

Xanthurenic acid as a preclinical diagnostic marker and drug target of Alzheimer's disease in rats and mice and associated effects of Schizandrin

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Abstract

Alzheimer's disease (AD), a multipathogenic and irreversible neurodegenerative disease, threatens human health because a well-developed and compelling 'theragnostic' marker for monitoring biochemical events in this disease is lacking. In this experiment, high excretion of xanthurenic acid was found quantitatively before neurodegeneration in two classic AD models, including APP/PS1 transgenic mice and a chemical-induced rat model, based on metabolomic analysis coupled with UPLC-TQD-MS/MS. The discovery was beyond the recorded pathological process, including A β ₄₂ deposition, astrocyte inflammatory hyperplasia and neuron loss, and it can be used not only as a diagnostic marker for the preclinical stage of AD but also as a target reflecting the disease-modifying effect of lead compounds for preventing AD. In this way, Schisandrin, a neuroprotective agent from *Schisandra chinensis* (Turcz.) Baill, was proven to adjust the level of xanthurenic acid in urine significantly in the present study. The predictability, treatment relevance, stability, reproducibility and accessibility of urine xanthurenic acid in our experiment indicates its feasibility as a potential diagnostic marker and drug target for the preclinical stage of AD.

Introduction

AD, a neurodegenerative disease that threatens the quality of life of nearly 300 million elderly people worldwide, has been perplexing clinicians for decades due to its complex, long-term, irreversible and invisible progression. Markers related to treatment and diagnosis with the properties of specificity, predictability, pathologic relevance, therapeutic relevance and accessibility are essential to cope with these challenges. In fact, a marker with all the above characteristics has not been found until now. Classic markers such as A β ^[1], tau^[2] and related secretases^{[3][4]} with specificity located in the brain were more beneficial to pathogenesis research but less beneficial to medical guidance^{[5][6]} because of the complexity of their controversial pathogenesis^{[7][8]}, which results in delayed treatment^[9]. Hence, predictive early AD markers are considered to be more valuable in preventing disease development. Since the National Institute on Aging-Alzheimer's Association working groups made recommendations on diagnostic guidelines for the staging diagnosis of AD in 2011, the long pathological process (5-10 years) without obvious symptoms before AD onset has been divided into preclinical AD and moderate cognitive impairment (MCI), which represent the optimal times for medical intervention^{[10][11]}. Major predictive markers were studied in these two stages^[12]. Among them, brain region-specific changes on positron emission tomography (PET) with tracers is one of the most accurate prediction methods for staging disease severity^{[13][14][15]}. However, the application of PET in AD is limited to basic research due to its expensive instrument maintenance, radiation exposure and off-target binding of tracers. Other predictive markers in cerebrospinal fluid (CSF), including the A β ₄₂/A β ₄₀ ratio and A β ₄₂ used for monitoring the amyloid deposition process; sugar metabolism associated with microglia and astrocyte activation^[16]; and T-tau^[17], P-tau^[18], neurofilament light (NfL)^[19] and dendritic protein neurogranin (Ng)^[20] related to synaptic dysfunction and neurodegeneration. Although their good sensitivity and specificity in MCI were confirmed in clinical trial, the invasiveness of CSF sampling and instability of the marker proteins restrict their application^[21], highlighting the accessibility of markers as another basic factor in clinical practice. Cost-effective blood markers, such as plasma A β ₁₋₄₀/A β ₁₋₄₂^[22], P-tau181^{[23][24]} and NfL^[25], which indirectly reflect brain pathology, are more desirable, and their diagnostic rates are similar to those of PET imaging. These results show the great potential of accessible fluid markers in clinical diagnosis. However, whether these can contribute to the evaluation of treatment still needs to be determined in large cohort studies.

A comparison of these characteristics indicates that a medically useful AD marker should be at least predictive for disease development, relevant for its treatment evaluation and accessible for implementation. This finding is consistent with the current conceptualization of a 'theragnostic' biomarker that can be used to monitor biochemical events during pathology and treatment evaluation^[26]. Therefore, we carried out a metabolomic analysis of the pathological changes over time in two classic AD models, including APP/PS1 transgenic mice^[27] and chemically induced rats^[28] and quantitatively analysed the dynamic changes of xanthurenic acid in the urine of these disease animal models by UPLC-TQD-MS/MS to determine the relationship between xanthurenic acid and AD-related pathological changes. Furthermore, we used an identified neuroprotective agent, schizandrin, to treat these AD models and verify the significance of xanthurenic acid in AD. Schizandrin is an active ingredient originating from the fruit of *Schisandra chinensis* (Turcz.) Baill, and it is the main component of the anti-AD agent that can penetrate the blood-brain barrier and showed a neuroprotective effect in our previous study^{[29][30]}. Its neuroprotection has also been demonstrated by other experiments^{[31][32]}. Subsequently, the properties of urine xanthurenic acid as a theragnostic marker for AD were validated through our experiment, which will play an important role in AD prevention and related drug discovery.

