

Ethanol extract of *Nepeta angustifolia* C. Y. Wu ameliorates hyperuricemia in fructose-induced mice

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Abstract: As a popular medicinal plant traditionally used in Tibet of China, *Nepeta angustifolia* C. Y. Wu is mainly administered to treat apoplexia, cerebral haemorrhage, fainting and epilepsy and other symptoms, while its effect on hyperuricemia is still unclear. In the present study, we evaluated the improvement of the 70% ethanol extract of *Nepeta angustifolia* C. Y. Wu in fructose-induced hyperuricemic mice. The results revealed that *Nepeta angustifolia* C. Y. Wu significantly decreased blood glucose and blood lipid levels, as well as lowering the urinary levels of uric acid, creatinine and urea nitrogen. Meanwhile, it effectively restored the serum levels of uric acid, creatinine and urea nitrogen and inhibited serum and hepatic XOD activities and renal oxidative stress, while suppressing the secretions of TNF- α , IL-1 β and IL-6 in kidney. *Nepeta angustifolia* C. Y. Wu also attenuated the infiltration of inflammatory cells and reduced the production and accumulation of glycogen and collagen, while restoring the dysregulated protein expressions of renal URAT1, GLUT9, OAT1 and OAT3. In summary, our results support the idea that *Nepeta angustifolia* C. Y. Wu is a promising agent for treating hyperuricemia.

Keyword: Fructose, hyperuricemia, *Nepeta angustifolia* C. Y. Wu, xanthine oxidase, uric acid transporters.

INTRODUCTION

Hyperuricemia refers to the presence of supersaturated urate in the extracellular fluid. The condition occurs when a male or female has a serum uric acid (UA) exceeding 7.0mg/dl or 5.7mg/dl has diagnosed as hyperuricemia (Chen-Xu *et al.*, 2019).

Over recent decades, the development of sweet drinks and fast food has dramatically increased intake of fructose riching in purines. Sufficient evidence demonstrated that one of the essential reasons for the increased prevalence of metabolic syndrome is the long-term consumption of fructose, which is mainly manifested in insulin resistance, such as elevated blood glucose levels and abnormal levels of TC, TG, LDL-c and HDL-c and the detection of their levels can generally act as indicative roles in the clinical diagnosis of hyperuricemia (Taskinen *et al.*, 2019). In addition, emerging evidence showed that high-fructose intake increased the incidence of hyperuricemia and as an inducer of hyperuricemia, high-fructose-diet has been widely used to establish hyperuricemic animal models (Zhang *et al.*, 2020; Zhu *et al.*, 2017).

UA is an end-product in the body, produced from purine metabolism and metabolized from hypoxanthine and

xanthine under the action of xanthine oxidase (XOD) (Wang *et al.*, 2020). The increased serum level of UA is generally accompanied by the elevated activity of XOD; therefore, the level of XOD in serum and in kidneys is an essential index for evaluating the UA level (Doehner and Landmesser 2011). As metabolic substances in the body, blood urea nitrogen (BUN) and creatinine (Cr) genuinely reflect the level of UA in blood and in urine from the side (Cai *et al.*, 2017). Urate transporter 1 (URAT1) and glucose transporter-like protein-9 (GLUT9) are efficient UA transporters, in the kidneys, it is mainly responsible for the reabsorption of UA and organic anion transporter 1 (OAT1) and organic anion transporter 3 (OAT3) are necessary transporters for excretion of UA in the body. Thus, regular expression of UA transporters is the crucial factor to maintaining the balance of UA between in serum and in urine and the imbalanced expression of UA transporters is another main reason for worsening hyperuricemia (Keenan 2020; Wang *et al.*, 2018).

Excessive fructose intake causes the imbalance of oxidative stress, which leads to inflammatory response and renal injury (Qiao *et al.*, 2017). As essential indexes to measure the degree of oxidative stress, it is necessary to detect the level of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and methane dicarboxylic aldehyde (MDA). A higher level of UA was

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considered to promote the development of inflammation and led to the recruitment of leukocytes and monocytes, which would further release numerous tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β) and interleukin-6 (IL-6) and aggravated the structure destruction and dysfunction of kidney cells, eventually caused damage to the microvessels and organs (Wang *et al.*, 2016).

