Original Article
Combination of astragaloside IV and ACEi ameliorates renal injuries in db/db mice

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Abstract: Evidences demonstrated that the effect on anti-proteinuria and renal protection of Chinese herbs combination with ACEi or ARB seemed to be better than ACEi or ARB alone. Astragaloside IV could decrease the urinary albumin excretion rate and could protect against renal injuries linking to its anti-oxidation ability. We aimed to investigate the effect of astragaloside IV combined with ACEi on diabetic nephropathy and to explore whether its underlying mechanism is dependent on anti-oxidation. 8-week-old male experiment mice were randomly assigned to five groups: lean wild type (wt) group, db/db group, db/db + astragaloside IV group, db/db + enalapril group, db/db + combination therapy with astragaloside IV and enalapril group. During the experiment, 24 hours urinary albumin, fasting glucose, body weight, and metabolic parameters were monitored in regular intervals. At the end of the study, tail blood pressure, serum H$_2$O$_2$, lipid, and liver function were measured and kidney histological injuries were evaluated. Results of the study indicated that combination therapy with astragaloside IV and ACEi further reduced 24 hours urinary albumin excretion rate, blood pressure, and body weight. Combination therapy reduced the foot process width, glomerular base membrane thickness, glomerular tuft cell proliferation, tubular cell atrophy, tubular base membrane thickness, and improved tubular cell proliferation. It modulated the body H$_2$O$_2$ metabolism and up-regulated the expression of the catalase in renal cortex. Astragaloside IV combined with ACEi exerted renal protective effects in db/db mice more significantly than their individual used. The mechanism possibly involved their synergistic effects on anti-oxidation.

Keywords: Diabetic nephropathy (DN), astragaloside IV (AS-IV), angiotensin-II converting enzyme inhibitor (ACEi), combination therapy, reactive oxygen species (ROS)

Introduction
Diabetic nephropathy (DN) is one of the most frequent comorbidities of diabetes, and is the leading cause of chronic kidney diseases (CKD) [1]. Proteinuria is the featured presentation of DN, and to reduce the proteinuria to the greatest extent might delay the progression of DN [2].

Utilizing the renin-angiotensin system (RAS) blockades, such as angiotensin converting enzyme inhibitor (ACEi) or angiotensin-II type 1 receptor blocker (ARB), is the cornerstone in reducing proteinuria in DN treatment [3]. Dual blocking using combination ACEi with ARB was introduced to further reduction of the proteinuria ever. However, a series of trails that investigated the dual blockade of RAS to prevent DN progression had provided negative or inconclusive data [4, 5]. These propel the development of additional therapeutic approaches beyond RAS blockades. In recent years, accumulating evidences had revealed that Chinese herbs could reduce proteinuria and ameliorate the renal injuries independent on RAS blocking [6, 7]. Some trials demonstrated that the effect on anti-proteinuria and renal protection of Chinese herbs combination with ACEi or ARB seemed to be better than ACEi or ARB alone [8, 9]. These provide us clues to explore more effective add-on therapy to RAS blockades in treatment of DN.

Astragaloside IV (AS-IV) is the derivate of Huangqi (Radix Astragali Mongolici) and it has
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a wide range of biological activities, including anti-inflammation, anti-viral, and anti-neoplasm [10]. Some studies indicated that AS-IV could decrease the urinary albumin excretion rate and could protect against diabetic renal injuries [11, 12]. However, effect of AS-IV combined with ACEi or ARB on renal protection has not been investigated. Oxidative stress had been linked to proteinuria and renal injuries [13]. Evidences demonstrated that several antioxidants could reduce inflammation and fibrosis in the diabetic kidney [14]. A previous study had shown that the protection of AS-IV on glucose-induced renal cells injury might be associated with reactive oxygen species (ROS) reduction [15]. Therefore, we aimed to investigate the effect of AS-IV combined with ACEi on diabetic nephropathy and to explore whether its underlying mechanism was dependent on antioxidation.

Materials and methods

Animal experiments

All the animal studies were approved by the Guangzhou University of Chinese Medicine Institutional Animal Care and Use Committee. Specific pathogen free 8-week-old male db/db mice (BKS.Cg-Dock7m/+Leprdb/Nju) and lean wild type control mice were purchased from the Model Animal Research Center of Nanjing University and were housed in the Central Animal Facility at Shenzhen Graduate School of Peking University according to relevant guidelines and regulations. Experiment mice were randomly assigned to five groups: Lean wild type (wt) group, db/db group, both group were fed a regular diet; db/db + astragaloside IV (db/db + AS-IV) group, db/db + enalapril (db/db + ACEi) group, mice from these groups were fed a regular diet supplement with 5 g/kg AS-IV (ChengDu ConBon Biotech Co., LTD, China), 0.8 g/kg enalapril (MedChemExpress, N.J, USA) respectively; db/db + combination therapy with AS-IV enalapril (db/db + Combined) group, fed a regular diet supplement with 5 g/kg AS-IV and 0.8 g/kg enalapril. The treatment lasted for 12 weeks.

