

The Therapeutic Effect and Possible Mechanism of Guluronic Acid in Copper-Loaded Mice

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Abstract Objective: To explore the therapeutic effect and possible mechanism of low molecular weight align--guluronic acid (PG) on the copper-loaded mice, so as to provide a new idea for the prevention and treatment of Wilson's disease. **Methods:** Fifty healthy male Kunming mice were fed copper-loaded diet to establish the copper-loaded mouse model. The mice in treatment group were given different concentrations of PG by gavage, while the mice in control group were orally given same amount of normal solution simultaneously 1 time per day for 60 days. The neurobehavioral function of mice was evaluated by animal behavior; the liver function indexes of mice such as serum levels of total protein (TP), albumin (ALB), globulin (GLB) were detected by biochemical assay, and the ratio of ALB/GLB (A/G) was calculated; the expression levels of ceruloplasmin (CER), adenine monophosphate activated protein kinase (AMPK) and phosphorylated-AMPK (p-AMPK) in hepatic cells were detected by Western blotting. **Results:** Compared with control group, the weight of mice in model (Copper) group decreased significantly ($P<0.05$); The time of rotating rod shortened significantly ($P<0.05$) and the climbing time prolonged significantly ($P<0.01$); The serum level of TB decreased significantly ($P<0.05$), while the ratio of A/G was significantly increased ($P<0.05$); The expression of CER enhanced significantly ($P<0.05$), and the level of p-AMPK/AMPK was significantly increased ($P<0.05$). After treated with different concentrations of PG, the weight of mice in Copper+PG-H group was significantly higher than that in Copper group ($P<0.05$); The time of rotating rod prolonged significantly ($P<0.01$) and the climbing time shortened significantly ($P<0.05$); The serum level of TB increased significantly ($P<0.01$), while the ratio of A/G was significantly dropped ($P<0.05$); The expression of CER reduced significantly ($P<0.01$) and the level of p-AMPK/AMPK was significantly decreased ($P<0.01$). **Conclusion:** PG could adsorb and excrete copper ions, reduce inflammation reaction, improve the hepatic metabolism of copper-loaded mice, so that to act a positive effect on Wilson's disease.

Keywords: guluronic acid, copper load, ceruloplasmin, phosphorylated-AMPK, mice, liver

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1. Introduction

Wilson's disease (WD), an autosomal recessive genetic illness of copper metabolism disease, was first identified by Wilson in the United States in 1911 [1]. Hepatolenticular degeneration (HLD) was the label given to a case that was described in China in 1932 and had clinical signs resembling those of WD. HLD is characterized by chronic liver damage, extrapyramidal symptoms and corneal pigmentation rings, accompanied by plasma cuprocytin (CER) deficiency and aminoaciduria [2]. The clinical manifestations of HLD are Copper ions (Cu^{2+}) are necessary to support biological activity, but concentrations exceeding the body's tolerance can lead to hepatocytotoxicity, inducing oxidative stress, mitochondrial dysfunction, and apoptosis in hepatocytes [3]. Impaired copper metabolism and synthesis in hepatocytes

cannot synthesize CER, leading to extensive copper deposition in liver, brain, kidney, cornea and other organs and tissues. The application of ^{64}Cu to study copper metabolism *in vivo* proved that the reduction of serum CER is the main reason for copper accumulation in WD [4]. CER concentration less than 200 mg/L is one of the diagnostic criteria for WD [5]. WD is a copper-loaded disease, and current existing animal models of WD carry loss-of-function mutations or deletion of the P-type copper-transporting ATPase (ATP7B) [6]. Copper-loaded models of chronic overfeeding of copper-containing feeds and copper water are similar to WD models [7,8]. Previous studies by our research group have shown that fucoidan has a certain effect on copper excretion, but the mechanism is not well understood [9,10]. However, the mechanism is not yet clear. The aim of this study is to further investigate the therapeutic effect of low molecular weight align--guluronic acid (PG) on copper-loaded mice, and to provide new ideas

for the prevention and treatment of WD.