As a popular medicinal plant traditionally used in Tibet of China, *Nepeta angustifolia* C. Y. Wu (NA) is mainly used to treat apoplexy, cerebral hemorrhage, fainting and epilepsy. Our previous phytochemical studies found that NA possesses an abundance of ursolic acid, oleanolic acid (Meng *et al.*, 2017), which has been confirmed that it could exert significant protective effects against glucose-induced inflammation in endothelial cells through modulating levels of inflammation and oxidative stress (Huang *et al.*, 2018). This plant can also suppress the apoptosis of the mesangial cells in type II diabetic rats, thereby ameliorating renal injury (Huang *et al.*, 2020). In view of the fact that there is no report about NA treatment on hyperuricemia, hyperuricemia mice induced by a high-fructose diet were used to probe into the pharmacological effects of NA extract and the possible mechanisms.

MATERIALS AND METHODS

Chemicals and reagents

Allopurinol (>98%, Catalog No.A0083) and fructose (\geq 99%, Catalog No.F0127) obtained from Sigma-Aldrich Shanghai Trading Co., Ltd. (Shanghai, PR China). Enzyme-linked immunosorbent assay (ELISA) kits for tumor necrosis factor- α (TNF- α , Catalog No. EK0527), interleukin-1 β (IL-1 β , Catalog No. EK0396) and interleukin-6 (IL-6, Catalog No. EK0421) purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Primary antibodies: mouse/rat anti-urate transporter 1 (URAT1, Catalog No. #DF12340), anti-organic anion transporter 1 (OAT1, Catalog No. #DF8582), anti-OAT3, the secondary antibodies: Actin (Catalog No. #AF7018) sourced from Affinity Biologicals, Inc. (Cincinnati, OH, USA) and anti-glucose transporter-like protein-9 (GLUT9, Catalog No. ab223470) purchased from Abcam (UK). Assay kits for the measurement of triglyceride (TG, Catalog No. A120-2-1), total cholesterol (TC, Catalog No. A113-2-1), low-density lipoprotein-cholesterol (LDL-c, Catalog No. A113-1-1), high-density lipoprotein-cholesterol (HDL-c, Catalog No. A112-1-1), xanthine oxidase (XOD, Catalog No. A002-1-1), uric acid (UA, Catalog No.C012-2-1), creatinine (Cr, Catalog No. C011-3-1), blood urea nitrogen (BUN, Catalog No. C013-2-2), superoxide dismutase (SOD, Catalog No. A002-3-3), methane dicarboxylic aldehyde (MDA, Catalog No. A003-2-3) and glutathione peroxidase (GSH-Px, Catalog No. A004-2-1) provided by Jiancheng Biotech (Nanjing, China).

Plant preparation

A voucher specimen (No. utibet-1608) kept in the herbarium of the Department of Medicament, College of Medicine, Tibet University. NA identified by Professor Jule Wang (College of Medicine, Tibet University, China) in August 2020. NA were crushed into powder and boiled twice with 70% ethanol before being processed under reflux for 2 h for each round. The obtained liquid was filtered with filter paper following suction filtration and the remaining decoction was evaporated in vacuo. The ethanol extract of NA was stored in the Component Bank of Tibetan Medicine, Lhasa, China (CBTM-210).

Experimental animals

Mice provided by Qingdao Institute for Food and Drug Control (approval number: SYXK (Lu) 2021 0009). Mouse-related experiments were designed according to the standards of the Animal Ethics Committee at Qingdao University of Science & Technology (approval number: ACQUEST-2020-056). Mice were housed in a room at 23 \pm 1 °C with a relative humidity of 55 \pm 5%, a light-dark cycle of 12h and normal diet and water.

