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A possible new mechanism and drug intervention for kidney damage of arsenic poisoning rats

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Abstract

Arsenic poisoning is a worldwide endemic disease that affects thousands of people. Currently, the aetiology of the disease is known, but its pathogenesis is uncharacterized and there is no specific treatment. We established a rat model of coal-burning arsenic poisoning by feeding the animals corn powder baked with high arsenic coal. By observing subsequent changes in kidney and immune function, we found that arsenic induces both kidney and immune damage. Furthermore, there is a significant correlation between kidney and immune damage. Moreover, Ginkgo biloba, a known immune enhancer, was used as an intervention agent in arsenic poisoned rats to validate the relationship between kidney and immune damage. Meanwhile, we also explored the mechanism of Ginkgo biloba treatment of kidney damage in burning-coal arsenic poisoned rats. We found that Ginkgo biloba enhanced immune function in rats with arsenic poisoning and ameliorated arsenic-induced kidney damage. These results suggest that immune suppression may be one of the mechanisms underlying arsenic-induced kidney damage and that Ginkgo biloba might relieve kidney damage by enhancing immune function.

Keywords: arsenic poisoning; kidney; ginkgo; immune function; rat.

Introduction

Arsenic is an environmental toxicant and a known carcinogen. Chronic exposure to arsenic continues to be a major public health problem worldwide, affecting thousands of people. In the Guizhou province of China, arsenic poisoning caused by the burning of coal in unventilated indoor stoves represents an unusual but common type of exposure^[1]. The aetiology of this disease is known, but its pathogenesis remains uncharacterized and no specific treatment exists. Thus, progress in the prevention and therapy of arsenic poisoning has been slow.

It is well known that the kidney is the organ that is primarily responsible for arsenic excretion; as such, its structure and function are vulnerable to damage. Many studies^[2-5] have shown that arsenic exposure is a risk factor for kidney damage. According to current research, there are two possible mechanisms of kidney damage by arsenic: oxidative stress and apoptosis. A functioning immune system is critical for mounting a response to foreign substances and pathogens, and an immunomodulator imbalance can affect the body's immune response, thereby resulting in disease. Accumulating lines of evidence^[6-7] indicate that arsenic may adversely affect the immune system, but the relationship between immune damage and kidney damage is poorly understood.

Ginkgo biloba is an ancient herbal medicine, the most important active compounds of which are flavonoids and ginkgolides. Some studies have found^[8-9] that ginkgo biloba can improve the body's immune function. Other studies have shown that ginkgo extract has a preventive effect on kidney damage and kidney interstitial fibrosis^[10-11]. Nevertheless, the role and mechanism of Ginkgo biloba in kidney damage by arsenic are poorly understood.

This study was designed to investigate the relationship between kidney and immune damage in a coal-burning arsenic poisoning model in rats. Ginkgo biloba was used as an intervention agent in arsenic poisoned rats to demonstrate that Ginkgo biloba may relieve kidney damage by enhancing immune function. This study provides an animal experimental basis for the application of Ginkgo biloba in the treatment of kidney and immune damage resulting from endemic arsenic poisoning.

Materials and methods

Drugs, reagents and instruments

Ginkgo biloba was produced by the Yangtze River Pharmaceutical Group Co. under the approval number Zhunzi Z20027949, China. Blood CD₃⁺, CD₄⁺ and CD₈⁺; urinary albumin (UALB); N-acetyl-beta-D-glucosaminidase (UNAG); beta-2-micro-globulin (Uβ₂-MG); and creatinine (UCr) kits were purchased from the Nanjing Jiancheng Bioengineering Institute. Arsenic standard stock (1.00 g/L) was purchased from the National Research Center (GB08611). All other reagents used were analytical grade and made in China. A Vista MPX ICP-OES spectrometer (Varian, Inc. USA), VGA-77 hydride generator (Varian, Inc. USA), MQX200 microplate reader (Olympus, Japan), Ethos high pressure microwave digestion system (Italy Milestone Company), AU400 automatic biochemical analyser (Olympus, Japan), AF-610D2 chromatography-atomic fluorescence spectrometer (Beifen-Ruili Company, China), T6 UV-visible spectrophotometer (Purkinje Company, China), and BX51 Upright System Microscope (Olympus, Japan) were used.

Experimental animals

A total of 140 weaned specific pathogen-free (SPF) Wistar rats [80-100 g, half male and half female, certificate of conformity for the SCXK (Chongqing) 2007-0003] were housed in a standard vivarium at ambient temperature (20-22°C) and standard relative humidity (60-70%) under a 12 h/12 h light/dark cycle. After one week of adaptive feeding, the rats were randomly assigned to a group. This study was performed according to the national and institutional guidelines and approved by Animal Experimental Ethical Committee of Guizhou Medical University (approval number is 1503145).