2. Materials and Methods

2.1. Main Instruments and Reagents

Bar Rotator (Med Associates, Inc, USA); CS-800 Automatic Biochemistry Analyzer (Shenzhen Yonglixin Technology Company); Eppendorf 5430R Refrigerated Centrifuge (Eppendorf, Germany); WIX-EP300 Electrophoresis Instrument and Membrane Transfer Instrument (Beijing WIX Technology Co., Ltd); Gel Imaging Analysis System (UVP, Analytik Jena US LLC, USA).

Guluronic acid (PG, purity > 95%, product No.: SAGKA, Qingdao Bozhihui Biotechnology Co. Ltd.); Copper sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, purity > 99.0%, product No.: 20210819, Sinopharm Chemical Reagent Co., Ltd.); Rabbit anti-mouse CER (product No.: 66156-1, Wuhan Sanying Biotech Co. Ltd.) Ltd; Rabbit anti-mouse AMPK (product No.: 2603S), p-AMPK (product No.: 2535S), Cell Signaling Technology USA; β -actin (product No.: bs-0061R, Beijing Bo Aosun Biotechnology Co. Ltd); Goat anti-rabbit horseradish peroxidase IgG secondary antibody (product No.: abs20002, Beijing Ebixon Biotechnology Co. Ltd.); RIPA lysate (Product No.: CW2333S), BCA protein quantification kit (Product No.: CW00145), Beijing Kangwei Century Biotechnology Co. Ltd.; High-sensitivity chemiluminescence detection kits (Immobilon ECL Ultra Western HRP substrate, ECL, Product No.: WBULS0500, Millipore, USA).

2.2. Experimental Animals

Specific Pathogen Free (SPF) grade male Kunming mice 50 cases, two months old, body mass 40-44 g, were purchased from Jinan Ponyue Laboratory Animal Breeding Co. Ltd. Production License No.: SCXK (LU) 20190003, and Certificate of Conformity No.: 370726221101107382. This experiment was approved by the Ethics Committee for Laboratory Animal Welfare of Qingdao University (No. 20221018KM5020230525082), and the experimental steps strictly complied with the ethical requirements of animal welfare.

2.3. Model Construction and Interventions

Experimental animals: 50 Kunming mice were kept in the SPF class laboratory animal room of Qingdao University Laboratory Animal Center at a constant temperature of 23-25 °C with natural light and free diet. After adapting to the environment for 1 week, 10 mice were randomly selected as the control group and continued to be fed with normal feed; the remaining 40 mice were fed with copper sulfate loading diet to establish a copper loading model [11,12]. The remaining 40 animals were applied the copper sulfate loading diet to establish the copper loading model. All the animals survived without death or detachment. The 40 successfully constructed copper-loaded mouse models were included in the statistics and randomly grouped for intervention.

2.3.1. Treatment Group (Control)

The control group (Control) consisted of 10 animals, which were routinely housed and gavaged with 1 mL of saline daily for 60 d. The control group consisted of 10 animals.

2.3.2. Treatment Group (Copper)

In the model group (Copper), 10 animals were gavaged with 1 mL of saline daily for 60 d after modeling.

2.3.3. Treatment Group (Copper+PG)

Referring to Xu et al. [13] reported doses, LMWA was divided into low (Copper+PG-L), medium (Copper+PG-M) and high (Copper+PG-H) dose groups, 10 animals in each group, 50 mg/kg body mass, 100 mg/kg and 200 mg/kg body mass, in a volume of 1 mL, were gavaged once a day for 60 d. The LMWA was used as a treatment group for the first 60 days.

2.4. Observation Indicators

2.4.1. Body Mass of Mice

mice were acclimatized to the environment for 1 week, and the body mass of mice in each group was recorded as the body mass of pre-modeling mice; after the completion of modeling, the body mass of mice in each group was again recorded as the body mass of post-modeling mice; and the body mass of mice in each group after the start of the drug treatment was recorded as the body mass of post-treatment mice. The body mass of each group was analyzed and compared with that of the other groups at different times.

2.4.2. Baton Twirling Test

Mice were pre-adapted to baton twirling for 3 d before the official test, once a day. The starting speed of the baton was 1 r/min, the final speed was 40 r/min, and the test cut-off time was 300 s. The time that the mice maintained balance on the baton, i.e., the time on the baton, was used to assess the motor coordination of the mice.

