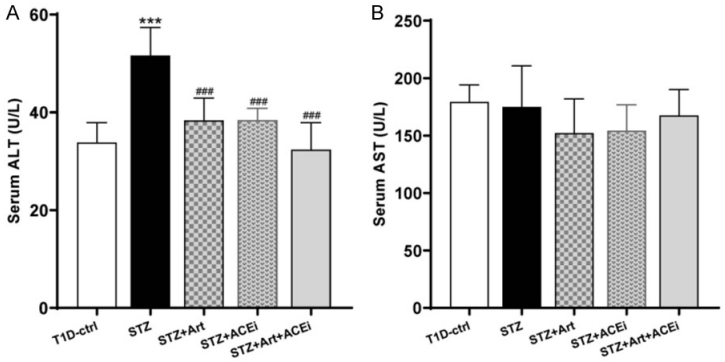

Figure 7. Administration of artemether or enalapril alone or their combination showed no hepatotoxicity. A, B: Bar graphs representing the levels of serum ALT and AST. n=8 per group. $^{***}P<0.001$ vs. the T1D-ctrl group; $^{\#\#\#}P<0.001$ vs. the STZ group.

therapy group. Conversely, the expression of catalase was significantly decreased in the STZ group compared with T1D-ctrl group, while combination therapy recovered its expression. These results were further supported by IHC staining (**Figure 6E**).

Combination therapy showed no hepatotoxicity

In order to assess the safety of these interventions, we detected the levels of serum ALT and AST. Compared with the T1D-ctrl group, the level of ALT was significantly higher in the STZ group. All interventions could decrease the level of ALT compared to the STZ group (**Figure 7A**). However, no difference in the level of AST was observed among all groups (**Figure 7B**).

Discussion

This study exhibited that the combination therapy of derivatives of artemisinin, artemether, and an agent that arrests the RAAS (enalapril), provided superior renoprotective effects and showed antioxidant property in T1D mice model. Albuminuria was regarded as a promising biomarker for detecting incipient DN for its ability to predict renal early structural changes [21]. Here, we found that administration of artemether alone or as a combination therapy improved glomerular injury. Furthermore, combination therapy could also alleviate tubular injury and showed superior effects on renal pathologic injuries. Such results were mostly in concert with the change in albuminuria. This indicated that combination therapy exerted superior effects on reducing albuminuria. This might be associated with amelioration of overall renal structural injuries.

Further, such renoprotective effects, as shown by improvement of albuminuria and kidney pathologic injuries, might be correlated with enhancing the antioxidant defense. Diabetic nephropathy, one of the common microvascular complications of diabetes, has the strongest association with mortality among all diabetic microvascular complications [22]. Among the molecular mechanisms implicated in the pathogenesis and development of DN, oxidative stress is important. A pilot trial conducted by Scaramuzza et al. concluded that an antioxidant diet could improve endothelial dysfunction in T1D adolescents [23]. ACEi is a first-line therapy in the treatment of DN. Many studies have confirmed its antioxidant property in experimental DN [12, 24]. Similarly, the antioxidant activity of artemether had also been demonstrated [17, 18].

In this study, we have demonstrated that catalase was exhausted in the STZ group compared with T1D-ctrl group. The administration of artemether or enalapril alone could slightly recover the level of catalase in the kidney, while combination therapy was effective. Catalase is an important antioxidant enzyme in charge of breaking down hydrogen peroxide [25]. Many studies have demonstrated that the expression of catalase in the kidney decreased in the experimental DN model [26-29]. SOD2 is also an important antioxidant enzyme, while acetylation at k68 and k122 of SOD2 decreased its activity [30, 31]. Previous reports showed that the expression of acetylated SOD2 (k68), concomitant with mitochondrial oxidative stress,

increased in diabetic rats [32, 33]. Acetylated SOD2 (k122) was also important for regulating mitochondrial redox. Activation of SOD2 through deacetylation at k122 could decrease mitochondrial oxidative stress [34]. Given the close relationship between SOD2 and acetylation of its lysine residues, we detected the SOD2 acetylation levels using anti-acetyl-lysine antibodies (acetyl k68 and acetyl k122) with immunoblotting and IHC analysis. Here, we found that increased expression of acetylated SOD2 (k68) in the kidney of STZ mice which was restored with administration of artemether or enalapril alone or their combination. We also showed a rising expression of acetylated SOD2 (k122) in the STZ group, although not significant, while the combination group exerted greater effects through down-regulating the expression of acetylated SOD2 (k122). Conversely, the expression of acetyl SOD2 (k122) was decreased in the kidney of high fat diet-fed Wistar rats [35]. Such a result might be correlated to the experimental model.

Hyperglycemia and hypertension are well-known driving factors initializing or/and exacerbating the development of DN. BP-lowering therapy is recommended for managing proteinuria since BP is associated with the degree of proteinuria [36]. An elevated blood glucose level, UAE, and increased kidney weight were found to characterize T1D mice complicated with kidney disease in the present study. This study showed that artemether exerted effects not only on improving FBG and diabetic symptoms, but also on renal pathological injuries, which is consistent with our previous study. Furthermore, combination therapy provides greater protective effects on aforementioned values and blood pressure. Such evidence implies that the beneficial effects of combination therapy on delaying the process of DN might, to some extent, correlate with its influence on the aforementioned risk factors.

In conclusion, this study found that combination artemether with enalapril provided greater renoprotection in T1D mice than their separate use, probably associated with their synergistic effects on enhancing antioxidant defense.

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Disclosure of conflict of interest

None.

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