Dose design and feed preparation

Based on preliminary test results (arsenic trioxide acute oral toxicity LD₅₀ of 43.0 mg/kg), low (2.5 mg/kg), middle (5.0 mg/kg) and high (10.0 mg / kg) arsenic groups were established.

Corn powder baked using high arsenic coal from an arsenism ward was used as the main raw material (average arsenic concentration: 164.67 mg/kg), in line with the quality requirements of "Laboratory animals—Mice and rats formula feeds" (GB 14924.3-2001) for formulated feeds. Based on dose and assuming a 10% body weight as food intake, the arsenic concentrations of the high, medium and low dose groups in feeds were calculated to be 25, 50, and 100 mg/kg, respectively. All feeds were formulated by the experimental animal centre of the Third Military Medical University in China by adjusting the proportion of corn powder baked using high arsenic

coal. Simple random sampling was used to extract arsenic-containing feed ($n = 6$ in each group) and determine its arsenic content. After digestion via the wet digestion method, hydride generation and inductively coupled plasma optical emission spectrometry were used to determine the arsenic content of the feed. Finally, the average arsenic contents in the feeds were used to calculate the actual exposure dose.

Animal groups and administration

This study includes two parts: model generation and treatment. By feeding corn powder baked with high arsenic coal, we established a coal-burning arsenic poisoning rat model. The model rats were divided into four groups, including a control (A) group and low, middle, and high arsenic groups. The treatment portion of the study included control (B), high arsenic, recovery and treatment groups. The actual feed consumption of rats was measured every day to calculate the actual exposure dose of the rats based on food consumption and body weight. Ginkgo biloba was administered by gavage. Specific animal groups and the administration methods used are shown in Table 1.

Sample collection

After the end of the experiment, 24 h of urine production was collected for each rat using a metabolic cage. A polyethylene plastic treated with 1:9 nitric acid was used to collect the urine. One millilitre of urine was acidified with hydrochloric acid to a final pH of less than 2 and was used for urinary arsenic (UAs) assay. One millilitre of the urine sample was alkalinized with 1 mol/L sodium hydroxide to a final pH of approximately 8 and was used to determine the concentration of $U\beta_2$ -MG. The rest of the urine sample was used untreated to measure UALB, UNAG and UCr levels. All samples were stored at -20°C until analysis.

Finally, the rats were anesthetized with 0.9% sodium pentobarbital, and two ml of heparin anticoagulated blood was collected through the heart and used to characterize T lymphocyte subsets.

After killing the rats by anaesthesia, the kidneys were immediately removed. The kidneys were slit, and half of the kidney was fixed in 10% neutral formalin for histopathological examination. The rest of the kidney was stored at -80°C and used to determine the arsenic content.

Validation of the animal model

To validate the rat model of kidney damage by arsenic poisoning, poisoning symptoms, body

weight changes, urinary arsenic content, kidney arsenic (KAs), kidney weight to body weight ratio (kidney weight / body weight) and kidney histopathology were characterized. Poisoning symptoms were observed daily, and the weight was determined weekly. UAs was determined by atomic fluorescence spectrometry, which is the national health industry standard in China and has an accuracy of 97.5 to 113.6% and a coefficient of variation (precision) of 7.0 to 9.1%. UAs were standardized to the concentration of UCr. After digestion via the wet digestion method, hydride production and inductively coupled plasma optical emission spectrometry were used to determine the arsenic content of the kidney. After fixation, dehydration, embedding in paraffin, sectioning (4 μm), and hematoxylin eosin staining, morphological changes of the kidney were observed by light microscopy. According to the extent and severity of disease, the lesions were divided into three categories: negative (-), positive (+), and strongly positive (++). If the extent of the disease was greater than or equal to 25% of the kidney, the sample was considered to be positive. Strongly positive sampled exceeded 50%. In the high power field ($\times 400$), the percentage of positive cells was determined to characterize the severity of the lesion. If the positive cell rate exceeded or reached 25%, it was characterized as positive. If it exceeded 50%, the sample was determined to be strongly positive. The highest detected severity was considered to be the final result.

Determination of kidney function

The concentrations of UALB, UNAG and U β_2 -MG were determined by enzyme linked immunosorbent assay. The concentration of UCr was determined by the Jaffe reaction method. All urinary parameters were standardized to the concentration of creatinine in the urine.

Determination of T lymphocyte subsets

Lymphocytes in rat blood were isolated, and T lymphocyte subsets (CD $_3^+$, CD $_4^+$ and CD $_8^+$) were quantified by the peroxidase labelled streptavidin avidin staining method. Two-hundred random cells were counted under a microscope to calculate the rate of CD $_3^+$, CD $_4^+$ and CD $_8^+$ cells.