2.4.3. Pole-climbing Test

At the end of treatment, mice were allowed to climb down from the top of a 50 cm, 1 cm diameter gauze-covered pole, and the time from the start of the downward climb to the forelimb touching the ground was recorded, and more than 60 s was recorded as 60 s. Acclimatization was performed 3 d before the formal test, which was carried out 1 week prior to the formal experiment.

2.4.4. Liver Function Index Test

After the end of treatment, 10% Aphrodite intraperitoneal injection (20 mL/kg) was used to anesthetize mice, 1.0 mL of blood was extracted via the heart, centrifuged at 4 000 r/min for 5 min, and the supernatant was taken to the EP tube. The serum total protein (TP, g/L), albumin (ALP, g/L), and globulin (GLP, g/L) were detected by applying an automatic biochemistry instrument, and the white globulin ratio (A/G) was calculated.

2.4.5. Western Blotting

3 mice were randomly selected from each group, 100 mg of liver tissue was placed in a 1.5 mL EP tube, lysed by adding 1 mL of lysis solution for 30 min, grinded and ultrasonically homogenized, centrifuged at 4 °C and 12 000 r/min for 20 min, and the supernatant was extracted, and the protein concentration was determined by BCA method. The protein concentration was determined by BCA. 20 µg of the protein sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and electrotransferred to a polyvinylidene difluoride membrane. 5% skimmed milk powder (1 × TBST) was closed at room temperature for 2 h. The membrane was washed, and then the primary antibody was added (AMPK 1:1,000, p-AMPK 1:1 000, CER 1:1 000, and β-actin 1:10 000), and the membrane was incubated overnight at 4 °C. The membrane was washed and incubated overnight at 4 °C. After washing the membrane, add sheep anti-rabbit secondary antibody (1:10 000), incubate at room temperature for 1.5 h, and develop the color with high sensitivity chemiluminescence detection reagent (ECL) luminescence reagent. analyze the grayscale value of each band by Image J software, and repeat it 3 times, take the average value. Calculate the relative gray value (target protein to β-actin ratio), AMPK phosphorylation degree = (p-AMPK content/AMPK content) × 100 %.

2.5. Statistical Analysis

GraphPad Prism 8.0.2 software was used for statistical analysis. The data information that conformed to normal distribution and chi-square was expressed as ($\bar{x} \pm s$), the number of experimental observation cases were 5 groups, $n = 10$ in each group, one-way ANOVA was used for comparison between multiple groups, and Turkey method was used between two groups, and $P < 0.05$ was used to represent that the difference was statistically significant.

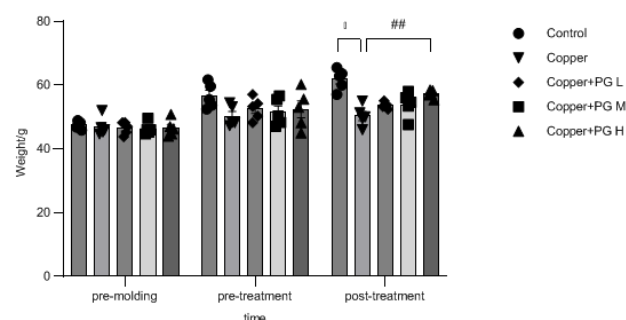
3. Results

3.1. Effect of LMWA-PG on Body Mass of Mice

Copper is an important trace element for the organism, but excessive intake can have some adverse effects on the organism. In Figure 1, the body mass of mice in the Control group increased to (56±3) g. The body mass of mice in the Control group increased to (56±3) g after modeling. After modeling, the body mass of mice in the Control group increased to (56±3) g. The body mass of mice in the remaining groups was lower than that in the Control group, but there was no significant difference in body mass among the groups ($P > 0.05$).

The body mass of mice in the Control group increased to (62±3) g after treatment. The body mass of mice in the Copper group was significantly lower than that in the Control group ($P < 0.01$). The body mass of mice in the Copper+PG-H group was significantly higher than that of the Copper group ($P < 0.05$), and the body mass of the treatment groups was higher than that of the Copper group,

but there was no significant difference between the Copper+PG -L, Copper+PG-M were not significantly different compared with Copper group, and no significant difference was observed in the comparison among the 3 treatment groups ($P > 0.05$).