Urine albumin determination

Urine was collected 24 hours using metabolic cages (Tecniplast S.p.a, Buguggiate, Italy) at 0, 2, 6, 9, 12 week post treatment. Urine albumin ELISA Kit was purchased from Bethyl Laboratories (Montgomery, TX, USA) and performed according to the manufacturer’s instructions.

Physiological and metabolic parameters

Fasting blood glucose was measured using blood glucose meter (Roche, Basel, Switzerland) every 2 weeks during the experiment, and the body weight was measured. After 12 weeks of treatment, blood HbA1c levels were measured using an Ultra2 HbA1c Analyzer (Primus, Kansas City, MO). Urine and serological indices (urine glucose and serum ALT, AST, TG) were detected using automatic biochemical analyzer (Roche, Basel, Switzerland). Serum H2O2 was measured by using Amplex UltraRed reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Blood pressure was measured via tail cuff using MRBP system (IITC Life Science Inc., CA).

Histological examinations

Kidneys were immediately harvested and processed. For light microscopic analysis, kidney tissues were fixed with 10% formalin. Paraffin-embedded kidneys tissues were cut into 4-μm sections then stained with Periodic acid-Schiff (PAS) and scanned by a slide scanner (Motic Easyscan Digital Slide Scanner, Xiamen, China) to evaluate renal morphological changes. A total of 30-50 renal glomerular mesangial matrix areas were measured. About 50-90 proximal tubules (PAS staining possessing brush border) were randomly selected for lumen cross-sectional area (CSA), tubular CSA measurement and for tubular cell nucleus count. The tubular cell CSA was calculated by using the following formula: Tubular cell CSA = (Tubular CSA-Lumen CSA)/nucleus count. The renal cortex (sized 1 mm3) was fixed in 2.5% glutaraldehyde and then post-fixed in 1% osmic acid for electron microscopic analysis. A total of 8-10 glomerular and tubular photographs in each sample were taken using electron microscopy (EM, JEM-1400, JEOL, Tokyo, Japan) to measure the glomerular basement membrane (GBM), foot process width (FPW), and tubular basement membrane (TBM) thickness. The average GBM and TBM thicknesses were estimated as the ratio of the area inside the recognized GBM and TBM segments to the length of the central line [16]. The FPW was calculated using a previously described method [11].
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ImageJ (National Institutes of Health, Bethesda, MD, USA) was used for all these histological images processing and analysis.

**Western blotting**

The snap-frozen renal tissues were stored at -80°C for western blotting analysis. Renal cortex were homogenized in lysis buffer and prepared in sample loading buffer (Bio-Rad, Hercules, CA, USA). The proteins were separated on SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Danvers, MA, USA). Then membranes were blocked with TBS buffer containing 5% nonfat dry milk at room temperature for 1 hour, and then were incubated with rabbit anti-SOD2 (1:1000 dilution C.S.T, Danvers, MA, USA), rabbit anti-catalase (1:1000 dilution, C.S.T, Danvers, MA, USA), mouse anti-GAPDH (1:1000 dilution, Proteintech, IL, USA) and mouse anti-β-actin (1:4000 dilution, Sigma Aldrich, St. Louis, MO, USA) primary antibodies overnight at 4°C. After membranes were washed with TBS, they were incubated with secondary antibodies for 1 h at room temperature with shaking. ChemiDoc™ MP Imaging System (Bio-Rad, Hercules, CA, USA) was used for protein bands detection and analysis. β-actin or GAPDH were used as the loading control. ImageJ Software was used for densitometric analysis.

**Statistical analysis**

Data were expressed as mean ± SD. Data analysis was performed using SPSS statistics software (IBM, NY, USA). Comparisons between two groups were analyzed by unpaired Student’s t test. Differences among multiple groups were analyzed by using one-way analysis of variance (ANOVA) followed by Bonferroni or Dunnett T3 post hoc analysis. Albuminuria and Serum H2O2 data were analyzed after logarithmic transformation.

**Results**

**Combination therapy with AS-IV and ACEi further reduced albuminuria in DN**

Urinary albumin excretion (UAE) rate of db/db mice was significantly higher than wt mice at the initial of the experiment and increased gradually. After 6 weeks of treatment, it showed a clear reduction of UAE in combination therapy group, while both AS-IV and ACEi therapy group showed no remarkable change compared with db/db control group. In AS-IV therapy group, UAE started to decline from the sixth week of treatment, but it showed statistical significance compared with db/db group until the twelfth week of treatment. ACEi therapy displayed UAE reduction effects earlier than AS-IV group at the ninth week of treatment. At the end of this study, for 12 weeks treatment, compared with db/db control group, all treatment groups showed a significant decrease in UAE. Importantly, the effects of combination therapy on UAE were much more obvious than groups treated with ACEi or AS-IV alone (Figure 1).