A total of 60 mice, 10 mice were randomly selected and fed normally for 12 weeks as the normal control group (NC, n=10); the remaining 50 mice were temporarily divided into one group, drank 30% fructose aqueous solution and fed normally for 6 weeks (Yang Y *et al.*, 2015). After 6 weeks, split into five groups (n=10): the hyperuricemic model control group (MC); the positive group treated with allopurinol (Allo, 5mg/kg/d, i.g.); low-dose NA group (L-NA, 86.7mg/kg/d, i.g.), medium-dose NA group (M-NA, 173.3mg/kg/d, i.g.) and high-dose NA group (H-NA, 346.7mg/kg/d, i.g.) (Huang S *et al.*, 2020). Body weight was recorded after 12 weeks and fasting blood glucose (FBG) was measured after fasting for 12 hours, concurrent with the oral glucose tolerance test (OGTT), in which gavaged with glucose solution (2.0 g/kg), starting from 0 minutes, the blood glucose of the mice were measured every 30 minutes for five times.

Samples collection

Mice were transferred to metabolic cages according to groups, without the restriction of diet and water, recorded 24h urine volume, retained urine samples for measurement, fasted for 12h and euthanized. The blood collection method was a cardiac puncture. The left and right kidneys were removed, fixed in 4% paraformaldehyde and homogenized respectively. After homogenization, the supernatant was stored at -80°C (Huang *et al.*, 2020).

Biochemical assays in serum and urine

Fresh blood and urine samples were centrifuged to separate the supernatant and stored at -80°C, centrifugation conditions: 3000 rpm/min for 15 min. The

serum concentrations of TC, TG, LDL-c and HDL-c, along with the serum and urinary levels of UA, BUN and Cr, the serum and hepatic level of XOD were assayed. Furthermore, renal SOD, MDA and GSH-Px levels were detected using the appropriate assay and the levels of TNF- α , IL-1 β and IL-6 in renal were measured by ELISA kit (Yu *et al.*, 2018).

Histopathological evaluation of renal tissue.

Freshly removed kidneys were fixed with 4% paraformaldehyde for more than 24hours, then washed with water, dehydrated by gradient elution with ethanol, dealcoholized with xylene to make the tissue transparent, and then embedded in paraffin middle, deparaffinization after sectioning, hematoxylin and eosin (H&E) staining, periodic acid-schiff (PAS) staining and Masson staining, and microscopic observation, recording of histopathological changes. Take a photograph of the slice. Image J software was used to analyze the following characteristics. Fifty glomeruli were randomly selected for calculation. Glomerular volume: (glomerular area)^{2/3}×1.25. Ten glomeruli were randomly selected from each layer, percentage of PAS-positive substances = the area of PAS-positive substances/cross-sectional area of glomeruli. Glomerular collagen content = collagen area/glomerular area (Hossain *et al.*, 2020).

Western blot analysis

Proteins obtained from renal tissue homogenates were loaded and separated on a 12% SDS-PAGE gel; after that, the gel is retained and the PVDF membrane, sponge pad and filter paper are attached in order to remove air bubbles and transfer the protein to the membrane. The membrane was sealed with Tris-buffered saline Tween-20 (TBST) blocking solution containing 5% non-fat dried milk for 3h, then cultured with an appropriate concentration of diluted primary antibody (1:1000 dilution in 1× antibody binding buffer) for 2h. After washing the membrane three times, the goat anti-rabbit/mouse antibody was added with horseradish peroxidase to the TBST solution to combine with the second antibody (1:5000 dilution in 1× antibody binding buffer) for 1.5h in the incubating membrane. Protein visualization using ECL western blotting substrate (Amersham Bioscience, Buckinghamshire, UK). Protein bands were calculated by Chemi Doc image analyzer (Tanon 4600, Tanon, China) (Tian *et al.*, 2020).

Ethical approval

Animal experiments were approved by the Ethics Committee at Qingdao University of Science and Technology.