RESULTS

Xanthurenic acid in a chemically induced rat model of AD

Xanthurenic acid is the terminal metabolite in the Tryptophan-Kynurenine- Xanthurenic acid (TKX) pathway, as shown in Fig. 2 a. After continuous administration of aluminium chloride (AlCl_3) and intraperitoneal injection with D-galactose (D-gal) for 90 days (Fig. 1a), the metabolic changes of xanthurenic acid in the TKX pathway and the results of the pathological process were collected for a correlation analysis (Fig. 1b). Under the constant stimulation of AlCl_3 +D-gal, the metabolism of tryptophan in the blood was disturbed, and then this abnormal fluctuation passed to kynurenine and tryptophan (Fig. 2e-h). This metabolic disorder was transmitted in the blood throughout the entire modelling process (Fig. 2h) when the chemically induced rats experienced oxidative stress, inflammation and $\text{A}\beta_{42}$ -positive expression in their brains. $\text{A}\beta_{42}$ was obviously highly expressed in both the hippocampal CA3 region and cerebral cortex by immunostaining (Fig. 1c and Extended Data Fig. 1). Peroxidation in brain tissue was also significantly increased (Fig. 1f), and continuous astrocyte proliferation was observed in the cortex (Fig. 1d). As the AD modelling process developed, the contents of kynurenine and tryptophan in the brain were both abnormal on the 45th and 60th days (Fig. 2c, d, h). Coincidentally, the disordered arrangement of neurons in the hippocampal CA3 region was detected on the 45th day, and more serious arrangement disorder and even nucleolar pyknosis were found on the 60th day (Fig. 1e and Extended Data Fig. 2). This serious neuronal damage finally resulted in learning and spatial memory impairment on the 90th day (Fig. 1g and Extended Data Fig. 3a-c). The results above indicated that the disturbed metabolism in the TKX pathway in the blood was related to the continuous stimulation of the neurotoxic substance AlCl_3 +D-gal, and the abnormal metabolism of the TKX pathway in the brain was connected with neuronal damage. More notably, these TKX metabolism changes in vivo and even their undetectable anomalies in the early stage could be cumulatively recorded by the excretion of xanthurenic acid in urine. When hippocampal neurons were induced on the 45th day, the excretion of xanthurenic acid in the urine began to increase, and it continued to be highly excreted during the process of neuronal damage (Fig. 1h and Fig. 2h, i). Therefore, urine xanthurenic acid could reflect the process of neuronal damage.

In the study of the pharmacodynamic correlation of xanthurenic acid and schizandrin, a neuroprotective agent was given to chemically induced-AD rats by intragastric administration every day (Fig. 1a). The results showed inhibited astrocyte proliferation (Fig. 1d), protection of the hippocampal neurons (Fig. 1e and Extended Data Fig. 2) and improved cognitive ability (Fig. 1g and Extended Data Fig. 3a-c), indicating significant intervention effects of schizandrin on AD, although the positive expression of $\text{A}\beta_{42}$ was not inhibited simultaneously (Extended Data Fig. 1); nevertheless, the disturbed metabolism of xanthurenic acid in the blood, brain and urine was rescued by schizandrin (Fig. 1h and Fig. 2d, g, i). Therefore, xanthurenic acid is more suitable as a drug target and treatment evaluation than $\text{A}\beta_{42}$.

Xanthurenic acid in APP/PS1 transgenic mouse model of AD

The experimental design and key results are shown in Fig. 3a and b. Disordered metabolic transmission in the TKX pathway in the blood of APP/PS1 transgenic mice was also observed, even though it was not as obvious as that in the chemically induced rats (Fig. 4d-g), but the reduction in xanthurenic acid concentration in the blood at multiple time points was consistent with that in the chemically induced rats (Fig. 4f). Mild intermittent abnormal metabolic transmission was found in the TKX pathway in the brains of APP/PS1 transgenic mice (Fig. 4a-c, g). When $\text{A}\beta_{42}$ first exhibited obvious positive expression in the cerebral cortex at the 6th month (Extended Data Fig. 4), the contents of tryptophan and kynurenine in the brain were both abnormal (Fig. 4a, b). In our study, the expression of $\text{A}\beta_{42}$ in the hippocampal CA3 region of APP/PS1 transgenic mice showed scattered positive expression at the 12th month (Extended Data Fig. 5) and was not as obvious as their aggregation in the cortex (Extended Data Fig. 4). As cortical $\text{A}\beta_{42}$ deposition gradually increased (Fig. 3c, d), obvious proliferation of astrocytes was found in the cortex at the 11th month (Fig. 3e and Extended Data Fig. 7), and disordered arrangement and nucleolar pyknosis of neurons in hippocampal CA3 were detected at the 10th and 11th months (Fig. 3f and Extended Data Fig. 6), when the content of kynurenine in the brain showed significant up and down fluctuations (Fig. 4b). Finally, when a large number of neurons were lost in the hippocampal CA3 region (Fig. 3f) and cognition was impaired at the 12th month (Fig. 3g, h and Extended Data Fig. 3d-f), the content of

xanthurenic acid in the brain was significantly increased (Fig. 4c). This finding was consistent with the evidence in chemically induced rats. These subtle dynamic changes in abnormal metabolism in the TKX pathway in the blood and brain were accumulated and amplified by the excretion of xanthurenic acid in the urine. While the abnormally expressed human A β ₄₂ in the brains of APP/PS1 transgenic mice did not cause obvious pathological changes in the nervous system at the 3rd month, the excretion of xanthurenic acid in the urine increased significantly (Fig. 4h). This increase in excretion was continuous over the entire modelling process (Fig. 3i and Fig. 4h). Therefore, urine xanthurenic acid excretion can predict and reflect the process of nervous system damage before AD.

In the correlation study of xanthurenic acid in AD treatment, when cerebral cortex gliosis (Fig. 3e), hippocampal neuron injury (Fig. 3f) and cognitive impairment (Fig. 3g, h) were improved significantly by schizandrin, the adjustment of abnormal metabolism of xanthurenic acid in urine (Fig. 4h) was more obvious than that of A β ₄₂-positive expression (Fig. 3c, d). This result was consistent with the finding in the chemically induced rats.

Method validation and experimental sample analysis

Quantitative analysis of biological samples in the chemically induced rat model and APP/PS1 transgenic mouse model were investigated in terms of specificity, accuracy, precision, stability, matrix effects, and limit of quantitation (LOQ), as shown in Extended Data Fig. 9 and Supplementary Tables 6-8, and all results met the quantitative analysis conditions. The accompanying QC samples were randomly assigned to the analysis process and quantitatively analysed by standard curves of xanthurenic acid, kynurenine and tryptophan (Supplementary Table 5). The coefficient of variation of the QC during the sample data collection was less than 9.68%, which guaranteed the stability of the quantitative analysis (Extended Data Fig. 8).