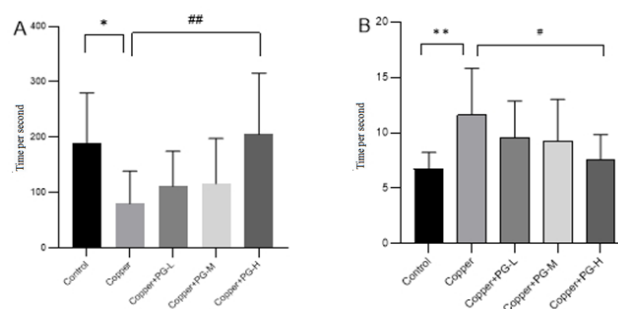


* $P < 0.01$, Control group vs Copper group; # $P < 0.05$, Copper group vs Copper+PG-H group

Figure 1. The comparison of body weight of mice in each group

3.2. Effects of LMWA-PG on Behavioral Functions in Mice

Figure 2A shows that mice in the Copper group had a significantly shorter ($P < 0.05$) residence time on the rotating rod compared to mice in the Control group, whereas mice in the Copper+PG-H group had a significantly longer ($P < 0.01$) residence time on the rotating rod compared to mice in the Copper group. Figure 2B shows that the rod-climbing time of mice in the Copper group was significantly longer ($P < 0.01$) than that of mice in the Control group, whereas the rod-climbing time of mice in the Copper+PG-H group was significantly shorter ($P < 0.05$) than that of mice in the Copper group.



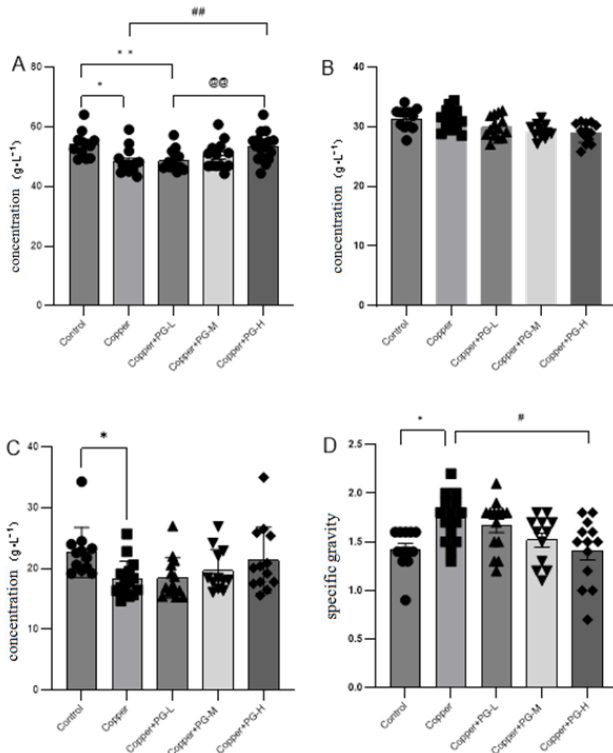
Compared with Control group * $P < 0.05$, ** $P < 0.01$, compared with Copper group, # $P < 0.05$, ## $P < 0.01$

Figure 2. Rotating rod experiment (A) and Pole climbing experiment (B)

3.3. Effect of LMWA-PG on Protein Metabolism in Mice

Figure 3A shows that the TP levels of mice in both Copper and Copper+PG-L groups were significantly lower than those in Control group ($P < 0.05$), and the TP levels of mice in Copper+PG-H group were significantly higher than those in Copper group Copper+PG-L group ($P < 0.01$). Figure 3B shows that the ALB levels of mice in the Copper group were decreased compared to the Control group ($P > 0.05$), and the ALB levels of mice in the

treatment group were both decreased compared to the Copper group, but none of the group comparisons were significant ($P>0.05$). Figure 3C shows that the GLB levels of mice in the Copper group were significantly lower than those in the Control group ($P<0.05$), and the GLB levels of mice in the treatment group were all increased compared with those in the Copper group, but none of the group comparisons were significant ($P>0.05$). Figure 3D shows that the A/G value was significantly higher in the Copper group compared to the Control group ($P<0.05$), while the A/G ratio was significantly lower in the Copper+PG-H group compared to the Copper group ($P<0.05$).