STATISTICAL ANALYSIS

All data were expressed as the mean±SD. Double-tailed t-tests were used to determine the differences between

groups. Statistical analysis was conducted using Prism 7 software (GraphPad, San Diego, CA, USA). A value of P<0.05 was assumed to be significant.

RESULTS

Effects of NA on body weight, kidney weight, kidney index and urine volume in hyperuricemic mice

In Figure 1A, a comparison of body weight changes in different groups of mice following a high-fructose diet. As expected, compared with the NC group, no significant increase or decrease in body weight was observed in mice following of high-fructose diet or NA treatment. Moreover, the kidney weight, kidney index and urine volume of the MC group were all increased (P<0.05 or P<0.01), but after NA intervention, the afore-mentioned indices all showed a dose-dependent decrease (fig 1B-1D) and the M-NA group and H-NA group showed significant difference compared of that in MC group (P<0.05). The above results demonstrate that NA could effectively reduce the kidney weight, kidney index and urine volume in high-fructose mice.

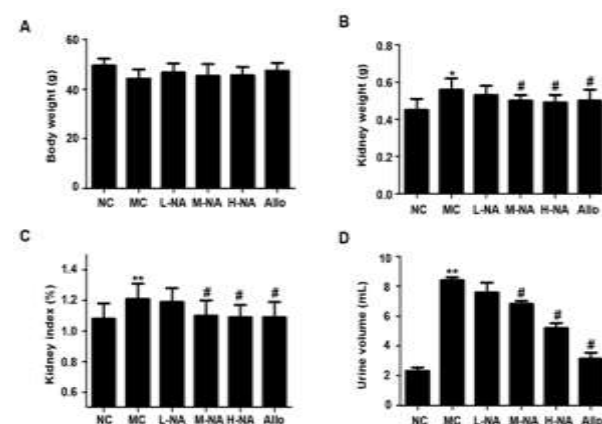


Fig. 1: Effects of NA on body weight, kidney weight, kidney index and urine volume in hyperuricemic mice. Notes: (A) Body weight. (B) Kidney weight. (C) Kidney index (The ratio of kidney weight to body weight). (D) Urine volume. Groups: NC: normal control group, MC: hyperuricemia model control group, L-NA: low-dosage of NA group, M-NA: medium-dosage of NA group, H-NA: high-dosage of NA group, Allo: allopurinol group. The data are expressed as the mean ± SD, n=10. *P<0.05 or **P<0.01 compared to NC group; #P<0.05 compared to MC group.

Effects of NA on FBG level, OGTT level and blood lipids levels in hyperuricemic mice

After 6-week treatment with NA, the FBG level of mice was examined and OGTT was performed. In fig 2A-2B show that blood glucose levels in the FBG and OGTT were significantly increased in the MC group compared with the NC group (P<0.05). These results suggest that the high-fructose diet has dramatically elevated the blood glucose level and maintained long-term. Consequently,

NA significantly decreases the elevated blood glucose levels and the magnitude of the fallen was positively correlated with the dosage. Furthermore, mice in the MC group developed severe dyslipidemia, with elevated serum TG, TC and LDL-c levels, along with decreased HDL-c levels (fig 2D-2G) ($P < 0.05$). A significant reversal in elevated or decreased blood lipid levels was observed following 6-week NA intervention ($P < 0.05$). Hence, NA exhibited significant hypolipidemic effects.