Statistical analysis

SPSS version 13.0 software were used for frequency calculations, F test, correlation analysis, trend chi-square test and Fisher's exact test. For quantitative data, one-way analysis of variance was used for comparisons of more than two groups. Additional pairwise comparisons were performed using the Least Significant Difference method. The data were expressed in terms of means and standard deviations. The relationship between kidney damage and immune damage

was determined using Spearman correlation analysis. Moreover, the trend chi-square test and Fisher's exact test were used to analyse the count data. Further pairwise comparisons were performed using the chi-square section method. The significance level was set at 0.05.

Results

Actual exposure amounts.

The average arsenic content of the control (A), low, middle and high arsenic groups was 1.58, 23.17, 47.28 and 94.99 mg/kg, respectively. According to the average daily feed consumption of the rats, the actual exposure amounts of the rats in the control (A), low, middle and high arsenic groups were calculated to be 0.13, 1.87, 4.12 and 8.81 mg/kg, respectively. These results are presented in Table 2.

An arsenic poisoning kidney damage rat model was successfully established.

To validate kidney damage via arsenic poisoning in rats, poisoning symptoms, body weight changes, urinary arsenic, kidney arsenic (KAs), the kidney weight to body weight ratio (kidney weight / body weight), and kidney histopathology were observed.

(1) Poisoning symptoms. The rats of the control (A) and low arsenic groups did not exhibit obvious poisoning symptoms. However, the middle and high arsenic groups presented varying degrees of fluffy hair, hair loss, listlessness, decreased activity, and slowed response. These symptoms in the high arsenic group were exacerbated compared with the middle arsenic group.

(2) Body weight changes. Throughout the experimental period, the difference in body weight between the low arsenic group and control (A) group was not statistically significant. From the beginning of the 7th week, the body weight of the high arsenic group was less than that of the control (A) group and the low arsenic group (P all < 0.05). Beginning in the 10th week, the body weight of the rats in the middle arsenic group was decreased compared with the control (A) group (P all < 0.05). In the 12th week, the body weight of the rats in middle arsenic group was higher than that of the low arsenic group ($P < 0.05$). These results are presented in Figure 1A.

(3) Urinary arsenic and kidney arsenic. With increasing doses of arsenic exposure, the level of UAs and KAs gradually increased. UAs and KAs in the high arsenic group were higher than in the control (A) group as well as the low and middle arsenic groups ($P < 0.05$ or 0.01). Compared

with the control (A) group, UAs in the middle arsenic group was increased ($P < 0.05$) and KAs in the low and middle arsenic groups were also increased ($P \text{ all } < 0.05$). These results are presented in Figure 1B.

(4) Kidney weight to body weight ratio. The difference in the kidney weight to body weight ratio for all groups was not statistically significant ($F = 0.260$, $P = 0.900$). These results are presented in Figure 1C.

(5) Histopathology of the kidney. Histopathology of the kidneys in the control (A) group rats and low arsenic group rats showed clear glomerular and tubular structures, tubular epithelial cells with normal morphology, lumen rules, and no interstitial inflammatory cell infiltration; very few tubular epithelial cells exhibited scattered vacuole degeneration. Tubular atrophy, granular casts, and a small amount of chronic inflammatory cell infiltration of the kidney interstitial space were observed in the rats of the middle arsenic group. In the high arsenic group, early glomerular sclerosis, tubular atrophy, and marked chronic inflammatory cell infiltration of the kidney interstitial space were observed. According to the extent and severity of disease, these results show that the incidence and severity of chronic inflammation, tubular atrophy and glomerular sclerosis increased with increasing doses of arsenic exposure ($\chi^2_{\text{correlation}} = 16.322, 18.605, 7.484$; $P = < 0.001, < 0.001, 0.006$). However, vacuole degeneration and granular casts did not exhibit this trend ($\chi^2_{\text{correlation}} = 3.413, 0.200$; $P = 0.065, 0.655$). These results are presented in Figure 1D.

