Pneumonia activates renal antioxidant defense function to reduce the damage of aristolochic acid I through Keap1/Nrf2/NQO1 signaling pathway

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Research Article

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Abstract

Aristolochic acids (AAs) are a group of compounds widely found in Aristolochiaceae, and the main toxic component of AAs is aristolochic acid I (AAI). AAI causes severe direct nephrotoxicity and carcinogenicity. Plants containing AAI are widely used around the world; for example, Asari Radix et Rhizoma (ARR) contains trace amounts of aristolochic acid and is still included in the Chinese Pharmacopoeia. Based on the theory of therapy for syndrome differentiation in traditional Chinese medicine, we established a 7-day mouse model of pneumonia and observed that kidney damage in the pneumonia group was significantly lower than that in the healthy group after the same treatment. Subsequently, the carcinogenic metabolite aristolochic acid-DNA adduct standards were synthesized, and assays were established. No adduct formation was detected in our limit of quantification (500 pg/ml) in the ARR group, while the adduct formation in the Caulis Aristolochiae Manshuriensis (CAM) group, whose content of AAI is 900 times that of the ARR, was significantly lower than that in the healthy group in the pneumonia state. Here, we demonstrate that the safety risk of ARR is not high at normal doses. Western blot analysis revealed that the level of the AAI metabolic enzyme NQO1 in the pneumonia group was significantly lower than that in the healthy group. Combined with the transcriptomic results, we speculate that kidney injury and adduct production in the pneumonia group were lower than those in the healthy group because of the ROS-Keap1-Nrf2-NQO1 pathway.

1. Introduction

Aristolochic acids (AAs), a group of compounds widely found in Aristolochiaceae, include more than 500 species. Plants containing AAs are widely used as herbal medicines in many countries around the world[1]. Most of these AAs-containing herbs have anti-inflammatory, analgesic, and rheumatism-dispelling effects. Many studies have shown that AAs cause severe direct nephrotoxicity, and the kidney damage caused by AAs is called aristolochic acid nephropathy (AAN)[2, 3]. The main toxic component of AAs is aristolochic acid I (AAI), and the products metabolized by AAI in vivo are mutagenic and carcinogenic[4, 5]. Although some herbal medicines containing AAs have been banned in some countries, many Chinese herbal medicines are still widely used[6, 7].

After years of revision, most of the drugs containing AAs in the Chinese Pharmacopoeia have been eliminated, and among the drugs included in the latest edition (2020 edition), only one drug, Asari Radix et Rhizoma (ARR), contains extremely trace amounts of AAI (less than 0.001%). However, at least 50 proprietary Chinese medicines containing AAs have been approved by the China Food and Drug Administration (CFDA), excluding those for private use and dietary supplements. Therefore, nephrotoxicity caused by herbal medicines containing AAs remains a serious and worthy public health concern.

The mechanism of kidney injury caused by AAs is complex and remains unclear. Previous studies have shown that AAI, the main toxic component of AAs, can directly damage renal tubular epithelial cells, especially proximal renal tubular epithelial cells, resulting in cell necrosis or apoptosis[5, 8]. The active
metabolites of AAI bind to DNA in vivo to form AAI-DNA adducts, which cause mutations in the tumor suppressor gene p53 in patients, leading to AAN upper urothelial carcinoma and adenocarcinoma of the colon in the digestive tract[4, 9]. Therefore, although AAI can cause severe nephrotoxicity, its most harmful effects are mutagenicity and carcinogenicity.

Xiao Xiaohe proposed a new concept based on the basic theory of traditional Chinese medicine "therapy with syndrome differentiation" - Disease-Syndrome-based Toxicology. The simple explanation is that the damage or side effects caused by the drug to the body in the state of illness should be less severe than the damage in a healthy state[10]. ARR has a wide range of pharmacological effects, including antibacterial, antiviral, anti-inflammatory, and immune-modulating effects[11–13]. The purpose of this study was to verify the safety of ARR containing extremely trace concentrations of AAI, to explore the differences in the damage or side effects caused by AAs-containing herbs to the body in terms of illness and health status, and to explore the reasons for the differences through metabolomics and transcriptomics.