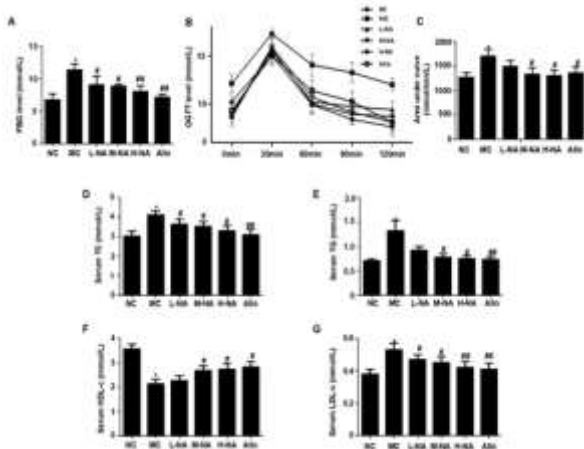


Fig. 2: Effects of NA on blood glucose level and blood lipid levels in hyperuricemic mice. Notes: (A) FBG level, (B) OGTT level, (C) Area under curve, (D) Serum TC, (E) Serum TG, (F) Serum HDL-c, (G) Serum LDL-c. FBG: fasting blood glucose; OGTT: oral glucose tolerance test; TC: triglyceride; TG: total cholesterol; HDL-c: high density lipoprotein cholesterol; LDL-c: high density lipoprotein cholesterol. The data are expressed as the mean \pm SD, n=10. * $P < 0.05$ or ** $P < 0.01$ compared to NC group; # $P < 0.05$ or ## $P < 0.01$ compared to MC group.

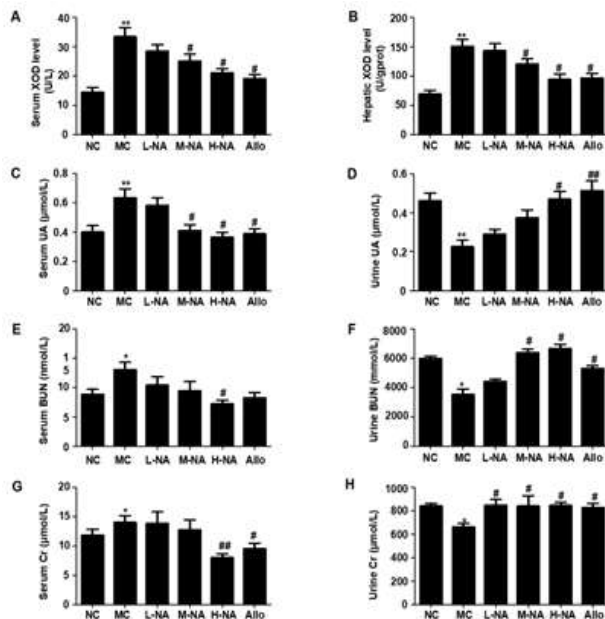


Fig. 3: Effects of NA on XOD activity and renal function related parameters in hyperuricemic mice. Notes: (A)

Serum XOD level, (B) Hepatic XOD level, (C) Serum UA level, (D) Urinary UA level, (E) Serum BUN level, (F) Urinary BUN level, (G) Serum Cr level, (H) Urinary Cr level. XOD: xanthine oxidase; UA: uric acid; Cr: creatinine; BUN: blood urea nitrogen. The data are expressed as the mean \pm SD, n=10. * $P < 0.05$ or ** $P < 0.01$ compared to NC group; # $P < 0.05$ or ## $P < 0.01$ compared to MC group.