Discussion

In this assay, we provided evidence for the chronology of xanthurenic acid metabolism changes in the Tryptophan-Kynurenine-Xanthurenic acid (TKX) pathway with other phenomena in the progression of AD. The main features in the two AD models are summarized in Fig. 1 and Fig. 3. Collecting their common phenomena, we found that a low level of xanthurenic acid in the blood (Fig. 2g and Fig. 4f) was related to AD preclinical risk factors such as peroxidation (Fig. 1f), A β ₄₂ deposition (Extended Data Fig. 1,4) and astrocyte hyperplasia (Fig. 1d and Fig. 3e). Clinical reports support that a low level of peripheral xanthurenic acid is found in AD and other mental illnesses, such as depression^[33] and psychosis^{[34][35]}. These mental illnesses are also accelerating factors of cognitive decline, as 40–60% of AD patients with psychotic symptoms deteriorated more rapidly^{[36][37]}. Second, the abnormal metabolism of kynurenine-xanthurenic acid in the brain (Fig. 2c, d and Fig. 4b, c) was associated with disordered arrangement of hippocampal neurons and nucleolar pyknosis (Fig. 1e and Fig. 3f). Other experiments have proven the distribution of xanthurenic acid in neurons and its correlation with cognitive function^{[38][39]}. During this process, interesting transmission in the TKX pathway was observed in the blood of chemically induced rats, in which abnormal metabolic fluctuation was observed from tryptophan to kynurenine and finally to xanthurenic acid (Fig. 2e-h). This was not obvious in the blood of APP/PS1 transgenic mice but was apparent in their brains (Fig. 4g), which may be related to the different model processing methods, as the direct effect on blood metabolism of chemical-induced rats was derived from irritation of the gastrointestinal tract by oral aluminium chloride and intraperitoneal injection of D-galactose. The abnormal expression of A β ₄₂ in the brains of APP/PS1 transgenic mice directly affects their brain metabolism. This abnormal transmission in a metabolic pathway caused by pathogenic stimuli has been reported in few previous studies and may be an interesting field of further metabolomics research. In addition, trends of metabolic abnormalities in the TKX pathway in the brain and blood of chemical-induced rats and APP/PS1 transgenic mice were not always consistent and continuous (Fig. 2h and Fig. 4g), which may be related to the self-regulation of metabolic disturbance.

Therefore, although the dynamic changes in xanthurenic acid in the blood and brain help us to understand its association with the pathological process of AD, they are not the best candidates for theragnostic markers. Both the transience of the metabolic disturbance in the TKX pathway and the instability of their intermittent fluctuations caused by self-regulation of abnormalities make them unsuitable as diagnostic markers of AD. Additionally, the narrow diagnostic threshold is another problem. As shown in Fig. 2g and Fig. 4f, the ratio of xanthurenic acid in the serum of the model and negative control groups was approximately

0.82~1.21 (the closer to 1, the smaller the difference). Such subtle differences could be discovered in the laboratory under strict controlled conditions, but its clinical application to individuals with complicated metabolic backgrounds is difficult. These may be common problems faced by most AD blood metabolism markers^{[19][20][40][41]}.

Conversely, xanthurenic acid is the end product in the TKX pathway^[42], and its content in urine after continuous collection for 12 h cumulatively could reflect the complex and dynamic metabolic disturbances in the TKX pathway during this period. The abnormal signal accumulated and amplified as the ratio of xanthurenic acid in the urine of the model and negative control groups was nearly 1.35~2.62 (Fig. 2i and Fig. 4h). Moreover, the relatively continuous and stable trend over the occurrence and development of AD is an important reason for xanthurenic acid as an AD predictive marker. Its high excretion in the preclinical stage of AD was proven in the two classic AD models in our research (Fig. 2i and Fig. 4h). Positive results were also found in the research on its relevance for treatment evaluation. When the high excretion of xanthurenic acid was inhibited by the neuroprotective agent schizandrin, the relevant AD pathological conditions, such as astrocyte proliferation and loss of hippocampal neurons and the final cognitive disorder, were all improved to some extent (Figs. 1 and 3).

In summary, urine xanthurenic acid has the following advantages as a candidate AD theragnostic marker: (1) predictability, with consistent correlation with the development of pre-AD pathology; (2) stability, with persistent elevation in the preclinical stage of AD; (3) treatment relevance, as its regulation is related to improved neuronal state and delayed cognitive decline; (4) good reproducibility, proven by two classic AD models; (5) signal amplification, as a cumulative record of abnormal metabolism in the TKX pathway *in vivo*; and (6) accessibility, with a noninvasive sampling method. Even though it is not perfect, the main problems are the impact of collection duration and urine volume on the content of metabolites. We accurately carried out a 12-hour continuous collection and calculated the volume to reduce the error in our experiment. However, when used in clinical applications, standardized operating procedures will still be required for patient urine collection. Finally, the relationship between xanthurenic acid metabolism and neuronal function^[43] and energy metabolism disorders^[44] in AD needs to be studied in future experiments to reveal its mechanism.

Conclusions

Collectively, we monitored the variation of xanthurenic acid *in vivo* to describe its correlation to the pathological phenomenon and drug regulation in the progression of AD by quantitative metabolomics method and verified the advantages of urinary xanthurenic acid as a theragnostic marker for AD from the multiple characteristics discovered in our experiment. These results reveal its promising ability in AD prediction and drug development for future clinical trials.

Methods

Reagents and material

Aluminium chloride and D-galactose were purchased from Sigma-Aldrich (Shanghai, PRC). Schizandrin was purchased from Must Biotechnology Co., Ltd. with a purity >98%. Standard tryptophan, kynurenine, xanthurenic acid and 2-chloro-L-phenylalanine (internal standard) were provided by Jacschem Co., Ltd. with a purity >97%. HPLC grade methanol, acetonitrile, and formic acid were purchased from Fisher Chemical (Waltham, USA). Deionised water (18.25 MΩ) was purified through a Milli-Q unique water system from RSJ Co., Ltd. (XiaMen, PRC).

Model preparation

All experiments were approved by the HeiLongJiang University of Traditional Medicine Animal Ethics Committee, and all applicable institutional and governmental regulations concerning the ethical use of animals were followed (SYXK 2018-0101).