2. Materials and methods

2.1. Animals

SPF male C57BL/6 adult mice (8 weeks old, weighing 21 ± 2 g) were obtained from Sibeifu Biotechnology Co., Ltd. (Beijing, China) (Certificate No. SCXK 20190010). The mouse were fed on different chests, provided food and drink freely, maintained at room temperature (25 ± 2°C), and disinfected regularly with an ultraviolet lamp.

All animal protocols were reviewed and approved by the animal ethics committee of the Fifth Medical Center of Chinese PLA General Hospital (IACUC No. 2021-0010). After adaptive feeding for six days, the 51 male mice were randomly divided into the normal group (n = 6), the GP group (n = 16), the GC group (n = 6), the XP group (n = 17) and the XC group (n = 6). According to previously described methods[14], the other two groups but not the normal group, GP group or XP group were forced to inhale 50 µL of 1 mg/mL LPS solution into the trachea on the first, third, fifth and seventh days, respectively (Fig. 1A). Then, in the XP and XC groups, 200 µL of gavaged ARR water decoction was administered daily from the second day to the eighth day. Similarly, in the GC and GP groups, the gavage agent was replaced with Caulis Aristolochiae Manshuriensis (CAM) water decoction. The dose of ARR was equivalent to the usual human dose of 6 g/day, and the dose was 0.05 g/mL according to the body surface area. The dosage of CAM is based on the content of aristolochic acid, which is equivalent to 900 times that of ARR.

At the end of the study, the lungs, blood, bronchoalveolar lavage fluid (BALF) and kidney were collected and stored at −80°C for use in the kit assay, transcriptomics or metabolomics assays or stored in 4% formalin for H and E staining.

2.2. Materials and chemicals
Asari Radix et Rhizoma (Chinese name XiXin) (ShanXi Province, China, No. 20210711) and Caulis Aristolochiae Manshuriensis (Chinese name GuanMuTong) (Jilin Province, China, No20210927) were purchased from the TCM Pharmacy of The Fifth Medical Center of Chinese PLA General Hospital (Beijing, China).

2.3. H&E staining and microscopic examination

Mouse kidney and lung specimens were fixed with formalin and embedded in paraffin. The specimens were cut into sections with a thickness of approximately 4 ~ 7 µm using a paraffin slicer, stained with hematoxylin for 5 minutes, dehydrated, and covered with neutral gum for microscopic observation.

2.4. Metabolomics analysis based on mouse serum

2.4.1. Metabolite extraction

Then, 50 µL of mouse serum sample was transferred to an EP tube, 200 µL of extraction solution (methanol:acetonitrile = 1:1, containing an isotope-labeled internal standard mixture) was added, and the mixture was vortexed for 30 seconds. The samples were ultrasonicated for 10 minutes in ice water and then incubated at -40°C for 1 hour. The sample was centrifuged at 12000 rpm (centrifugal force 13800×g) at 4°C and 8.6 cm for 15 minutes. The supernatant was collected and placed in an autosampler vial for testing.

2.4.2. UHPLC/MS conditions

LC–MS/MS analyses were performed using a UHPLC system (Vanquish, Thermo Fisher Scientific) with a Waters ACQUITY UPLC BEH Amide (2.1 mm × 50 mm, 1.7 µm) coupled to an Orbitrap Exploris 120 mass spectrometer (Orbitrap MS, Thermo). The A phase of liquid chromatography consisted of an aqueous phase containing 25 mmol/L ammonium acetate and 25 mmol/L ammonia water, while the B phase consisted of acetonitrile. The sample disk temperature was 4°C, and the injection volume was 2 µL. The Orbitrap Exploris 120 mass spectrometer can collect MS1 and MS2 data under the control of control software (Xcalibur, version 4.4, Thermo). The detailed parameters were as follows: sheath gas flow rate, 50 Arb; aux gas flow rate, 15 Arb; capillary temperature, 320°C; full MS resolution, 60000; MS/MS resolution, 15000; collision energy, 20/30/40; and spray voltage, 3.8 kV (positive) or -3.4 kV (negative).