The relationship between immune damage and kidney damage in arsenic poisoned rats

Effect on cellular immunity in arsenic poisoned rats. By observing the changes in cellular immunity in coal-burning arsenic poisoned rats, we showed that the CD_3^+ rate in the control (A) group was higher than in the low, middle and high arsenic groups ($P = 0.047, 0.002, < 0.001$). Furthermore, the rate of CD_3^+ cells in the high arsenic group was lower than in the low and middle arsenic groups ($P = < 0.001, 0.008$). The CD_4^+ cell rate was higher in the control (A) group than in the middle and high arsenic groups ($P = 0.006, < 0.001$), and the low arsenic group was higher than high arsenic group ($P = 0.010$). The difference in the rate of CD_8^+ cells in all groups was not statistically significant ($F = 0.700$, $P = 0.558$). The CD_4^+/CD_8^+ ratio in the control (A) group was higher than in the low, middle and high arsenic groups ($P = 0.046, 0.001, < 0.001$). In addition, the CD_4^+/CD_8^+ ratio in the high arsenic group was lower than in the low arsenic group ($P = < 0.001$). Differences in the rate of CD_3^+ and CD_4^+ cells as well as the CD_4^+/CD_8^+ ratio in other groups

were not statistically significant. These results are presented in Figure 2A.

The effect on kidney function in arsenic poisoned rats. We found that the UALB content in the middle and high arsenic groups was higher than in the control (A) group ($P = 0.003$, <0.001), but this difference was not significant for UALB between the low arsenic group and control (A) group ($P=0.125$). The UNAG content in the middle and high arsenic groups was higher than in the control (A) group ($P = 0.005$, <0.001), but there was no significant difference between the low arsenic group and the control (A) group ($P = 0.211$). Compared with the low arsenic group, the UNAG content of the high arsenic group was increased ($P = 0.007$). The U β_2 -MG contents of the low, middle and high arsenic groups were higher than that of the control (A) group ($P = 0.024$, 0.003 , <0.001), but there were no significant differences between the other groups. These results are presented in Figure 2B

The relationship between cellular immunity and kidney function. A correlation analysis of kidney function and cellular immunity revealed that there is a clear correlation between UALB, UNAG, U β_2 -MG and CD $_3^+$ ($r = -0.864$, -0.857 , -0.932 ; P all <0.001), CD $_4^+$ ($r = 0.883$, -0.884 , -0.904 ; P all <0.001), and the CD $_4^+$ /CD $_8^+$ ratio ($r = -0.326$, -0.425 , -0.353 ; $P = 0.002$ <0.001 , 0.001). However, there are no correlations between UALB, UNAG, U β_2 -MG and CD $_8^+$ ($r=-0.308$, -0.103 , -0.172 ; $P=0.053$, 0.527 , 0.289). These results are presented in Figure 2C.

The therapeutic effect of Ginkgo biloba on kidney damage in arsenic poisoned rats

Due to its known role as an immune enhancer, Ginkgo biloba was used as an intervention agent in arsenic poisoned rats to further validate the relationship between kidney damage and immune damage. Meanwhile, we aimed to explore the mechanism of Ginkgo biloba treatment of kidney damage in rats.

The effect of Ginkgo biloba on cellular immunity. By observing changes in cellular immunity, we found that the rate of CD $_3^+$ and CD $_4^+$ cells as well as the CD $_4^+$ /CD $_8^+$ ratio in the treatment group were higher than those high arsenic group ($P=0.002$, 0.013 , 0.019) and recovery group ($P=0.004$, 0.033 , 0.042). Compared with the control (B) group, the positive rate of CD $_3^+$, CD $_4^+$ and CD $_4^+$ /CD $_8^+$ ratio in the high arsenic group and recovery group were decreased ($P < 0.05$ or 0.01), but there were no significant differences between the treatment group and control (B) group ($P=0.340$, 0.708 , 0.172). The difference in the rate of CD $_8^+$ cells in all groups was not statistically significant ($F = 0.275$, $P = 0.843$). These results are presented in Figure 3A.

The effect of Ginkgo biloba on kidney function. By monitoring changes in kidney function, we found that the UALB and U β_2 -MG contents in the treatment group were decreased compared to those of the high arsenic group ($P = 0.018, <0.001$). Compared with the control (B) group, the UALB and U β_2 -MG contents of the high arsenic group and recovery group were increased ($P < 0.05$ or 0.01), but there are was no significant difference between the treatment group and control (B) group ($P = 0.372, 0.363$). Moreover, the UNAG contents in the high arsenic, recovery, treatment groups were higher than that that of the control (B) group ($P \text{ all } < 0.001$). These results are presented in Figure 3B.

The effect of Ginkgo biloba on histopathology of kidney. Histopathology of the kidney in the control (B) group showed clear glomerular and tubular structures, tubular epithelial cells of normal morphology, lumen rules, and interstitial with no inflammatory cell infiltration; only some tubular epithelial cells were found with scattered vacuole degeneration. In the high arsenic group, early glomerular sclerosis, tubular atrophy, a large number of chronic inflammatory cell infiltrations of the kidney interstitial space were observed. After 45 days of recovery, the histopathology of the kidney in the recovery group still showed tubular atrophy and a large number of chronic inflammatory cell infiltrations of the kidney interstitial space. However, in the treatment group, no inflammatory cell infiltration in the interstitial space was observe and only some tubular atrophy was observed.