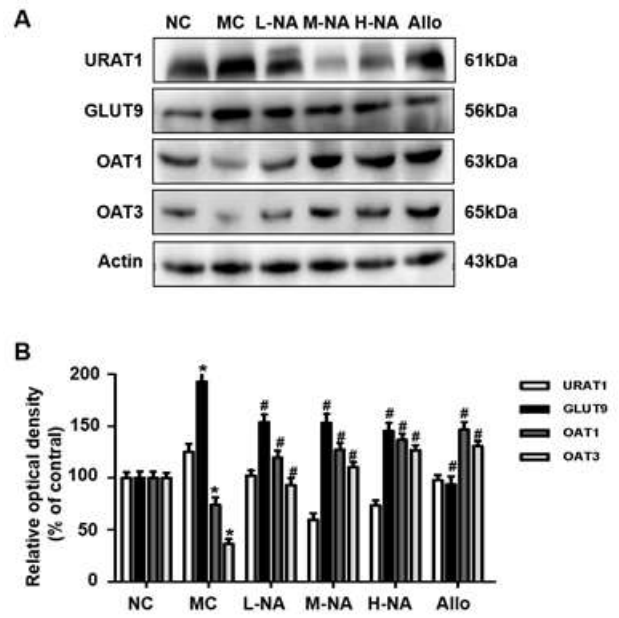


Fig. 4: Effects of NA on protein expression of renal UA transporters in hyperuricemia mice. Notes: (A) The protein expressions of UA transporters, (B) Relative optical density. Values are mean \pm SD of 3 independent experiments with triplicate samples. * $P < 0.05$ or ** $P < 0.01$ compared to NC group; # $P < 0.05$ or ## $P < 0.01$ compared to MC group.

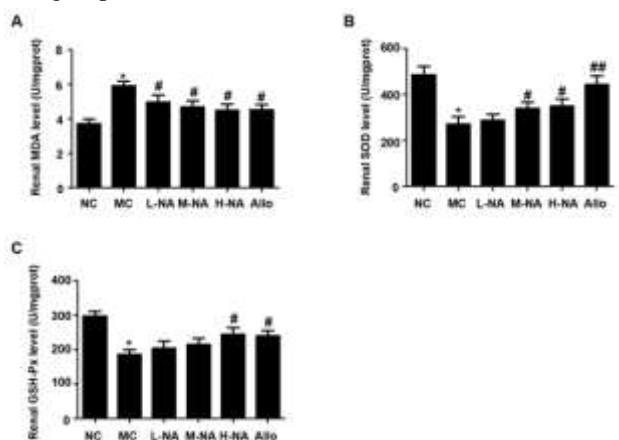


Fig. 5: Effects of NA on renal oxidative stress in hyperuricemic mice. Notes: (A) MDA, (B) SOD, (C) GSH-Px. MDA: malondialdehyde; SOD: superoxide dismutase; GSH-Px: glutathione peroxidase. The data are expressed as the mean \pm SD, n=10. * $P < 0.05$ or ** $P < 0.01$ compared to NC group; # $P < 0.05$ or ## $P < 0.01$ compared to MC group.

Effects of NA on XOD activity and biochemical parameters related to renal function.

As shown in fig 3A-3B, compared to that in the NC group, a high-fructose diet increased serum and hepatic XOD activities with statistical significance ($P < 0.01$). However, NA effectively inhibited the elevated XOD activities, while the M-NA group and H-NA group exhibited a significant differences compared to that in the MC group ($P < 0.05$). Fig 3C-3H presents the data provided by the experiments; the changes in biochemical parameters related to renal function were analyzed in serum and urine. High-fructose diet leads to increased levels of UA, Cr and BUN *in vivo* and inhibits their excretion *in vitro* ($P < 0.05$ or $P < 0.01$). After intervention, compared with MC group, NA significantly reversed the levels of UA, BUN and Cr in serum and urine. ($P < 0.05$ or $P < 0.01$)

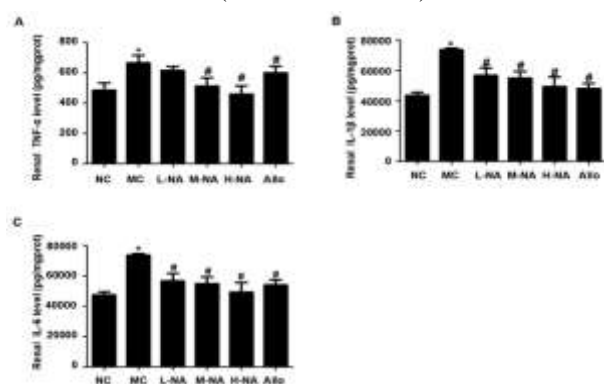


Fig. 6: Effects of NA on renal inflammatory responses in hyperuricemic mice. Notes: (A) TNF- α , (B) IL-1 β , (C) IL-6. TNF- α : tumor necrosis factor- α ; IL-1 β : interleukin-1 β ; IL-6: interleukin-6. The data are expressed as the mean \pm SD, n=10. * $P < 0.05$ or ** $P < 0.01$ compared to NC group; # $P < 0.05$ or ## $P < 0.01$ compared to MC group.