2.4.3. Data processing

The raw data were converted to the mzXML format using ProteoWizard and processed with an in-house program, which was developed using R and based on XCMS, for peak detection, extraction, alignment, and integration. Then, an in-house MS2 database (BiotreeDB) was used for metabolite annotation. The cutoff for annotation was set at 0.3.

2.5. Transcriptomic analysis of mouse kidney tissue

We first used the TRiZol method to extract total RNA from mouse kidney tissue and then carried out strict quality control on the RNA samples. The quality control method was mainly to detect RNA quality
through agarose gel electrophoresis and finally to construct a transcriptome library. AMPure XP beans were used to screen cDNA of approximately 250–300 bp, PCR amplification was performed, and the PCR products were purified again using AMPure XP beans to ultimately obtain a library. After the construction of the library was completed, preliminary quantification was performed using a Qubit 2.0 fluorometer, and the library was diluted to 1.5 ng/µl. Then, the insert size of the library was tested using an Agilent 2100 bioanalyzer. After the insert size reached the expected value, qRT–PCR was used to quantify the effective concentration of the library (the effective concentration of the library was greater than 1.5 nM) to ensure the quality of the library. After passing the library inspection, the different libraries were pooled according to the effective concentration and target sequencing data volume requirements, and then Illumina sequencing was performed. Four fluorescently labeled dNTPs, DNA polymerase, and adaptor primers were added to the sequencing flow cell for amplification. When each sequencing cluster extends the complementary chain, each fluorescently labeled dNTP can release the corresponding fluorescence. The sequencer captures the fluorescence signal and converts it into a sequencing peak through computer software, thereby obtaining the sequence information of the tested fragment.

2.6. Western blot analysis

One milliliter of RIPA lysis buffer (containing protease inhibitors) was added to the liver tissue (25 mg), which was subsequently homogenized at a frequency of 100 Hz for 90 s. The tissue lysate was placed on ice and allowed to stand for 30 minutes before the supernatant was aspirated. The protein level was quantified by the BCA Protein Assay Kit (No. BN27109, BIORIGIN Co., Ltd., Beijing, China). The collected protein was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane, which was blocked with 5% skim milk for 1 h. Then, the cells were incubated with antibodies against CYP1A1, CYP1A2, Keap1, Nrf2, and NQO1. β-Actin and HSP90 were used as loading controls. After incubation with the linked horseradish peroxidase (HRP)-conjugated secondary antibody, the bands were observed using enhanced chemiluminescence (ECL) detection reagent and then developed together with the film.

2.7. Statistical analysis

Statistical analysis and mapping were carried out using GraphPad Prism 9.5.0 software (GraphPad Software Co., Ltd., California, USA). Differences between each group were statistically compared using an unpaired t test. One-way analysis of variance was utilized for multiple comparisons. P < 0.05 was considered to be statistically significant.

3. Results

3.1. The mouse pneumonia model was successfully constructed

In this study, bronchial instillation of LPS was used to establish a mouse pneumonia model. According to the pre-experiments, a single bronchial instillation of LPS can only cause a mild pneumonia state that
lasts for approximately two days, so the method of instilling LPS every other day is used to maintain the mice in a pneumonia state during the dosing cycle (Fig. 1A). To verify whether the mice remained in a state of pneumonia during the 7-day experimental period, we randomly selected 3 mice from the GP or XP groups (model group) every day, sacrificed them, and collected BALF to detect inflammatory factors. We used ELISA to measure the levels of IL-6 and TNF-α in BALF collected from three randomly sacrificed mice daily. The results are shown in Fig. 1C and 1D, and it is clear that the content of IL-6 and TNF-α in BALF was significantly increased from the first day of modeling compared with that in the BALF of the control group without modeling, and the content of IL-6 and TNF-α also increased with the increase in the number of models, which means that the pneumonia state of the mice was also aggravated. Inflammatory cytokine testing alone did not fully confirm that the mice were in a state of pneumonia, so we also performed H&E staining on the mouse lungs (Fig. 1B). As shown in Fig. 1B, H&E staining revealed obvious inflammatory cell infiltration in the GP and XP groups compared with the nonmodeled GC and XC groups. From the morphological photographs of the lungs, it can also be seen that compared with those of the control groups (GC and XC groups), the lung tissues of the GP and XP groups were slightly enlarged and slightly larger, and some parts were dark red. Based on the above results, a 7-day mouse model of pneumonia was successfully established in our study.