According to the extent and severity of the disease, cases with different degrees of kidney disease for each dose group show that there is no significant difference between the groups for vacuole degeneration ($\chi^2 = 5.406, P = 0.056$). The incidences and severities of chronic inflammation in the treatment group were decreased compared to the high arsenic group and recovery group ($P = 0.003, 0.020$), and there was no significant difference between the treatment group and control (B) group ($P = 1.000$). Compared with the control (B) group, the incidence and severity of chronic inflammatory in the treatment group were increased ($P = 0.003, 0.020$). For tubular atrophy, there were no significant differences between the recovery, treatment and high arsenic groups ($P = 0.827, 0.073$). Compared with the control (B) group, the incidence and severity of tubular atrophy in the high arsenic, recovery and treatment groups were increased ($P = 0.001, 0.003, 0.047$). Compared with the control (B) group, the incidence and severity of glomerular sclerosis were not significantly different than the recovery and treatment groups ($P =$

0.231, 1.000), but the high arsenic group was demonstrated an increased incidence and severity ($P = 0.014$). These results are presented in Figure 3C.

Discussion

Arsenic is a ubiquitous, naturally occurring metalloid that may contaminate drinking water, food, cigarettes, industrial products, the occupational environment, and air^[12]. Among the various routes of arsenic exposure, arsenic poisoning can occur via drinking water and burning coal. Currently, the aetiology of the disease is known, but its pathogenesis remains uncharacterized and there is no specific treatment. Guizhou Province, China, is rich in Ginkgo biloba, which is known to improve immune function^[13-15]. In recent years, immunotherapy has become a topic of interest for many major diseases and refractory disease. However, little is known about the treatment of arsenic poisoning with Ginkgo biloba.

We established a rat model of arsenic poisoning by feeding the rats corn powder baked with high arsenic coal. To characterize these rats as a kidney damage model, poisoning symptoms, body weight changes, urinary arsenic, kidney arsenic (KAs), and kidney histopathology were observed. Body weight is a conventional non-specific indicator that can be integrated to reflect toxic effects on an animal *in vivo*. In this study, increasing doses of arsenic exposure resulted in a greater decrease in body weight, indicating an obvious dose - response relationship. Combined with the poisoning symptoms of rats in the middle and high arsenic groups, different levels of fluffy hair, hair loss, listlessness, decreased activity, slow and other symptoms, including fluffy hair, hair loss, listlessness, decreased activity, and slowed responses, indicated that exposure to different levels of arsenic result in different degrees of toxic effects. UAs is considered to reflect the recent arsenic exposure level, whereas KAs may reflect arsenic accumulation in the kidney^[16]. Our results showed that UAs and KAs in the low, middle and high arsenic groups were higher than in the control (A) group. This demonstrated that with increasing doses of arsenic exposure, arsenic absorption gradually increased and the excretion of arsenic in the urine and the accumulation of arsenic in the kidney increased. Excessive accumulation of arsenic in the kidney can affect the excretion of arsenic and its metabolites, thereby damaging kidney function. The kidney weight to body weight ratio is a sensitive indicator of kidney damage, but is susceptible to anatomical

factors and in this study did not produce a good result. Histopathological examination is viewed as the “gold standard” to determine organ damage. In this study, the exposure to arsenic produced different degrees of histological alteration, including vacuole degeneration, tubular atrophy, granular casts, early glomerular sclerosis, and chronic inflammation. According to the extent and severity of the disease and statistics describing the degree of kidney disease for each dose group, we found that the incidence and severity of chronic inflammatory, tubular atrophy and glomerular sclerosis were increased with increasing doses of arsenic exposure, but vacuole degeneration and granular casts were unaffected. These results suggest that vacuole degeneration and granular casts may be spontaneous diseases that are unrelated to arsenic exposure. In summary, a kidney damage model of arsenic poisoning in rats was successfully established.

The relationship between immune damage and kidney damage in arsenic poisoned rats

Albumin is a normal protein in blood. Under normal circumstances, large proteins cannot pass through glomeruli, but when the kidneys are not functioning properly, such proteins occasionally pass through the glomeruli and find their way into the urine [17]. N-Acetyl-beta-D-glucosaminidase is mainly found in kidney proximal tubule epithelial cells and is an important lysosomal hydrolase. Beta-2-micro-globulin is a low molecular weight protein in the kidney tubular structures that can be reabsorbed by kidney tubular structures, where it is degraded. When the kidney tubular structures are damaged, the UNAG and U β_2 -MG contents will rise [18]. In this study, upon increasing doses of arsenic exposure, the UALB, UNAG and U β_2 -MG contents increased, exhibiting an obvious dose – response relationship. These results suggest that arsenic exposure may cause damage to the reabsorption of the kidney tubular and filtration function of the glomeruli. Combined with the UAs and KAs results, this demonstrated that arsenic exposure is the direct cause of kidney damage.

