Protection effect of intracellular melanin from *Lachnum* YM156 and Haikunshenxi capsule combination on adenine-induced chronic renal failure in mice

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Materials and methods

Preparation of LIM

The fruiting bodies of *Lachnum* YM156 were collected from Yunnan Province, China. *Lachnum* YM156 was isolated and preserved in the Microbial Resource and Application Laboratory of the Hefei University of Technology. The intracellular melanin of *Lachnum* YM156 was fermented, extracted and purified according to the method of Ye et al. with slight modification.[1] The crude melanin was obtained by the method of alkali extraction and acid precipitation, then 15 mg extracted melanin were dissolved in 1 mL 0.5% $NaHCO_3$ solution for chromatography on sephadex G-15 column (1.6 cm × 60 cm), with 0.5% $NaHCO_3$ solution as eluant, injection volume of 1 mL and the flow rate of 200 L/min. The samples were sequentially collected with tube that each containing 5 mL. And the main component was collected.

Reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Biosharp (Anhui, China), phosphate buffered saline, fetal bovine serum, dulbecco's modified

eagle medium (DMEM) and penicillin-streptomycin solution were purchased from HyClone company (HyClone，USA). Adenine was provided by Luoyang Desheng Chemical Co.,Ltd. (Henan, China), Haikunshenxi capsules from Huinan ChanglongBiochamical Pharmacy Co., Ltd (Jilin, China), serum creatinine (SCR) kit, blood urea nitrogen (BUN) kit, total protein (TP) kit and albumin (ALB) kit were obtained from Nanjing Jiancheng BioengineeringInstitute (Jiangsu, China). The kits of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-PX), glutathione reductase (GSH) and malonaldehyde (MDA) were acquired from Nanjing Jiancheng Bio-engineering Institute (Jiangsu, China). ELISA kits for the determination of tumor necrosis factor (TNF)-α; interleukin-1β (IL-1β); interleukin (IL)-6; inducible nitric oxide synthase (iNOS) were purchased from Shanghai Yansheng Bio-Technology Co. Ltd (Shanghai, China). All the other reagents used were analytically pure and bought from Shanghai Zhenqi Chemical Reagent Co., Ltd. (Shanghai, China).

Laboratory animals

One hundred Kunming mice (male), weighing 20 ± 5 g, were purchased from the Experimental Animal Center of Anhui Medical University (Certificate number: no. 1 license of the Medical Laboratory Animal of Anhui), and were kept under standard conditions, which include: 12 h light/dark cycle, temperature: 18–20 °C and a standard diet. This project was reviewed and approved by the Committee for Protection of Animal Care Committee at the Hefei University of Technology.

Cell culture and cytotoxicity of LIM

MTT test was performed on HepG2 cells which were seeded at 1×10^5 cells/well in 96-well plates and maintained at 37 °C in an atmosphere of 5% CO_2 and DMEM supplemented with 1% penicillin-streptomycin solution and 10% fetal bovine serum. The LIM were dissolved in phosphate buffered saline solution. Then the LIM at different concentrations (300, 150, 100, 50,

10, 5 and 1 μg/mL) were used and phosphate buffered saline was used as control group. Cells were incubated with LIM solution. After 24 h incubation, cells were incubated with 5 mg/mL MTT solution for 4 h in a CO_2 incubator to allow the transformation of MTT dye to formazan salt. After 4 h, the medium was removed, then 150 μl dimethyl sulfoxide was added and incubated for 10 min at 37 °C. The optical density at 490 nm was measured by microplate reader (Bio-Rad iMark).[2,3]