3.2. Mice in the pneumonia group had less kidney damage and less dA-ALI production

The 7-day body weight curve of each group showed a downward trend (Fig. 2A), which may be caused by the stimulation of the mice by daily gavage. There were significant differences in the body-to-kidney weight ratio (Fig. 2B) between the GC group and the control group, and there were also significant differences between the GC group and the GP group (pneumonia CAM group). There were no significant differences among the XP, XC, and control groups. Based on these results, we can say that the GC group may have some kidney injury compared with the GP group and the control group, but the degree of injury needs to be further tested and confirmed. Therefore, we not only measured the serum creatinine (CR) and urea nitrogen (BUN) concentrations (Fig. 2D&2E) in the mice but also performed H&E staining (Fig. 2C) on the kidneys of the mice to further confirm the degree of kidney damage in each group of mice. As shown in Figs. 2D and 2E, the serum CR and BUN levels in the mice in each group exhibited the same high and low trends; the levels in the GP group were slightly lower than those in the GC group, and there was a significant difference. The levels in the XP group were significantly lower than those in the XC group, there was a significant difference, and the levels of GC, GP and XC were significantly greater than those in the control group. A comparison of the XC vs. XP and GC vs. GP groups revealed that the CR and BUN levels of the XP and GP groups in the pneumonia model group were lower than those in the corresponding nonmodel XC and XP groups.

According to the H&E staining results (Fig. 2C), the glomerular structure in the field of vision of the GP, XP and XC groups was intact, the outline was clear, the cyst cavity was not expanded, the number of mesangial cells was clear, the number was normal, no necrosis was found, and there was no obvious inflammatory cell infiltration in the tissue. In addition, only the GP and XC groups exhibited brush border...
injury in a small number of renal tubules. In the GC group, individual glomeruli exhibited atrophy, mesangial cells were significantly reduced, the cyst cavity was slightly dilated, a small number of brush border injuries of renal tubules occurred, a small amount of protein mucus was visible in the renal tubular lumen, and no obvious inflammatory cell infiltration was found in the tissues. In summary, we preliminarily concluded that the ARR and CAM cause less kidney damage in pneumonic mice than in healthy mice. The XP group were even at the same level as those in the control group.

Finally, we extracted DNA from mouse kidney tissue, hydrolyzed it into a single nucleotide, and measured the concentration of the resulting DNA adduct (dA-ALI). The results are shown in Fig. 2F, within our detection limits, with dA-ALI detected only in the GC and GP groups. The dA-ALI content in the GP group was significantly lower than that in the GC group. Since pneumonia was the only variable between the GP and GC groups, we believe that pneumonia may have protected the body, resulting in less drug toxicity, less dA-ALI production, and less kidney damage.

3.3. **NQO1 protein in the healthy group was significantly greater than that in the pneumonia group**

Both CYP1A1/2 and NQO1 are enzymes involved in the metabolic process of AAI. WB analysis revealed no difference in CYP1A1/2 expression between the pneumonia group and the healthy group (Fig. 3B&3C), while NQO1 expression was significantly greater in the healthy group than in the pneumonia group (Fig. 3D). This result suggested that the reduction in kidney injury and adducts may be related to NQO1.