The immune system is of great importance in maintaining the steady state in the. CD $_3^+$ T cells are a sign of maturity, as they play an extremely important role in the antigen recognition and immune response of T cells. CD $_4^+$ T cells release signals by contacting the target cells and secrete cytokines, which have immunomodulatory effects. CD $_8^+$ T cells are the major effector immune cells and contact target cells and may play a role in killing these cells. In this study, the positive rate of CD $_3^+$ and CD $_4^+$ as well as the CD $_4^+$ / CD $_8^+$ ratio in arsenic exposure groups decreased. The level of CD $_8^+$ among all groups did not change significantly, and the positive rate of CD $_4^+$ in the

arsenic exposure groups gradually decreased; therefore, the ratio of CD_4^+ / CD_8^+ decreased. This is consistent with population studies [19]. These results suggest that exposure to arsenic can cause imbalance of lymphocyte subsets, which affects the body's immune response (immune dysfunction), resulting in damage of the target organs.

Numerous studies [20-22] have shown that the occurrence and development of some diseases are closely linked with the immune status; when the body's immune function is suppressed, the incidence of disease will increase, which may contribute to the development of the disease. Interestingly, there is a clear negative correlation between immune damage (including CD_3^+ , CD_4^+ and CD_4^+ / CD_8^+ ratio, unless CD_8^+) and kidney damage (UALB, UNAG and $U\beta_2$ -MG) (C). The results suggest that cellular immunity may be involved in the occurrence and development of kidney damage that is caused by arsenic and that immune suppression may be one of the mechanisms of arsenic-induced kidney damage; however more evidence is needed to support this conclusion

The therapeutic effect of Ginkgo biloba on kidney damage in arsenic poisoned rats

Ginkgo biloba is a known immune enhancer. As such, we used it as an intervention agent in arsenic poisoned rats to further validate the relationship between kidney and immune damage. We aimed to explore the mechanism of Ginkgo biloba treatment of kidney damage in arsenic poisoned rats. By observing changes in cellular immunity, we showed that the rate of CD_3^+ and CD_4^+ cells as well as the CD_4^+ / CD_8^+ ratio in the treatment group were increased after treatment and that there was no significant difference between the treatment group and the control (B) group. These results demonstrated that Ginkgo biloba increases the number of T lymphocytes and adjusts the balance of T lymphocyte subsets, thereby enhancing immune function. In addition, compared with the high arsenic group, the rate of CD_3^+ , CD_4^+ , and CD_8^+ cells as well as the CD_4^+ / CD_8^+ ratio in the recovery group increased slightly, but there were no significant differences between the high arsenic group and the recovery group. These results suggest that the effect of natural recovery is poorer following immune damage in arsenic poisoning rats.

By observing changes in kidney damage in arsenic poisoned rats, we demonstrated that the UALB and $U\beta_2$ -MG contents of the treatment groups decreased after treatment, whereas there was no significant difference between the treatment and control (B) groups. Combined with the histopathology of the kidneys in the recovery group, we found that chronic inflammatory and

glomerular sclerosis in the treatment group was restored and that tubular atrophy improved. Furthermore, we observed no significant differences between the high arsenic group and recovery group in UALB and U β 2-MG contents. These results demonstrated that (1) Ginkgo biloba might relieve kidney damage and (2) the effect of treatment with Ginkgo biloba is an improvement over natural recovery. Although, there were no significant differences between the control (B) group and treatment group, the content of UNAG gradually decreased, suggesting that the time of treatment with Ginkgo biloba is sufficient to reverse the kidney damage caused by arsenic poisoning in rats.

In summary, we conclude that immune suppression may be a mechanism of arsenic induced kidney damage and that Ginkgo biloba may relieve kidney damage by enhancing immune function. These results provide important clues toward a mechanistic understanding and prevention research of endemic arsenic poisoning. The next step must be to verify the results of this research in a larger cohort.

Conflict of interest statement

Nothing to declare.