Chemically induced rat

Eighteen-week-old Wistar male rats weighing 300±20 g were provided by LiaoNing Changsheng Biotechnology Co., Ltd. (BenXi, China). Rats were excluded from analysis if there were signs of cognitive deficiencies. The animals were fed commercial rat food that amount to 10% of their body weights at room temperature and allowed ad libitum water. After 7 days of environmental adaptation, we randomly divided the rats into 1-5 control groups (NC, n=10), 1-5 AD model groups (D-gal+AlCl₃, n=10) and 1-5 schizandrin intervention groups (SIN D-gal+AlCl₃, n=10). The schedule of model preparation and schizandrin intervention lasted 90

continuous days with model rats given aluminium chloride (AlCl_3) (28 mg/kg/day) by gavage and D-galactose (D-gal) (63 mg/kg/day) by intraperitoneal injection every morning 08:00. The control groups were given the same amount of distilled water and normal saline at the same time point. At 14:00 each day, the medication intervention rats were gavaged with schizandrin (50 mg/kg/day), and the control and model groups were given the same amount of distilled water every day (Fig. 1a).

APP/PS1 transgenic mouse

Seven-week-old APP^{swe}/PS1^{dE9} transgenic and littermate wild-type control male mice (weight, 25 ± 3 g) were provided by Nanjing University Institute of Biomedical Sciences (Nanjing, China). These mice were maintained at room temperature with free access to commercial mouse food, which amounted to 10% of their body weights at room temperature, and water. After acclimatization for 1 week in separate cages, the mice were randomly allocated to different groups. Briefly, transgenic mice were assigned to 20 groups, including 10 groups for model evaluation (APP/PS1 1-10 groups, $n=10$) and 10 groups for schizandrin intervention (SIN APP/PS1 1-10 groups, $n=10$). Wild-type mice were assigned to 10 control groups (WT 1-10 groups, $n=10$). Mice in the SIN APP/PS1 group were treated with schizandrin (7 mg/100 g/day, formulated daily in 3% CMC-Na) by gavage once daily for 10 months continuously, and the APP/PS1 and WT mice were given the same amount of vehicle (3% CMC-Na) (Fig. 3a).

Model evaluation

Morris water maze test

The Morris water maze (MWM) test was performed in a dark room according to a protocol published previously with the aim of measuring spatial learning and memory^[27]. Briefly, the animals underwent 5 days of navigation tests, and each animal performed 4 trials each day. The animals were released into the water at different starting positions, and the latency to find the platform was timed for learning ability evaluation. After 5 days of training, the platform was removed on the 6th day, and the trace and the number of platform-site crossovers were recorded by Ethvision XT 11.5 (Noldus, Netherlands) for the spatial memory ability evaluation. The MWM tests were carried out on the 10th-15th, 25th-30th, 40th-45th, 55th-60th and 85th-90th days in the chemical damage-induced AD rat experiment, and these tests were scheduled in the last week of each month for the APP/PS1 transgenic mouse model.

Histopathology of brain sections

For the histopathology analysis, rat whole blood was drawn from the aorta ventralis after anaesthetization, and the mice were decapitated. Then, all brains were gently extracted, and the left hemispheres were stored in 10% formalin until use. The brain hemispheres were gradually manipulated with dehydration and transparent operation, followed by embedding, cutting and staining. For haematoxylin-eosin, immunohistochemistry or immunofluorescence staining, brains were sagittally sectioned by a sliding microtome at 40 μm thick. Primary antibodies used for immunostaining were $\text{A}\beta_{1-42}$ (AB216504, Abcam) and GFAP (AB7260, Abcam and GB11096, Servicebio). Staining was visualized with a microscope (Dmi1, Leica), and photomicrographs were taken with an attached digital camera and associated software (Spot InSight).

Detection of clinical biochemical indicators

MDA levels in the rat brain were determined using ELISA kits following the manufacturer's instructions (Jiancheng Bioengineering). The right brain hemispheres from each group ($n=10$) were homogenized with a FastPrep-24™ 5 homogenizer (MP, America) in normal saline, the resultant homogenates were centrifuged at 4000 rpm for 10 minutes, and the supernatant of the homogenates was transferred to an ice-cold tube for subsequent detection.

Instrumentation

A Waters Acquity™ UPLC coupled with tandem triple quadrupole detector (TQD) mass spectrometry (LC-MS/MS) was used for quantitative analysis. A reversed-phase Waters ACQUITY UPLC™ HSS T3 column (100 mm×2.1 mm i.d., 1.8 μm) and C18 column (100 mm×2.1 mm i.d., 1.8 μm) were equipped for different biological samples with mobile phases A (0.1% formic acid in acetonitrile, v/v) and B (0.1% formic acid in water, v/v) (Supplementary Tables 1 and 2). The column was kept at 40°C, and the flow rate at all times was 0.4 ml/min. The column effluent was delivered to an MS detector (Supplementary Table 3) with no split, and all analytes

were detected in positive ion multiple reaction monitoring (MRM) mode (Supplementary Table 4). The injection volume was 5 µl. All quantification processing was conducted with the QuanLynx™ programs from the MassLynx™ software package (version 4.1, Waters).

Biological sample collection and storage

Twelve hours of continuous urine samples were collected every 5 days for rats and every month for mice. The urine volume of each animal was recorded, and it was discarded if the urine was mixed with faeces. After centrifugation, the urine was immediately put into a -80°C refrigerator until use. On the 15th, 30th, 45th, 60th and 90th days of rat model preparation, blood was obtained from the abdominal aorta, while for mice, blood was obtained by decapitation on the last day of every month. All serum samples were processed within 1 h, after which they were first placed quietly on the table for 30 min. Serum was then collected directly after 10 minutes of centrifugation and divided into tubes for -80°C storage until use. Normal saline was added to the right brain hemispheres to make a certain volume (1.5 ml for mice and 10 ml for rats). The mixture was homogenized with a FastPrep-24™ 5 homogenizer (MP, America) in normal saline, the resultant homogenates were centrifuged at 4000 rpm for 10 minutes, and the supernatant of the homogenates was transferred to an ice-cold tube for subsequent detection.

Sample preparation

Urine

Rat urine samples were first thawed at room temperature. Then, 300 µl of urine was added to 900 µl of solution (20 µg/ml internal standard in distilled water, v/v), vortexed for 1 min, and centrifuged at 13000 rpm at 4°C for 15 min. Then, 100 µl to 900 µl of the supernatant solution (20 µg/ml internal standard in distilled water, v/v) was mixed and shaken for 1 min to obtain the analysis sample.