* $P<0.05$, ** $P<0.01$ vs Control group; # $P<0.05$, ## $P<0.01$ vs Copper group; @ $P<0.01$ vs Copper+PG-L group

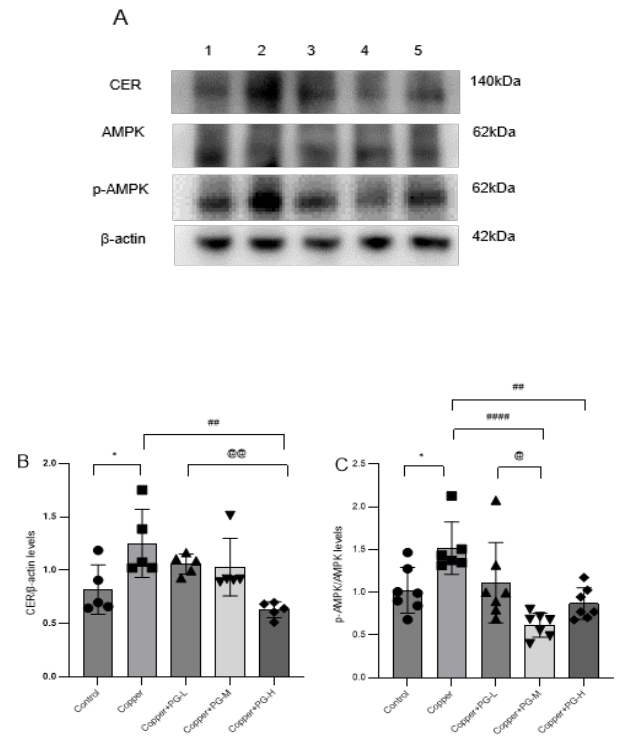
Figure 3. Comparison of protein metabolism in total protein concentration (A), albumin concentration (B), globulin concentration (C) and white globulin ratio (D) in each group of mice

3.4. Effect of LMWA-PG on hepatic CER and p-AMPK expression in mice

Shown as Figure 4, the hepatocyte CER expression level of mice in Copper group was significantly higher than that of Control group ($P<0.05$), and the hepatocyte CER expression level of treatment group was reduced compared with that of Copper group in both groups, in which the hepatocyte CER expression level of Copper + PG-H group was significantly lower than that of Copper group ($P<0.01$), and the Copper + CER expression level was significantly lower in the Copper + PG-H group than in the Copper + PG-L group ($P<0.05$).

There was no significant difference in the AMPK expression level among all groups ($P>0.05$). The p-AMPK expression levels of hepatocytes in Copper group was higher than that in Control group; while p-AMPK

expression levels of hepatocytes in Copper + PG-H group, Copper + PG-M group and Copper + PG-L group were all significantly lower than those in the Copper group ($P<0.05-0.01$); but there was no significant difference between the Copper + PG-H and Copper + PG-M groups ($P>0.05$). Since there was no significant change in AMPK in all groups, the trend of phosphorylation degree of AMPK (p-AMPK/AMPK) was basically the same as that of p-AMPK.



Lanes 1-5: Control group, Copper group, Copper+PG-L group, Copper+PG-M group, Copper+PG-H group

* $P<0.05$ vs Control group, ## $P<0.01$, #### $P<0.0001$ vs Copper group; @ $P<0.05$, @@ $P<0.01$ vs Copper+PG-M group

Figure 4. The comparison of the expression levels of CER and p-MAPK in each group. Western Blot (A), CER/β-actin levels (B) and p-MAPK/MAPK levels (C)

4. Discussion

The clinical manifestations of WD mainly involve the liver, nerves, kidneys, blood and skeletal muscular system, among which the liver and the basal ganglia nucleus pulposus are most commonly involved [14]. The liver and basal ganglia nuclei are most commonly involved. Copper toxicity is the main cause of organ damage in WD patients [15]. Copper toxicity is the main cause of organ damage in patients with WD, and the liver accumulates a large amount of Cu^{2+} and severely impairs liver function [16]. Neurologic symptoms include tremor, dystonia, ataxia, dysarthria and dysphagia [17]. Fucoidan is a substance from brown algae. Fucoidan is a polysaccharide extracted from brown algae and has a different selectivity for ions [15,18]. previous studies reported that PG has good

adsorption of metal ions and has a therapeutic effect on copper loading related diseases [19].