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Fig. 1 A kidney damage model for arsenic poisoning in rats was successfully established. By feeding corn powder baked with high arsenic coal, we established a kidney damage model of arsenic poisoning in rats. These results show that the body weight of rats in the middle (starting from the 10th week) and high arsenic (from the beginning of the 7th week) groups decreased (A). With increasing doses of arsenic exposure, UAs and KAs gradually increased (B). Pathological diagnosis is the gold standard for the identification of an animal model; although the difference in the kidney weight to body weight ratio in all groups was not statistically significant (C), the histopathology of the kidney revealed that the incidence and severity of chronic inflammation, tubular atrophy and glomerular sclerosis were increased with increasing doses of arsenic exposure. Vacuole degeneration and granular casts were unaffected (D). Thus, a kidney damage model for arsenic poisoning rats was successfully established. *, $P < 0.05$; **, $P < 0.01$.

Fig. 2 The relationship between immune and kidney damage in arsenic poisoned rats. It is clear that cellular immunity (i.e., CD_3^+ , CD_4^+ and CD_4^+/CD_8^+ ratio, but not CD_8^+) gradually decreased (A) and that kidney function (UALB, UNAG and $U\beta_2$ -MG) gradually increased (B) with increasing doses of arsenic exposure. To explore the relationship between kidney damage and immune damage in arsenic poisoned rats, Spearman correlation analysis was used. The results show that there is a clear correlation between immune damage (i.e., CD_3^+ , CD_4^+ and CD_4^+/CD_8^+ ratio, but not CD_8^+) and kidney damage (UALB, UNAG and $U\beta_2$ -MG) (C). These results suggest that immune suppression may be one of the mechanisms underlying arsenic-induced kidney damage. *, $P < 0.05$; **, $P < 0.01$.

Fig. 3 The therapeutic effect of Ginkgo biloba on kidney damage in arsenic poisoned rats. As a recognized immune enhancer, Ginkgo biloba is used as an intervention agent for arsenic poisoning in rats to further validate the relationship between kidney damage and immune damage. Meanwhile, the treatment mechanism of Ginkgo biloba on kidney damage in the arsenic poisoning in rats was explored. The results shown that the positive rate of CD_3^+ , CD_4^+ and the CD_4^+/CD_8^+ ratio in the treatment group were increased after treatment and there were no significant differences between the treatment group and control (B) group (A). This result suggests that the immune function in arsenic poisoned rats has been gradually restored. By observing the changes to kidney damage in arsenic poisoning rats, it was shown that the contents of UALB and $U\beta_2$ -MG in the treatment group were decreased after treatment and that there are no significant differences between the treatment group and control (B) group, but the content of UNAG was significantly

different (B). Furthermore, the histopathology of the kidney showed that chronic inflammatory and glomerular sclerosis in the treatment group had been restored and that the tubular atrophy has been improved (C). These results indicate that immune suppression may be a mechanism of arsenic-induced kidney damage. Moreover, they provide an important clues that suggest that Ginkgo biloba may relieve kidney damage by enhancing immune function. *, $P < 0.05$; **, $P < 0.01$.

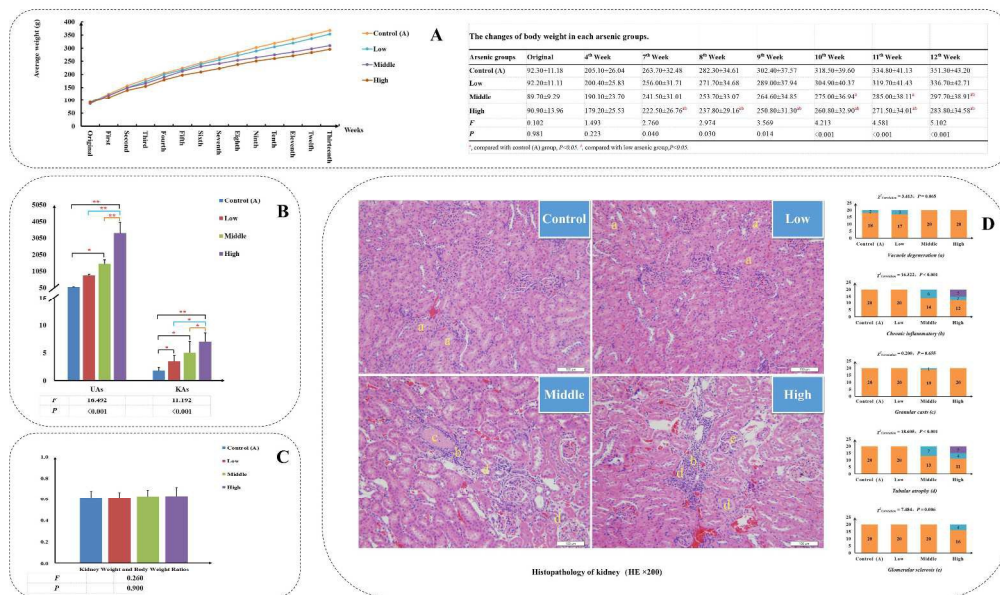


Fig. 1 A kidney damage model for arsenic poisoning in rats was successfully established. 686x411mm (150 x 150 DPI)