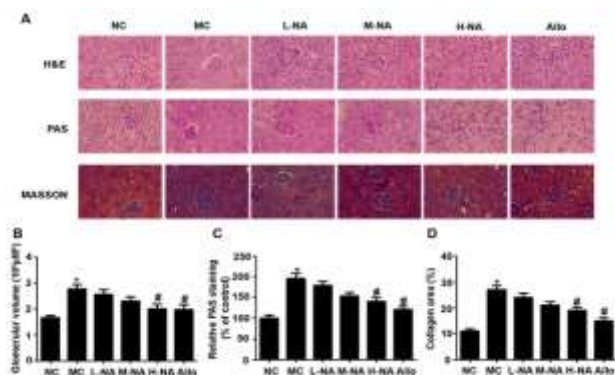


Fig 7: Effect of NA on histopathological changes in kidney. Notes: (A) H&E staining, PAS staining and Masson staining (200 \times). (B) Glomerular volume, (C) Relative PAS staining, (D) Collagen area. H&E: hematoxylin-eosin. PAS: periodic acid-schiff. * $P < 0.05$ or ** $P < 0.01$ compared to NC group; # $P < 0.05$ or ## $P < 0.01$ compared to MC group.

Effect of NA on UA transporters in hyperuricemic mice

The dysregulation of renal UA transporters was observed

in MC group mice as intended. Fig 4 shows that the expression of URAT1 and GLUT9 was inhibited in M-NA and H-NA ($P < 0.05$), and up-regulates the expression of OAT1 and OAT3 anion transporters ($P < 0.05$), showing the opposite trend to the MC group.

Effects of NA on renal antioxidants in hyperuricemic mice

The levels of oxidative stress are shown in fig. 5A-5C. The renal MDA level was significantly increased in the MC group after a high-fructose diet ($P < 0.05$) and intervention with NA decreased renal MDA level compared to the MC group ($P < 0.05$). Similarly, SOD as an antioxidant enzyme of the renal and its level was significantly reduced ($P < 0.05$). Similar trends were also observed in renal GSH-Px levels, whereas treatment with NA effectively increased the levels of these enzymes ($P < 0.05$). It can be concluded that NA can significantly inhibit high-fructose-induced renal oxidative stress.

Effects of NA on renal pro-inflammatory cytokines in hyperuricemic mice

In figs. 6A-6C, it can be observed that long-term fructose intake leads to increased levels of TNF- α , IL-1 β and IL-6 in the renal *in vivo*, while NA intervention can reduce their levels with statistical significance ($P < 0.05$).

Effect of NA on renal histopathological changes in hyperuricemic mice

The histopathological changes in the kidneys caused by high-fructose consumption were studied using H&E, PAS and Masson staining. The features obtained using these different staining methods are shown in the fig. 7. Compared with the normal diet, high-fructose consumption caused prominent infiltration of inflammatory cells to the kidney. It dilated the glomeruli in the MC group, while generating significant thickening of the basement membrane. Meanwhile, an abundance of glycogen, collagen and fibrin were observed in the MC group. However, treatment with NA markedly restored glomerulus structure and renal tissue damage, while reducing the accumulation of glycogen and collagen fibre protein.

DISCUSSION

Combined with previous pharmacological research and our material analyses on 70% ethanol extract of NA, we found that it possesses an abundance of ursolic acid, oleanolic acid, betulinic acid and β -sitosterol, which are generally considered to be the main biological components present in the treatment for many diseases, showing unique hypoglycemic, hypolipidemic, anti-inflammatory, antioxidant and anti-apoptosis activities (Mlala *et al.*, 2019; Wang *et al.*, 2018; Taiwo *et al.*, 2017), which also have been substantiated in studies (Huang *et al.*, 2018; Huang *et al.*, 2020). In this study, in order to

explore whether NA intervention has a certain protective effect on hyperuricemia, we established a hyperuricemia mice model and obtained a preliminary positive result.