**Combination therapy with AS-IV and ACEi ameliorated renal pathological injuries**

At the end of the study, characterized renal pathological injuries, including wider foot process, thicker glomerular base membrane (GBM), thicker tubular base membrane (TBM), tubular cell proliferation and tubular cell atrophy were observed in db/db mice (Figures 2A-E, 3A-E). All therapies in this experiment reversed these featured pathological changes of DN, especially in ultra-structure of the kidney (Figures 2B-E, 3C, 3E). However, compared with AS-IV or with ACEi therapy groups, combination therapy group did not show greater...
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In this experiment, all therapies did not reduce extracellular matrix expansion in db/db mice.

**Combination therapy with AS-IV and ACEi modulated the body H$_2$O$_2$ metabolism**

To determine effects of combination therapy on the body H$_2$O$_2$ metabolism, we detected the serum H$_2$O$_2$ concentration in the end of the experiment. Compared to that in wt group, serum H$_2$O$_2$ concentration was elevated in db/db group significantly (Figure 4A). As shown in the figure, AS-IV therapy, including AS-IV only or combined with ACEI, reduced serum H$_2$O$_2$ concentration. Combination therapy with AS-IV and ACEI reduced the serum H$_2$O$_2$ concentration further. The catalase in renal cortex, an enzyme decomposing H$_2$O$_2$ to water and oxygen, also decreased in db/db group (Figure 4B, 4C). There is no change of SOD$_2$ in all groups (Figure 4B, 4D).

**Combination therapy with AS-IV and ACEi reduced body weight without improving hyperglycemia and diabetic symptoms**

Compared with wt group, heavier body weight, hyperglycemia, polydipsia, polyuria, and increased feces production were observed in db/db group from their initial treatment and continued to the end of the whole experiment. During the first 6 weeks of treatment, combination therapy with AS-IV and ACEI group showed lighter body weight compared to db/db group which was significant in statistics and the effects continued through the end of study (Figure 5A). However, in this study, all therapies did not show effects on improving blood glucose (Figure 6A), urinary glucose (Figure 6C, 6D), HbA$_1c$ (Figure 6B), and some other diabetic symptoms including polydipsia, ployuria and increased feces production (Figure 5B).

**Combination therapy with AS-IV and ACEi decreased blood pressure but not serum lipid level**

Compared with wt group, blood pressure did not show any obvious changes in the db/db mice group at the end of the study. However, with ACEi treatment, mice from ACEi therapy group and combination group showed decrease in blood pressure (Figure 7A). Furthermore, greater effects on lowering the blood pressure was observed in the combination therapy group although it had no obvious significant statistics. No change of blood pressure was seen in the db/db group.

Figure 2. Combination therapy with AS-IV and ACEi attenuated GBM thickening and reduced podocyte FPW. A. Representative PAS staining images of glomerulus. Scale bars, 20 μm. B. Typical glomerulus EM images of the GBM and FPW. Scale bars, 1 μm. C. Bar graph representing the quantification and statistical analysis of extracellular matrix area of glomerulus. n=6 per group. D, E. Bar graph representing the quantification and statistical analysis of GBM thickness, and FPW. n=3 per group. *P<0.05 and **P<0.001 vs. the wt group. ***P<0.01 and ****P<0.001 vs. the db/db group.
Figure 3. Combination therapy with AS-IV and ACEi ameliorated tubular injuries. A, B. Bar graph representing the quantification and statistical analysis of tubular cell cross sectional area, tubular cell nuclei count. n=6 per group. C. Bar graph representing the quantification and statistical analysis of TBM width. n=3 per group. D. Representative PAS staining images of tubules. Scale bars, 20 μm. E. Typical EM images of the TBM. Scale bars, 1 μm. n=3 per group. *P<0.05, **P<0.01 and ***P<0.001 vs. the wt group. #P<0.05, ##P<0.01 and ###P<0.001 vs. the db/db group. ^P<0.05 and ^^P<0.01 vs. the db/db + ACEi group.

Figure 4. Combination therapy with AS-IV and ACEi reduced serum H$_2$O$_2$ level, regulated renal cortical intrinsic antioxidant protein expression. A. Serum H$_2$O$_2$ level in each group. n=5 per group. B. Western blot images of catalase, β-actin, SOD$_2$ and GAPDH. C. Quantification of catalase and SOD$_2$ expression. D. Quantification of GAPDH expression.
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SOD, β-actin and GAPDH in the renal cortex of mice in various groups. C, D. Bar graphs showing the fold change in protein expression after normalization to the internal control. n=5-6 per group. **P<0.01 vs. the wt group. *P<0.05, **P<0.01 and ***P<0.001 vs. the db/db group. P<0.05 vs. the db/db + ACEi group. †P<0.05 vs. the db/db + AS-IV group.