3.4. **Metabolomic analysis of pneumonic mice and healthy mice**

Serum metabolomics analysis of pneumonia mice and healthy mice was performed by multivariate analysis of the serum metabolic profiles of mice in the healthy (GC, XC group) and pneumonia (GP, XP group) groups. The PCA score plot showed that healthy mice and pneumonia mice were completely different (Fig. 4A&4B). As shown in the orthogonal projections to latent structures-discriminant analysis (OPLS-DA) score plot (Fig. 4C&4D), the metabolite profile distributions for the healthy (XC, GC) and pneumonia groups (XP, GP) were clearly separated, with both $R^2_Y$ and $Q^2$ greater than 0.5. Each point in the volcano plot (Fig. 4E&4F) represents a peak, and the graph contains all the materials measured. The abscissa represents the fold change of the group of contrasted substances (taking the logarithm with 2 as the base), the ordinate represents the P value of Student's t test (taking the negative number with 10 as the base logarithm), and the scatter size represents the VIP value of the OPLS-DA model; the larger the scatter is, the greater the VIP value. Metabolites that are significantly upregulated are indicated in red, metabolites that are significantly downregulated are shown in blue, and metabolites that are not significantly differentiated are shown in gray. Figure 4E shows the metabolite levels in the healthy group administered CAM compared to those in the pneumonia group (GC vs. GP), and Fig. 4G shows the
metabolite levels in the healthy group administered ARR compared to those in the pneumonia group (XC vs. XP).

According to the criteria OPLS-DA VIP (variable importance in the projection) > 1.0 and P value < 0.05, we identified 430 differentially abundant metabolites (123 upregulated and 307 downregulated) and 286 differentially abundant metabolites (83 upregulated and 203 downregulated) in mice administered CAM and ARR, respectively, that were significantly affected by pneumonia. The differentially abundant metabolites were mainly lipids and lipid-like molecules, organic acids and derivatives, and organic oxygen compounds, such as glucosylceramide, trans-octadecanoic acid, myristoleic acid, methyl dihydrojasmonate, and sebacic acid. Most metabolites differed between CAM-treated healthy mice and pneumonic mice, suggesting that these specifically altered metabolites may be related to the alleviation of the side effects of the drug caused by pneumonia.

We analyzed the metabolic pathways of mice that were administered CAM or ARR. The results are shown in Fig. 4I&5J; the abscissa represents the RichFactor corresponding to each pathway, and the ordinate is the name of the KEGG metabolic pathway. The size of the dot indicates the number of differentially abundant metabolites enriched in the pathway. The color indicates the size of the p value, and the smaller the p value is, the more reddish the color, and the more significant the enrichment. Because CAM and ARR are both herbs that contain AAI, we believe that there is a certain similarity in the mechanism by which pneumonia alleviates toxic side effects, so we directly examined the metabolic pathways in which they were enriched. As shown in Fig. 4I&4J, glycerophospholipid metabolism, thiamine metabolism and sphingolipid metabolism are all pathways that may be involved in alleviating the toxic side effects of pneumonia.

3.5. Transcriptomic analysis based on pneumonic mice and healthy mice

To explore the transcriptomic changes underlying the differences in the effects of CAM and ARR on healthy mice and pneumonic mice, RNA-seq data were obtained from healthy and pneumonia samples. The PCA score plot (Fig. 5A&5B) showed that there was a large difference between the pneumonia group and the healthy group, and the reproducibility within the group was acceptable. The heatmap was obtained by taking the DEGs of all comparison groups and taking them as the differential gene sets and then performing cluster analysis on the differential gene sets. In the figure, the abscissa is the sample name, and the ordinate is the normalized value of the differential gene FPKM; the redder the color is, the greater the expression level, and the greener the color is, the lower the expression level (Fig. 5C). The volcano plot clearly reflects the genes that are significantly expressed in both groups and is indicated in red and blue. The results showed that the expression of 236 genes in the CAM group (GP vs. GC) significantly changed in the pneumonia group, of which 212 genes were upregulated and 24 genes were downregulated (p ≤ 0.05, Fig. 5D). The expression of 779 genes in the ARR group (XP vs. XP) significantly changed during pneumonia, of which 562 genes were upregulated and 779 genes were downregulated (p ≤ 0.05, Fig. 5F). These results indicated that pneumonia status had a significant
effect on transcription in both the CAM and ARR groups. Since CAM and ARR are both herbs that contain AAI, we believe that there is a certain similarity in the mechanism of reducing their toxic side effects, so we directly searched for the same pathway through KEGG pathway enrichment analysis. As shown in Fig. 5E and 5G, chemical carcinogenesis—reactive oxygen species—was the only pathway enriched in the CAM-administered group and the ARR-administered group.