The changes in body mass of mice can reflect the general condition of mice to some extent. This experiment shows that PG is therapeutic for copper-loaded mice. The results of neurobehavioral tests proved that the results of rod-twirling and pole-climbing experiments were statistically significant, suggesting that PG can alleviate the damage to the nervous system caused by copper loading by promoting the metabolism of Cu^{2+} and thus improve the motor coordination function of mice [8,9].

Plasma proteins are mainly synthesized in the liver, and plasma protein levels reflect the liver's ability to synthesize and metabolize substances and the body's systemic condition. Plasma proteins mainly include TP, ALB, GLB, and the albumin/globulin ratio (A/G). ALB binds and transports many endogenous and exogenous molecules, and has various physiological properties such as anti-inflammatory, antioxidant, and anticoagulant properties. GLB is the main component of immunoglobulin, which is related to immune response, and an elevated A/G is indicative of the presence of chronic inflammation in the body [20]. The results of the present study showed that hepatocyte inflammatory injury was alleviated after PG treatment. This may be attributed to PG adsorption and excretion of Cu^{2+} , which favors the recovery of liver function [8,9,10].

Ceruloplasmin (CER), also known as copper oxidase, is synthesized in hepatocytes and binds to Cu^{2+} , which is excreted from the body via bile or urine. The antioxidant effect of CER may be important in neurodegenerative diseases [21] and low serum CER levels are a diagnostic test for WD [22]. The results of the present study showed that the model group of small children had a low serum CER level. The results of the present study showed that the expression of CER in hepatocytes of mice in the model group was significantly enhanced compared with that in the control group, which suggesting successful modeling. After PG treatment, hepatocyte CER expression decreased in all groups of mice compared with the model group, in which the hepatocyte CER expression level was significantly lower in the Copper+PG-H group compared with the Copper group. This is not consistent with the results of the previous study [8,9,10], probably because the present experiment is not an APT7B gene-deficient model, but a copper-loaded mouse model, and there is no CER synthesis disorder in the mice, but rather the Cu^{2+} excess caused oxidative stress and inflammatory injury in the hepatocytes, and thus the hepatocyte CER expression level was irritability increased, and the CER expression was reduced after the treatment. The exact mechanism remains to be confirmed by further studies with the ATP7B gene defect model.

Adenine monophosphate activated protein kinase (AMPK) is a sensor that coordinates eukaryotic cell metabolism and specific energy requirements (ATP/AMP ratio), also known as an "energy receptor" [23]. AMPK is rapidly activated after almost all mitochondrial stresses [24]. During nutrient and energy depletion, when the AMP/ATP ratio is elevated, ATP depletion activates the energy-sensitive liver kinase B1 (LKB1), which in turn activates AMPK [25]. It has been demonstrated that AMPK stimulates apoptosis and autophagy signaling, and

that Cu^{2+} induces autophagy [26,27]. The results of the present study showed that the expression of p-AMPK in hepatocytes of mice in the model group was significantly higher than that in the control group, suggesting a high degree of apoptosis and autophagy in hepatocytes, and stress factors such as energy deficiency or inflammation, which proved that the modeling was successful, and that Cu^{2+} caused hepatic damage to mice. After treatment with PG intervention, p-AMPK expression was significantly reduced compared with the model group, among which, p-MAPK expression was more significant in the middle-dose group, suggesting that PG has a role in alleviating the hepatocyte damage caused by Cu^{2+} .

In conclusion, PG can promote the excretion of Cu^{2+} , reduce inflammatory injury, and improve hepatic metabolic function, thus improving neurobehavioral function in the Cu^{2+} -load model.

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Ethics Approval and Consent to Participate

This experiment was approved by the Ethics Committee for Laboratory Animal Welfare of Qingdao University (No. 20221018KM5020230525082).

Conflicts of Interest Statement

The authors declare that there are no conflict of interest.

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