Discussion

Our study indicated that the combination of AS-IV and ACEi alleviated the proteinuria and delayed the progression of renal injuries of DN in db/db mice better than using AS-IV or ACEi alone. The underlying mechanisms might be associated with the enhanced antioxidative effects of combination therapy.

Albuminuria is the sensitive biomarker and outcome predictor of DN, which correlated to the renal structural lesions including GBM thickening, wider FPW, and tubular injuries et al [17, 18]. In this present study, we found that all of the AS-IV, ACEi and combination therapy improved glomerular and tubular injuries. But no one specific pathological injury index was ameliorated much remarkable in combination therapy group than in AS-IV or ACEi used alone. These results were not fully consistent with the change of urinary albumin levels. It suggested that greatest effects on albuminuria reduction of combination therapy might benefit from overall morphological injuries amelioration. Oxidative stress enhanced in the diabetic nephropathy [19]. Imbalance between ROS generation and elimination may enhance ROS accumulation in the kidney both directly and indirectly injuries, including glomerular fibrosis [20], tubules atrophy [21], or vessels sclerosis [22]. In our experiment, along with elevating serum H$_2$O$_2$ concentrations, both glomerular and tubular injuries were observed in db/db mice.

Angiotensin-II (Ang II) was elevated in kidney diseases with hypertension or normotension, and it could increase the circulating ROS with
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Figure 6. Combination therapy with AS-IV and ACEi had no effects on blood glucose level. A. Fasting blood glucose of mice at 0, 2, 6, 9, and 12 weeks in the indicated groups. B-D. Bar graphs representing the quantification and statistical analysis of serum HbA1c, urinary glucose excretion rate, urinary glucose concentration. n=6 per group. ***P<0.001 vs. the wt group.

Figure 7. Combination therapy with AS-IV and ACEi decreased blood pressure but not serum lipid level. A, B. Bar graphs representing the quantification and statistical analysis of systolic blood pressure, serum triglyceride. n=6 per group. **P<0.01 vs. the wt group. *P<0.05 and ##P<0.01 vs. the db/db group. $P<0.01$ vs. the db/db + AS-IV group.

or without the presence of angiotensin II type 1 (AT1) receptors [23]. Furthermore, ROS-induced renal injuries would generate Ang II which reinforced the feedback of promoting further ROS generation [24]. Evidence showed that ACEi treatment could reduce ROS production in the kidney [25]. In our experiment, we also found that ACEi treatment lowered the serum H2O2 concentration and protected the mice from renal injuries. Previous studies revealed that AS-IV had the property of antioxidation [26], and present study also showed AS-IV could reduce the serum ROS production. Interestingly, combination therapy further decreased the serum ROS production along with reduced albuminuria, compared to those
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treated with AS-IV or ACEi alone. There are some studies demonstrated that reduced SOD and catalase content, the two intrinsic important antioxidants, might lead to ROS overload in the kidney with diabetes or hypertension [27, 28]. Increasing the expression of SOD and catalase could promote the H$_2$O$_2$ degradation [28]. In this experiment, SOD$_2$ expression showed no differences among all groups, and the changes of the catalase seemed complicated. Catalase was depletion in db/db mice significantly and was restored by AS-IV or ACEi therapy. According to the serum H$_2$O$_2$ levels, catalase expression in combination therapy would be much more than in AS-IV and in ACEi group. Actually, it was just more than db/db group and less than both AS-IV and ACEi group alone. Taken into consideration its effects on improving renal pathological injuries and proteinuria and serum ROS, we inferred that after treated with AS-IV combined with ACEi, the kidney might need no more intrinsic catalase to catalyze the conversion of H$_2$O$_2$ to water and oxygen.

Hyperglycemia, hypertension, and obesity are factors initializing or/and deteriorating the diabetic nephropathy [29]. In this present study, combination with AS-IV and ACEi decreased the systolic blood pressure, body weight. These implicated that combination therapy slowing down the development of the DN might partially, due to its effects on these risk factors. Hypoglycemia effect of AS-IV is quite controversially [30, 31]. Our study showed that AS-IV has no effects on improving fasting blood glucose, HbA$_1c$ or diabetic symptoms consistent with some studies [32]. The conflicts might be associated with the dose of AS-IV, or diabetic animal model used.

In conclusion, the results of these experiments suggested that combination AS-IV with ACEi exerted renal protective effects in db/db mice more significantly than their individual used. It likely involves their synergistic effects on antioxidation. However, further investigations are needed to elucidate the underlying mechanisms.

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Disclosure of conflict of interest

None.

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