Mouse urine samples were first thawed at room temperature, and then 100 µl of sample was added to 400 µl of solution (20 µg/ml internal standard in distilled water, v/v). The mixtures were vortexed for 1 min and centrifuged at 13000 rpm for 10 min to obtain the supernatant.

Serum and brain

Two hundred microlitres of rat plasma or brain homogenate was added to 800 µl of methanol to denature the interfering macromolecular proteins. The mixture was vortexed for 1 min, and then the supernatant was centrifuged and dried using a vacuum evaporator. The residue was redissolved in 100 µl of solvent (20 µg/ml internal standard in distilled water/methanol, v/v) and centrifuged at 13000 rpm at 4°C for 10 min to obtain a clear extract.

Internal standard preparation and stock solutions

All stock solutions were prepared in either water, methanol, or water/methanol with 0.1 M NaOH and 100 µmol/L L-3-chlorophenylalanine internal standard according to varying analyte solubilities. The stock solutions of L-tryptophan, kynurenine, and xanthurenic acid were 500 µmol/L, 60 µmol/L and 50 µmol/L, respectively. They were mixed and diluted into different concentrations of working solutions (Supplementary Table 5).

QC samples preparation

QC samples of different matrices (brain, serum and urine) were prepared separately by mixing equal volumes of all analysed samples for each sample type. The mixtures were equally aliquoted and stored at -80°C. Before analysis, the mixtures were thawed in a water-ice bath, and 1 ml of each sample was separately added to 50 µl of standard working solution at different concentrations. After the sample preparation mentioned above, they were used for method validation and randomly inserted into the sample batch to monitor the stability of the instrument.

Statistical analysis

Unpaired two-tailed t test was used to analyze significant differences between each group. Coefficient of Variation (CV) was used for method validation.

Declarations

Conflicts of interest

The authors declare no competing financial interests.

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Figures

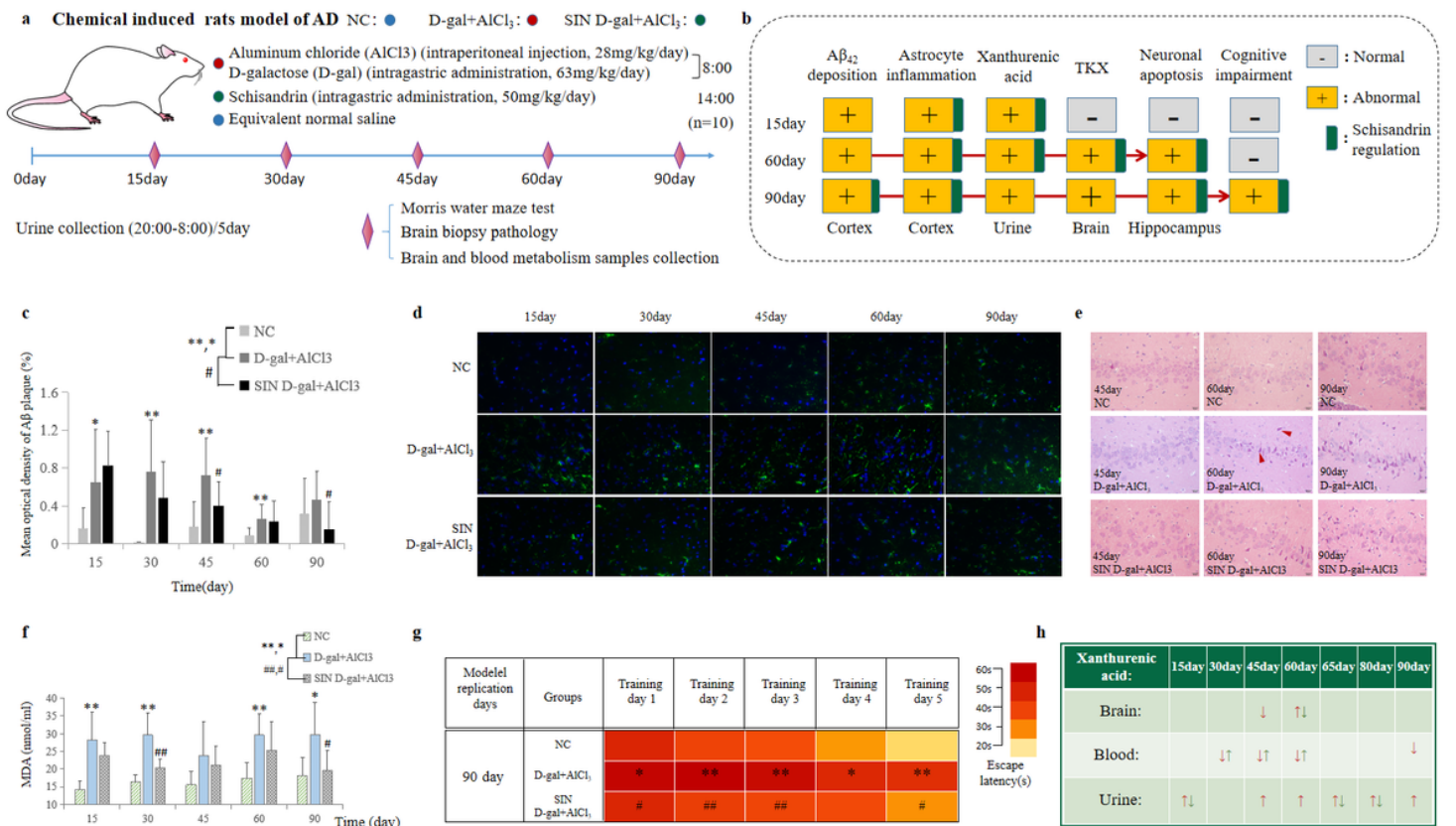


Figure 1

Relevance of xanthurenic acid to the diagnosis and treatment of a chemically induced rat model of AD.

