Supplementary materials:

Involvement of mitochondrial dysfunction in hepatotoxicity induced by *Ageratina adenophora* in mice

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Materials and methods

Animals and treatment

Forty 8-week-old Kunming mice were purchased from Chengdu Da-shuo Experiment Animal Technology Ltd. (Chengdu, China). Mice were randomly divided into four groups. Mice in those groups were administrated with 0 g/kg (as control), 100 g/kg, 200 g/kg and 300 g/kg level of *A. adenophora* pallet diet for 42 d, respectively. Mice were housed under standard conditions at (25±2) °C, ambient humidity of (55±5)% and in a 12 h light/dark cycle. *A. adenophora* was collected from Xichang city of Sichuan Province, Southwest China. The plant was dried in shade and broken into pieces. And the ground material was stored in a dry environment prior to experiment.

Histopathological examination

After euthanasia, the organs in different groups were carefully dissected out, washed with cold phosphate buffer solution (PBS, pH 7.2–7.4) and fixed 48 h with 4% formalin. Then a small piece of organs were rinsed with water for 12 h, dehydrated in serial dilutions of ethanol before embedding in paraffin wax. Paraffin blocks of organs were sectioned at 5 μm thickness, which were rehydrated in distilled water and stained with Hematoxylin-Eosin (HE) before served for histopathological tissue images under Olympus Microscope BX43 (Japan).

Mitochondria Isolation

At the end of experiment, mice in all groups were sacrificed according to the approved guidelines and the livers were immediately removed. Mitochondria isolation from liver was performed according to the manufacturer's instructions (Solarbio, Beijing, China). Briefly, liver (200 mg) was incubated in the ice-cold mitochondrial lysis buffer (1 mL) and were then ground with a glass homogenizer for 20 strokes. The homogenate was centrifuged 2 times at 1000 g for 5 min at 4 °C. The supernatant was collected and subjected to a centrifugation again at 12000 g for 10 min at 4 °C to obtain the mitochondria fraction.

Degree of Mitochondrial swelling

Mitochondria extracted from lives were used to detect the swelling degree. Mitochondria were dissolved in lysis buffer and then subjected to a flow cytometry (FCM) (Becton Dickinson, USA). The ratio of forward scatter (FSC) to side scatter (SSC) was used to measure the degree of mitochondrial swelling as previously described (Zheng et al., 2015).

Transmission electron microscopy (TEM) assay

Animals are executed in a humanitarian manner. The liver was collected and a volume of 1mm^3 tissue was fixed at 4 °C in a solution containing 2.5% (v/v) glutaraldehyde immediately for 4 h. Liver was stained with 1% (0.01 g/mL) osmium acid buffer at 4 °C for 15 min. Liver was dehydrated with different concentration of acetone, infiltrated and embedded in epoxyresin at room temperature. Ultrathin sections (100 nm) were cut and imaged by a H-7650 TEM (Hitachi, Tokyo, Japan).

Detection of ATPase activity

The mitochondria ATPase activity in liver was measured using a commercial assay kit from Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturer's instruction. Briefly, after 42 d of *A. adenophora* administration, mitochondria of liver from each group was isolated and 100 μL 2% concentration of mitochondria suspension was mixed with 100 μL ATP detection working solution. Absorbance value was measured at 660 nm wavelength. Protein concentration in each suspension was determined using a Bradford Protein Assay Kit (Sagon Biotech, Shanghai, China). The ATPase levels were presented as U/mg protein.

Real time RT-PCR to determine the copy number of mtDNA

Total genomic DNA was extracted using a Rapid Animal Genomic DNA Isolation Kit from Sagon Biotech (Shanghai, China). The mtDNA copy number was measured by detecting by quantitative real time PCR (qRT-PCR) as previous described (Tiao et al., 2007; Koh, et al., 2018). The mtDNA copy number were measured using SYBR Premix ExTaq TM (TAKARA, Japan) with a CFX96Thermal Cycler (Bio-Rad, Hercules, USA). PCR reaction mixture contained 5 μL SYBR-Green, each primers 0.5 μL, DNA 1 μL, and ddH_2O 3 μL. The real-time PCR condition was as follows: initial 95 °C for 1 min, 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C 30 s. The forward and reverse primers complementary to β-actin were 5'-CCTAGGCACCAGGGTGTGAT-3' and 5'-TCCATGTCGTCCCAGTTGGT-3'. The primers for detect mtDNA were complementary to Cytb, which were 5'-TGTCGGACGAGGCTTATATTATGG-3' and 5'-TGTGGCTATGACTGCGAACAG-3'.
The threshold cycle number (Ct) values of Cytb and β-actin were measured by individual real-time PCR. The copy relative number was represent as $2^{\Delta Ct}$ (ΔCt is $Ct_{\beta\text{-actin}} - Ct_{Cytb}$).

Statistical analysis

In each assay, the experiment data represents as means ± standards deviations. These data was analyzed using a one-way analysis of variance (ANOVA) complemented with the Turkey-Kramer multiple comparison test. And computations were performed by SPSS 22.0 software package (SPSS Inc., USA).

Reference

Zheng G, Lyu JJ, Liu S, et al., 2015. Silencing of uncoupling protein 2 by small interfering RNA aggravates mitochondrial dysfunction in cardiomyocytes under septic conditions. *INT J MOL MED*, 35(6):1525-1536. https://doi.org/10.3892/ijmm.2015.2177

Data

Table 1 Na^+K^+-ATPase activities of mitochondria in all groups

Control	Dose of *A. adenophorum*		
	100 g/kg	200 g/kg	300 g/kg
4.053 ± 0.665^{D}	2.587 ± 0.257^{C}	1.602 ± 0.239^{B}	0.610 ± 0.144^{A}

The data with different capital letters show extremely significant difference ($p<0.01$)

Table 2 $Ca^{2+}Mg^{2+}$-ATPase activities of mitochondria in all groups

Control	Dose of *A. adenophorum*		
	100 g/kg	200 g/kg	300 g/kg
3.567 ± 0.511^{D}	2.330 ± 0.598^{C}	1.602 ± 0.326^{B}	1.488 ± 0.179^{A}

The data with different capital letters show extremely significant difference ($p<0.01$)

Table 3 Change of copy number of mtDNA in all groups

Control	Dose of *A. adenophorum*		
	100 g/kg	200 g/kg	300 g/kg
2.039 ± 0.137^{D}	1.706 ± 0.212^{C}	1.256 ± 0.149^{B}	0.742 ± 0.114^{A}

The data with different capital letters show extremely significant difference ($p<0.01$)

Table 4 Swelling degree of mitochondria in all groups

Control	Dose of *A. adenophorum*		
	100 g/kg	200 g/kg	300 g/kg
0.770 ± 0.009^{Cd}	0.850 ± 0.035^{Cc}	0.941 ± 0.144^{Bb}	1.191 ± 0.049^{Aa}

The data with different capital letters show extremely significant difference ($p<0.01$), lowercase letters show significant difference ($p<0.05$)