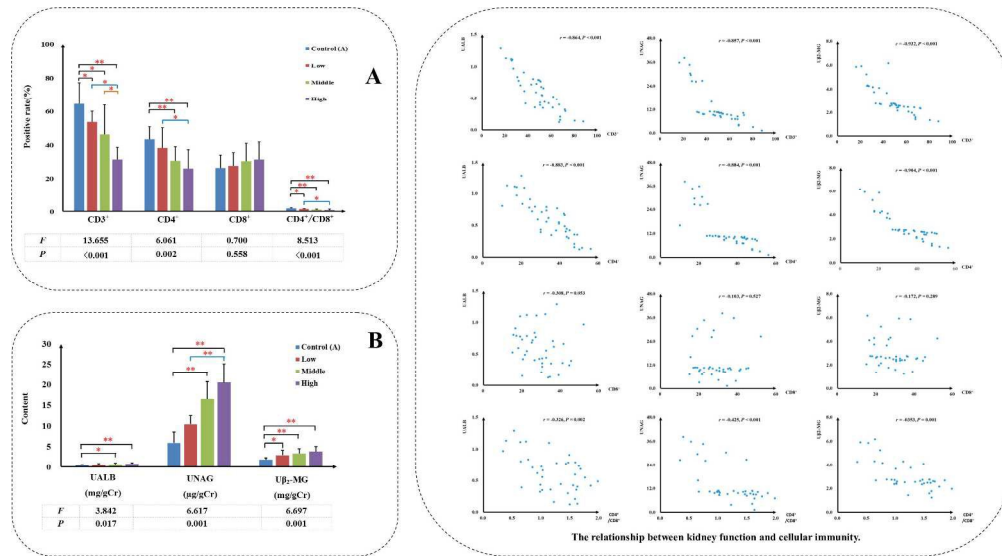


Fig. 2 The relationship between immune and kidney damage in arsenic poisoned rats. 540x296mm (150 x 150 DPI)

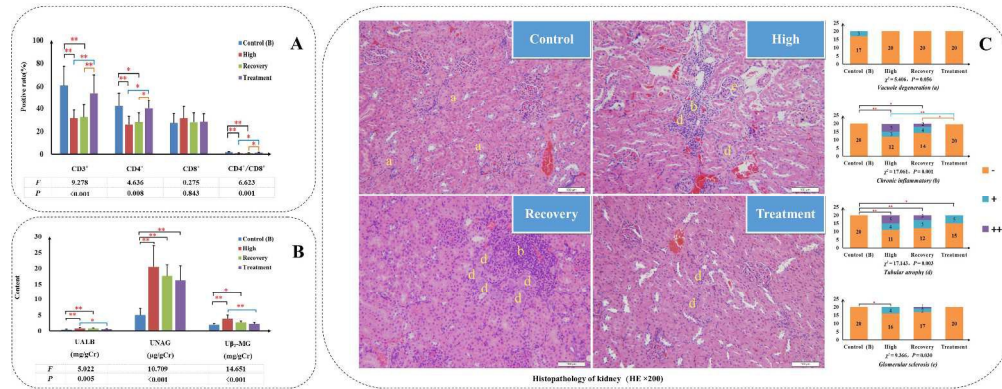


Fig. 3 The therapeutic effect of Ginkgo biloba on kidney damage in arsenic poisoned rats. 685x263mm (150 x 150 DPI)

Table 1 Specific animal groups and the administration methods

Groups	Administration methods
Control (A)	Fed 0mg/kg arsenic feed, 90d
Low arsenic	Fed 25mg/kg arsenic feed, 90d
Middle arsenic	Fed 50mg/kg arsenic feed, 90d
High arsenic	Fed 100mg/kg arsenic feed, 90d
Control (B)	Fed 0mg/kg arsenic feed, 135d
Recovery	Fed 100mg/kg arsenic feed, 90d; after then, fed 0mg/kg arsenic feed, 45d
Treatment	Fed 100mg/kg arsenic feed, 90d; after then, fed 0mg/kg arsenic feed and treatment with Ginkgo biloba (25mg/kg · bw), 45d

Table 2 Actual exposure amount

Arsenic groups	N	Average daily feed consumption (g/kg)	The average arsenic content in feed (mg/kg)	Actual exposure amount (mg/kg)
Control(A)	20	82.28	1.58	0.13
Low	20	80.71	23.17	1.87
Middle	20	87.14	47.28	4.12
High	20	92.75	94.99	8.81



408x298mm (150 x 150 DPI)