The reactive oxygen species pathway in chemical carcinogenesis refers to a carcinogenic mechanism mediated by reactive oxygen species molecules. Reactive oxygen species, including oxygen radicals and peroxides, are highly reactive and can oxidize biomolecules, leading to cell damage and mutations, thereby increasing the risk of cancer. According to the KEGG differential gene enrichment analysis of GP vs. GC and XP vs. XC, the chemical carcinogenesis-reactive oxygen species pathway showed a downward trend. It is likely that pneumonia can alleviate the toxic side effects of AAI-containing herbal medicines by inhibiting this pathway.

### 3.6. Determination of oxidative stress markers in the serum and kidney tissues of mice

We measured the following oxidative stress-related indicators [15, 16] in the serum and kidney tissues of the mice: reactive oxygen species (ROS), malondialdehyde (MDA), superoxide dismutase (SOD), oxidized glutathione (GSSG), reduced glutathione (R-GSH), and total glutathione/oxidized glutathione (T-GSH/GSSG). We first measured ROS in kidney tissue, as shown in Fig. 6A. The ROS in the healthy group (GC, XC group) were significantly greater than those in the pneumonia group (GP, XP group), which was also consistent with our transcriptomic enrichment results. Figure 6B shows that the SOD concentration in kidney tissue was significantly greater than that in serum in the pneumonia group, while there was no significant difference in the healthy group. This may indicate that the kidneys have a strong antioxidant capacity in the pneumonic state. Figure 6D shows that the MDA concentration in kidney tissue was lower than that in serum in all groups, suggesting that the degree of oxidative damage in kidney tissue may be lower than that in other organs. Figure 6C shows that the R-GSH concentration in the kidney tissue of the pneumonia group was significantly greater than that in the healthy group, suggesting that the kidneys of the pneumonia group may have experienced greater antioxidative stress. Figure 6E shows that the T-GSH/GSH ratio in the renal tissue of the pneumonia group was significantly greater than that in the renal tissue of the healthy group, while the opposite was true for the serum. These results indicated that the reduction in renal tissue in the pneumonia group was greater than that in the control group, which was conducive to normal metabolism and functional maintenance of cells, while the oxidative state of renal tissue in the healthy group was greater, which may have led to cell damage and abnormal function.

### 3.7. Pneumonitis can attenuate mouse kidney injury and dA-ALI adduct production through the Keap1-Nrf2 pathway

Based on the results of transcriptome KEGG enrichment and the results of oxidative stress indicators in section 3.5, we deduced that pneumonia may reduce kidney injury and dA-ALI production through the
Keap1-Nrf2 pathway. Therefore, we performed WB analysis of Keap1 and Nrf2 proteins in mouse kidney tissue. WB results are shown in Fig. 6G. Grayscale analysis revealed that Keap1 in the pneumonia group (GP, XP) was significantly lower than that in the healthy group (GC, XC), while Nrf2 was significantly greater than that in the healthy group (Fig. 6F). This finding is consistent with our hypothesis that pneumonitis may attenuate kidney injury and adduct production via the ROS-Keap1-Nrf2 pathway.

4. Discussion

AAs-containing herbs have been used for centuries to treat a variety of diseases[17], but in recent decades, AAs have been shown to cause nephrotoxicity and carcinogenicity[18–20], and such herbs have been widely banned. For example, in China, since 2003, the CFDA has cancelled the drug standards for three medicinal materials containing AAs—Caulis Aristolochiae Manshuriensis (CAM), Radix Aristolochiae Fangchi and Saussurea Costus—and in the new edition of the 2020 Pharmacopoeia, Aristolochia Debilis and Fibraurea Recisa Pierre were no longer included; the only medicinal material containing AAs is Asari Radix et Rhizoma (ARR), and its medicinal parts were changed from whole herbs to roots and rhizomes. Moreover, the AAI content of its medicinal parts should not be greater than 0.001%[21–23]. Although the AAI content of the ARR is extremely low, people are still concerned about its safety. In addition, during the epidemic of COVID-19 in Wuhan, QingFeiPaiDuTang (which contains 6 g of ARR as a drug) showed good therapeutic efficacy and could effectively reverse the incidence of severe disease[24, 25]. However, the trace amount of AAI in the ARR has seriously affected its promotion in overseas markets and the reputation of traditional Chinese medicine. Therefore, it is necessary to evaluate the safety of the ARR in the treatment of pneumonia.