A chemically induced rat model of AD (D-gal+AlCl₃) was generated via oral administration of aluminium chloride (AlCl₃, 28 mg/kg/day) and intraperitoneal injection with D-galactose (D-gal, 63 mg/kg/day) for 90 days, and the SIN D-gal+AlCl₃ treatment group was orally administered schisandrin (SIN) at dose of 50 mg/kg/day for the same duration. The negative control group (NC) was obtained by treating homologous Sprague-Dawley rats with an equal dose of saline. All groups were evaluated at corresponding time points, as shown in (a). Summary of the significant situation (b) that the elevated excretion of xanthurenic acid in urine (h) was synchronized with the positive expression of early AD risk factors, such as cortex A β ₄₂ depositions coloured by immunohistochemistry (c), astrocyte proliferation (green area, originally ×400) imaged by immunofluorescence staining (d) and

malondialdehyde (MDA) peroxidation tested by ELISA on the 15th day (f). The abnormal fluctuations of xanthurenic acid in the brain (h) were related to the disordered neuronal arrangement and nucleolar pyknosis (red triangle position, originally ×400) in the hippocampus imaged by haematoxylin-eosin staining from the 45th to the 60th day (e). Significant cognitive decline was detected by navigation experiment assay on the 90th day (g). In the treatment evaluation correlation study, when the pathological condition was improved, such as proliferation of astrocytes (d), MDA peroxidation (f), hippocampal neurons (e) and cognitive decline (g) by schisandrin, the abnormal metabolism of xanthurenic acid in the urine, blood and brain was also regulated (h).

(*P<0.05; **P<0.01, compared with the NC group; #P<0.05; ##P<0.01, compared with the D-gal+AlCl₃ group; Red arrow: the statistically significant abnormal metabolic trend in the D-gal+AlCl₃ group; Green arrow: the statistically significant regulation trend of schizandrin; panel d: the green area indicates proliferating astrocytes and the blue area indicates the neuron nucleus.)

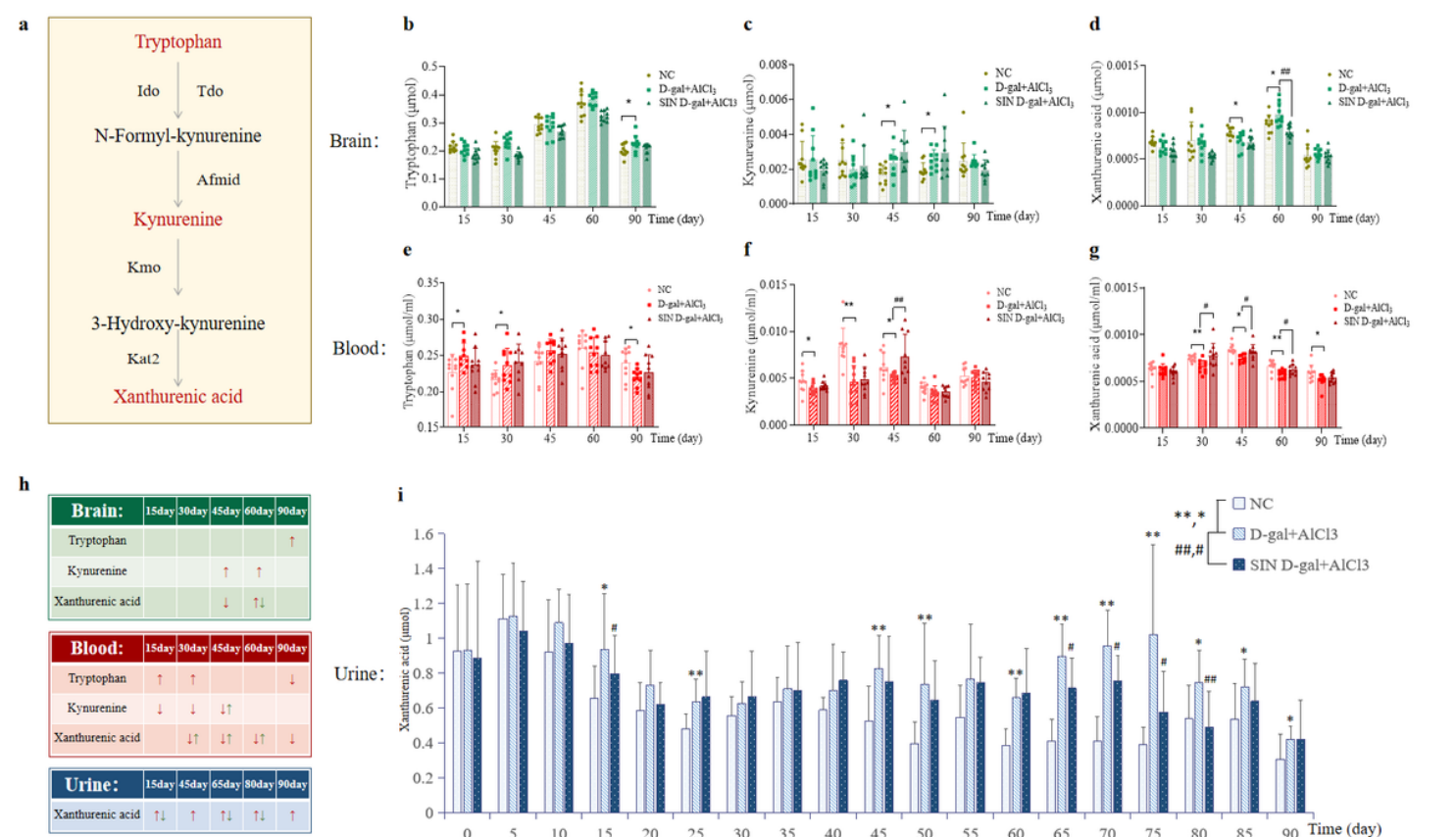


Figure 2

Metabolic changes in the tryptophan-kynurenine-xanthurenic acid pathway of the chemical-induced rat model of AD.