Long-term or excessive fructose intake will impair glucose homeostasis, which is incipiently manifest by increased blood glucose levels and damaged glucose tolerance. Excessive glucose was converted into lipids by the liver and the terrible result is that a large amount of lipids accumulates in the liver, resulting in dyslipidemia, which is manifested by the increase of TC, TG and LDL-c and decrease of HDL-c in the blood (Hannou *et al.*, 2018; Sever *et al.*, 2012). As confirmed in this study, excessive fructose intake could cause hyperglycemia and hyperlipidemia in mice. At the same time, NA intervention effectively improved these symptoms, which was consistent with our previous findings, that is, NA has the hypoglycemic and hypolipidemic effects, which has been confirmed in both type II diabetic rats induced by high-fat-diet combined with streptozotocin and hyperuricemic mice induced by fructose.

XOD, synthesized in the liver, is one of the main rate-limiting enzymes which could catalyze xanthine and hypoxanthine to produce UA (Yu *et al.*, 2020). The experimental results showed that high-fructose consumption promoted XOD activity in hyperuricemic mice, while NA intervention dose-dependently inhibited XOD activity both in blood and liver. Based on these results, we speculate that a particular component of NA may be a potential inhibitor of the XOD enzyme, which can reduce UA production by inhibiting its activity. Further experiments are needed to confirm this hypothesis. The abnormal expression of UA transporters in hyperuricemia is another major cause of the development and deterioration of hyperuricemia (Wang Z *et al.*, 2019). The experimental results showed that as the most essential proteins in the process of UA reabsorption into blood, the protein expression of URAT1 and GLUT9 were increased by long-term fructose stimuli, while NA treatment effectively protein expression of them. OAT1 and OAT3 play crucial roles in the process of UA excretion into urine. Long-term fructose intake decreased their expression, while NA treatment significantly reversed their expression. Thus, the hypothesis deduced from this experiment is that NA may improve hyperuricemia by inhibiting the activity of XOD and regulating the expression of UA transporters.

Oxidative stress and inflammatory response are the main culprits of renal injury caused by a higher level of UA. Several studies showed that excessive fructose intake promotes the imbalance of reactive oxygen species and down-regulates the antioxidant defense mechanism, such as the decrease of SOD and GSH-Px activity (Bernardes *et al.*, 2017; Nakagawa *et al.*, 2020). In addition, the accumulation of UA in the kidney is the primary source of

toxicity, which trigger inflammatory cascade reaction and the release of inflammatory cytokines due to stimulation, further promoting the vicious circle of vascular endothelial dysfunction and eventually leading to renal vascular injury (Braga *et al.*, 2019; Wang *et al.*, 2018). In this experiment, glycogen accumulation and renal fibrosis were significantly concentrated in the renal of hyperuricemic mice and high levels of oxidative stress and inflammatory responses were detected, while NA treatment significantly promoted the activities of SOD and GSH-Px and reduced the MDA production and inhibited the release of TNF- α , IL-1 β and IL-6 and effectively ameliorated the glycogen accumulation and fibrosis degree in the kidney, which indicated that NA might reduce the renal injury *in vivo* by affecting oxidative stress and inflammatory reaction. Compared with previous studies, we believe that NA has anti-inflammatory and antioxidant effects and can improve renal damage. These changes were also explained by the content changes of UA, Cr and BUN in blood and in urine.

Taken together, NA shows protective effects against high-fructose-induced hyperuricemia and renal injury and modulation the expression of the UA-related transporters and inhibiting the activity of XOD may be the underlying mechanisms. These findings have significant implications for a natural product and NA can be considered to be a new source for drug development in the therapy of hyperuricemia. However, the molecular mechanism of NA in treating hyperuricemia is still unclear, which is a limitation of our study and further studies are needed to elucidate fully.

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