In this study, taking the ARR as an example, combined with the idea of therapy combined with syndrome differentiation via traditional Chinese medicine, it was found that the kidney damage caused by the ARR in patients with pneumonia was milder than that in healthy individuals. Due to the small amount of AAs contained in the ARR, kidney damage was not very obvious, so CAM, whose AAs content was 900 times that of the ARR, was selected for observing changes in kidney injury. The results were consistent; that is, the kidney damage caused by taking CAM in the pneumonia state was less than that in the healthy state. In addition to nephrotoxicity, the carcinogenicity of AAs is an even more feared factor. The active metabolites of AAI in the body combine with DNA to form AAI-DNA adducts, which are the main substances that cause genetic mutations and carcinogenesis. In this study, we used WuXi AppTec Co., Ltd., to synthesize and purify a dA-ALI standard and establish a method for the detection of adducts. The results of adduct detection in the kidneys of the mice showed that no adduct was detected in the ARR group (limit of quantification 500 pg/mL), while the amount of adduct produced in the pneumonia group was significantly lower than that in the healthy group in the CAM. Therefore, we believe that pneumonia can reduce kidney damage and the production of the carcinogenic metabolite dA-ALI.

After AAI enters the body, it undergoes two biotransformation pathways: metabolic efflux and activation of toxin production. NQO1, CYP1A1, and CYP1A2 are involved[20, 26, 27]. Based on the previously observed low levels of kidney injury and adduct production in the pneumonia model, we performed WB
analysis of three enzymes involved in AAI-induced toxin production. There was no difference in the expression of CYP1A1/2, and the level of NQO1 was significantly lower in the pneumonia group than in the healthy group. To further explore the underlying mechanism, we combined metabolomics and transcriptomics analyses to identify the relevant pathways that affect NQO1 expression. We used a comparison strategy between the pneumonia group and the healthy group to screen for differentially abundant metabolites and DEGs between the CAM group and the ARR group. We then identified the same differentially abundant metabolites and DEGs in both the CAM and ARR groups, which were most likely associated with the alleviation of kidney injury and a reduction in DNA adduct production in the pneumonia state. The results of KEGG enrichment showed that only the chemical carcinogenesis-reactive oxygen species pathway was differentially expressed between the CAM group and the ARR group, and this pathway was downregulated in the pneumonia group compared with the healthy group. Reactive oxygen species refer to biochemical pathways mediated by reactive oxygen species molecules such as superoxide anions, hydrogen peroxide, and hydroxyl radicals. The reactive oxygen species pathway plays an important role in intracellular regulation, participating in the regulation of biological processes such as cell proliferation, apoptosis, cell signal transduction and gene expression, and can regulate the expression of NQO1[28, 29].

In this study, analysis of ROS, SOD, MDA and T-GSH/GSG, which are oxidative stress-related indicators, revealed that the antioxidant stress capacity of the kidney in the pneumonia group was greater than that in the healthy group, while the antioxidant content in the serum of the pneumonia group was significantly lower than that in the healthy group. Therefore, we deduced that the pneumonia state can activate the negative feedback regulatory mechanism of the whole body, which increases the levels of antioxidant enzymes and antioxidant substances in the kidney, the target organ of AAI, so that the ROS in the kidney tissue are relatively low. The relatively low levels of ROS in the chemical carcinogenesis-reactive oxygen species pathway cause Keap1 to undergo less oxidative modification, which allows Keap1 to bind more tightly to Nrf2 and promote the ubiquitination and degradation of Nrf2, limiting the expression of Nrf2 and inhibiting its nuclear translocation. As a result, the expression of NQO1, which is regulated by Nrf2, decreases[30, 31].