Xanthurenic acid was synthesized by a consequent transamination of the amino acid precursors in tryptophan-kynurenine pathway and eventually excreted in the urine in the form of end products (a). The quantitative analysis of metabolites by UPLC-MSMS is shown in this figure, and their trend is summarized in (h). The pathological process of AD was initially accompanied by the abnormal metabolism of tryptophan in the blood (e). Then, this abnormality was passed to the downstream kynurenine (f) and xanthurenic acid (g). The reduction of their concentration in the blood was related to the high excretion of xanthurenic acid in the urine (i). Finally, not the tryptophan (b) but its metabolites kynurenine (c) and xanthurenic acid (d) were disturbed in the brain induced by their advancing variation in the blood. These metabolic abnormalities in the brain were implicated in the disordered arrangement and nucleolar pyknosis of neurons in the hippocampus on the 45th and 60th days. All neuropathological phenomena were recorded by the high excretion of xanthurenic acid in urine at the same time. In xanthurenic acid's treatment-related research, the improvement of nerve damage in hippocampus on the 45th and 60th days by schisandrin was interrelated with the regulation of the xanthurenic acid metabolism as shown in the result of brain (d), blood (g) and urine (i).

(*P<0.05; **P<0.01, compared with the NC group; #P<0.05; ##P<0.01, compared with the D-gal+AlCl₃ group; The red arrow represents the significant trend in the D-gal+AlCl₃ group compared with the NC group, the green arrow represents the significant trend in the SIN D-gal+AlCl₃ group compared with the D-gal+AlCl₃ group)

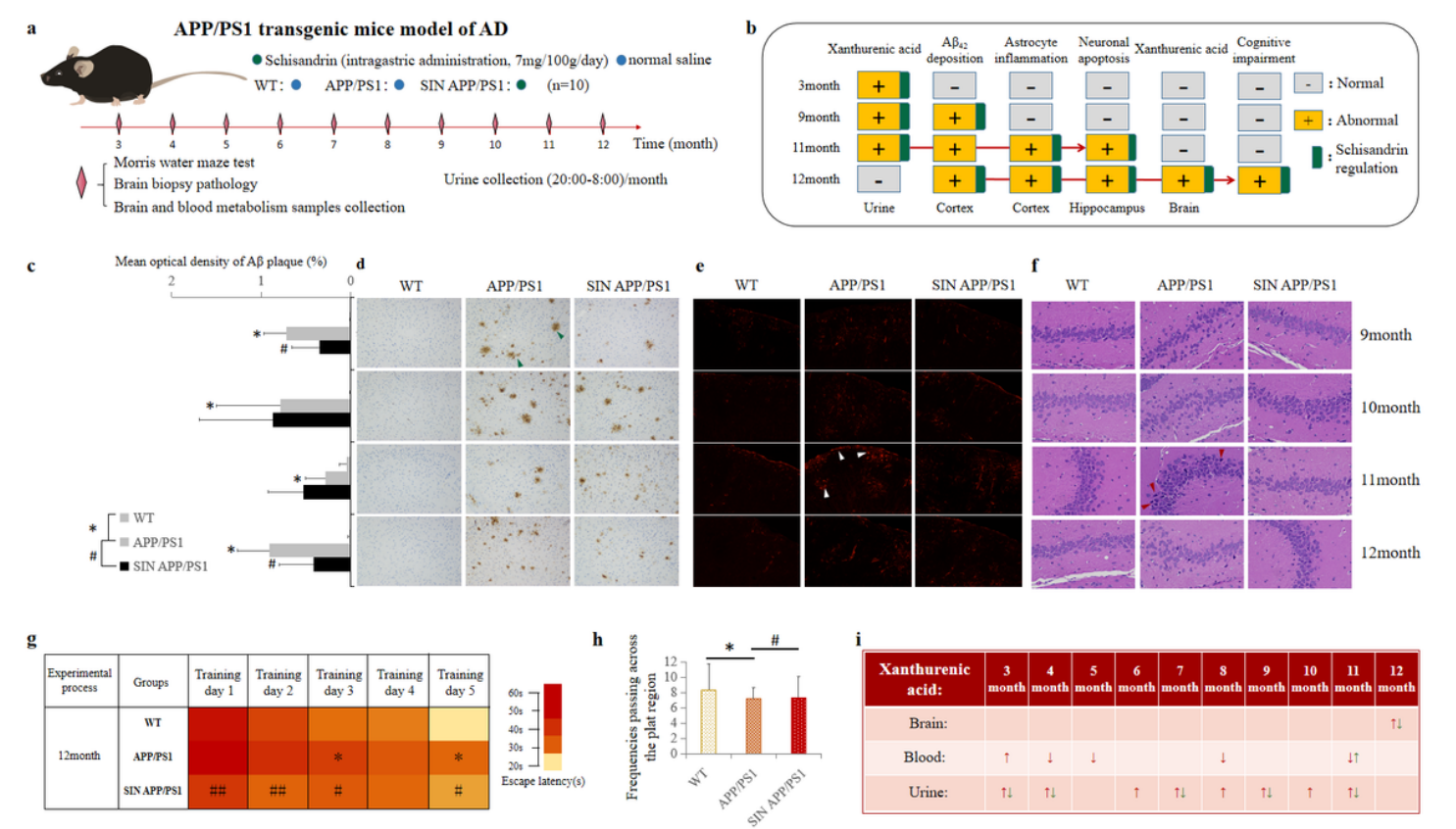


Figure 3

Relevance of xanthurenic acid to the diagnosis and treatment of an APP/PS1 transgenic mouse model of AD.

Three-month-old APP/PS1 transgenic mice were randomly divided into two groups: the SIN APP/PS1 group was treated with schisandrin by intragastric administration at a dose of 7 mg/100 g/day until 12 months of age, and the APP/PS1 group was treated with isometric saline. Their littermate wild-type controls were treated with isometric saline as a negative control group (WT). All groups were evaluated at corresponding time points, as shown in (a). The critical results at the time points were collected and compared in (b). Xanthurenic acid in urine was extremely significantly elevated in APP/PS1 mice from the 3rd month to the 11th month (i). Serious Aβ₄₂ deposition (c)&(d) (green triangle position, originally ×200) in the cortex was found by immunohistochemistry from the 9th month to the 12th month, and astrogliosis (white triangle position, originally ×200) was detected at the 11th month by immunofluorescence staining (e). With the stimulation of these risk factors, neuronal cells in the hippocampus imaged by haematoxylin-eosin began to be arranged disorderly at the 9th month, and the nucleolus exhibited obvious pyknosis (red triangle position, originally ×400) at the 12th month (f). Finally, the 12-month-old mice showed a significant decline in learning (g) and cognitive abilities (h) when the content of xanthurenic acid in their brains was increased significantly (i). In the treatment evaluation correlation study, the elevated excretion of xanthurenic acid was obviously inhibited by schisandrin in the urine of the SIN APP/PS1 group throughout the progression of AD, and this situation also appeared in their brains at the 12th month (i). Consistent with this, astrocyte proliferation (e), hippocampal neuron damage (f) and cognitive decline (g)&(h) were all improved.