Animal grouping and experiment design

After acclimatization for one week, 10 of the 100 healthy male Kunming mice were selected randomly as the normal group (control) and later intragastrically administered with normal saline at the ratio of 10 mL/kg. According to A. Nemmar's previous report with slight modification,[4] adenine-enriched diet (containing 10 g adenine per kg feed) administration was adopted for 3 weeks to build the CRF mice model. Then SCR and BUN concentrations in serum on the mice were measured. Mice reached modeling requirements (SCR>115 mM/L and BUN>8.05 mM/L) were randomly divided into 6 groups (10 mice per group), including no drug treatment model control (normal saline: 10 mL/kg), HC alone positive control (HC: 150 mg/kg), low dose LIM alone (LIM: 100 mg/kg), high dose LIM alone (LIM: 200 mg/kg), low dose LIM plus HC (LIM: 100 mg/kg; HC: 150 mg/kg) , and high dose LIM plus HC (LIM: 200 mg/kg; HC: 150 mg/kg), respectively. All supplements were freshly prepared every day and intragastric administration was performed once a day for 4 successive weeks. The mice were weighed before the beginning of the treatment weekly during the treatment period. And doses were administered once a day and adjusted based on the body weight.

statement

The study was approved by the Committee for the Protection of Animal Care Committee at the Hefei University of Technology. All experimental protocols were in accordance to the Guidelines of Experimental Animal Administration published by the State Committee of Science and Technology of People's Republic of China.

Sample collection

At the end of the experimental period, the mice were fasted for 12 h after the last treatment administration. Before the cervical dislocation of the mice, blood was sampled from the mice eyes and kept at room temperature for half an hour while it coagulated naturally. Serum was obtained from blood samples after being centrifuged at 10 000g for 10 min at 4 °C and were stored at −80 °C prior to analysis. The kidneys were removed and weighed which were homogenated along with 0.9% normal saline at a rate of 1 g:10 mL (weight: volume), and then stored at −80 °C for future analyses.

Determination of blood biochemical indexes of mice with CRF

The levels of BUN, SCR, TP and ALB in serum were determined using test kits, with diacetyl oxime method, picric acid colorimetric method, coomassie brilliant blue method and bromocresol green colorimetric method respectively according to the kit instructions.

Determination of weight growth rate and kidney index

Food intake, water consumption, furs, and movement of mice were monitored during the experiments. Body weights were measured initially and 24 h after the last drug dosage. Kindneys

were dissected and weighed, and then the kidneys index was calculated as: kidneys weight/body weight × 100%.[5]

Determination of antioxidant indicator of the kidney tissue homogenate

For examining treatment effects on oxidative stress, SOD, CAT, GSH-PX, GSH levels and the MDA concentrations in the kidney homogenate solution were determined with hydroxylamine method, visible spectrometry method, colorimetric method, colorimetric method, and thiobarbituric acid method respectively according to the kit instructions. About the hydroxylamine method, the final product of oxyhydroxylation of .O2 is nitrite, and the latter under the action of sulfanilic acid and methyl naphthylamine present purple, the maximum absorption peak at a wavelength of 530 nm, it determined by spectrophotometer. The visible spectrometry is a very popular method. The colorimetric method for GSH-PX is due to GSH-PX can promote the reaction of hydrogen peroxide with reduced glutathione to produce H2O and oxidized glutathione, so the activity of enzyme is obtained. Finally, the colorimetric method for GSH is due to GSH and 2-thio acid of nitrobenzene were used to produce 5-thio acid of two nitrobenzene anion, which showed a stable yellow. The absorbance of GSH could be calculated by measuring the absorbance at 412 nm.

Determination of cytokine production of the kidney tissue homogenate

Nephridial levels of TNF-α, IL-1β, IL-6, and activities of iNOS were analyzed using kits according to the manufacturer's protocols.

Histological analysis

Kidney samples were collected and fixed in 10% neutral buffered formalin, embedded in paraffin, followed by Haematoxylin and Eosin dyeing, and finally observed and imaged under an optical microscope.

Statistical analysis

All data were presented as mean ± standard deviation after being statistically processed. Software SPSS 13.0 was used for t-test analysis of the inter group deviation. $p < 0.05$ means significant difference while $p < 0.01$ means very significant difference.

References

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