5. Conclusion

Our study showed that ARR at the usual dose did not cause severe kidney damage and did not produce adducts (or generate adducts below our limit of detection of 500 ng/mL). The amount of kidney damage and adduct production caused by CAM in mice in the pneumonic state was lower than that in healthy mice. We attempted to elucidate this mechanism through metabolomics and transcriptomic analysis. Based on these results, we propose the following hypothesis: pneumonia activates the negative feedback regulatory mechanism in the body, which reduces ROS in the kidney, decreases the oxidative modification of Keap1, limits the activity of Nrf2 and promote it degradation, and decreases the expression of NQO1.
Declarations

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Conflict of interest:

The authors declare no conflicts of interest. We take full responsibility for the contents of this study.

Author contributions:

Yinkang Wang: Investigation, Writing - Original Draft Chengxian Li: Writing - Review & Editing Shuanglin Qin: Validation, Jiabo Wang: Data Curation Xianling Wang: Visualization Xinyu Li: Validation Ming Niu: Methodology, Supervision Zhaofang Bai: Conceptualization, Founding acquisition Xiaohe Xiao: Conceptualization, Founding acquisition

Convention on biodiversity.

We have obtained authority to access plant samples used for research and that this has been authorised by the appropriate agent of the government of the Chineses country as required under the framework of the United Nations Convention on Biodiversity.

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**Figures**
Figure 1

The mouse pneumonia model was successfully constructed. A. Schematic diagram of animal experiment modeling and administration process. B. H&E staining and morphological photographs of mouse lungs. C. Detection results of IL-6 content in mouse BALF D. Detection results of TNF- content in mouse BALF.
Figure 2

Mice in the pneumonia group had less kidney damage and less dA-ALI production. A. Trend of body weight of mice in each group during animal experiments. B. Renal weight/body weight ratio in each group. C. H&E staining results of mouse kidney. D. Urea BUN concentration in the serum of mice in each group. E. Urea CR concentration in the serum of mice in each group. F. Renal dA-ALI concentrations in mice in each group.
Figure 3

NQO1 protein in the healthy group was significantly greater than that in the pneumonia group. A. The results of WB experiment in kidney tissue of mice in each group. B. Grayscale analysis of CYP1A2 normalized to β-Actin in kidney tissues of mice in each group. C. Grayscale analysis of CYP1A1 normalized to β-Actin in kidney tissues of mice in each group. D. Grayscale analysis of NQO1 normalized to β-Actin in kidney tissues of mice in each group.
Figure 4

Metabolomic analysis based on pneumonic mice and healthy mice. A. Score scatter plot of PCA model for group GC vs GP. B. Score scatter plot of PCA model for group XC vs XP. C. Score scatter plot of OPLS-DA model for group GC vs GP. D. Score scatter plot of OPLS-DA model for group XC vs XP. E. Volcano plot for group GC vs GP. F. Donut Plot of metabolite classification and proportion for GC vs GP. G. Donut Plot
Transcriptomic analysis of pneumonic mice and healthy mice. A. Score scatter plot of PCA model for group GP vs GC B. Score scatter plot of PCA model for group XP vs XC C. Heat map of differentially expressed gene clustering D. Volcano map of differential genes for group GP vs GC E. KEGG enrichment
scatter plot for group GP vs GC F. Volcano map of differential genes for group XP vs XC G. KEGG enrichment scatter plot for group XP vs XC.

Figure 6

Determination of oxidative stress markers in the serum and kidney tissues of mice and renal tissue WB experiment validation results. A. The relative content of ROS in the kidney tissue of mice in each group. B. SOD concentrations in serum and kidney tissues of mice in each group. C. R-GSH concentrations in...
serum and kidney tissues of mice in each group. D. MDA concentrations in serum and kidney tissues of mice in each group. E. The ratio of T/GSSH in serum and kidney tissues of mice in each group. F. Grayscale analysis of Nrf2 and Keap1 normalized to β-Actin in kidney tissues of mice in each group. G. The results of WB experiment in kidney tissue of mice in each group.

**Supplementary Files**

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