(*P<0.05; **P<0.01, compared with the WT group; #P<0.05; ##P<0.01, compared with the APP/PS1 group; The red arrow represents the significant trend in the APP/PS1 group compared with the WT group, the green arrow represents the significant trend in the SIN

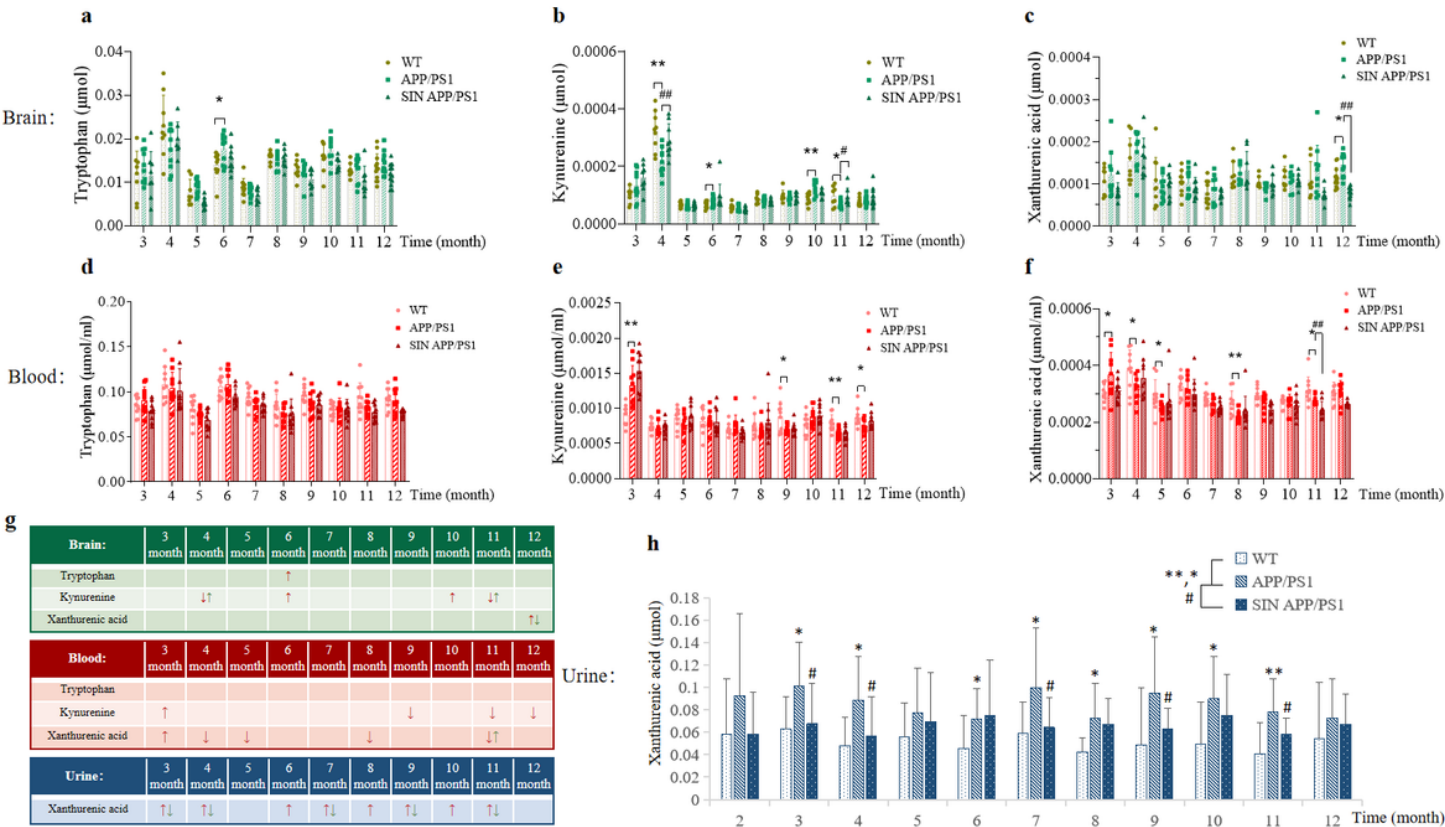


Figure 4

Metabolic changes in the tryptophan-kynurenine-xanthurenic acid pathway of the APP/PS1 transgenic mouse model of AD.

As shown in the sketch of metabolic changes in the TKX pathway of APP/PS1 transgenic mice (**g**), not tryptophan (**d**) but the metabolism of kynurenine (**e**) and xanthurenic acid (**f**) in the blood was disturbed in pathological progression. This persistent metabolic abnormality in the blood affects the TKX pathway in the brain. When abnormal levels of tryptophan and kynurenine were detected in the 6th month (**a**)&(**b**), A β_{42} had obvious positive expression in the cortex for the first time. The fluctuations of kynurenine on the 10th and 11th months and xanthurenic acid on the 12th month (**b**)&(**c**) were consistent with progressive pathological phenomena, including astrocyte hyperplasia, hippocampal neuron damage and cognitive impairment. This series of neuropathological events was accumulated, amplified and recorded by the end product xanthurenic acid in urine, as high excretion was observed from the 3rd to 11th months (**i**). Moreover, in xanthurenic acid's treatment-related research, when the nerve damage above was improved by schisandrin, the disturbed metabolism of xanthurenic acid in the brain (**c**), blood (**f**) and urine (**h**) was regulated.

(*P<0.05; **P<0.01, compared with the NC group; #P<0.05; ##P<0.01, compared with the D-gal+AlCl₃ group; The red arrow represents the significant trend in the D-gal+AlCl₃ group compared with the NC group, the green arrow represents the significant trend in the SIN D-gal+AlCl₃ group compared with the D-gal+AlCl₃ group)

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