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The protective effect of the earthworm active ingredients on hepatocellular injury induced by endoplasmic reticulum stress

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ABSTRACT

The earthworm is a widely used Chinese herbal medicine. There are more than 40 prescriptions including earthworms in the "Compendium of Materia Medica". TCM theory holds that earthworms exert antispasmodic and antipyretic effects through the liver meridian to calm the liver. However, the clinical effect of earthworms on liver injury has not been clearly demonstrated. We have previously established a method to extract the active ingredients from earthworms (hereinafter referred to as EWAs) [1]. In the present study, we observed protective effect of the EWAs on tunicamycin-induced ERS (endoplasmic reticulum stress) model in human hepatic L02 cells. The results showed that the EWAs promote proliferation and reduced apoptosis of ERS model in L02 cells ($P < 0.01$). The up-regulation of ERS-related proteins, including PERK (protein kinase RNA-like endoplasmic reticulum kinase), eIF2a (eukaryotic translation initiation factor 2a), ATF4 (activating transcription factor 4) and CHOP (CCAAT/enhancer binding protein homologous protein), in L02 cell under ERS was inhibited by treatment of the EWAs ($P < 0.01$). In summary, our data suggest the EWAs can significant attenuate ERS-induced hepatocyte injury via PERK-eIF2a-ATF4 pathway.

1. Introduction

Increasing investigations show that the earthworm extract has various beneficial pharmacological activities, including fibrinolytic [2] and anticoagulative activity [3], anti-inflammatory activity [4], anti-oxidative activity [5,6], peripheral nerve regeneration [7], bone regeneration [8] and wound healing [9,10]. Earthworms have many advantages, such as beneficial pharmacological activities, abundant resources and low-cost and so on. However, in-depth studies on the active ingredients of earthworms and their pharmacological action are limited. Thus, there is a great potential and prospect for development in this field. In the pre-animal experiments, we observed that the EWAs (earthworm active ingredients) can significantly reduce the levels of ALT (serum alanine aminotransferase) and AST (aspartate aminotransferase) in the acute liver injured mice. Such results suggest that the EWAs may have a protective effect on the liver.

The main functions of the endoplasmic reticulum in eukaryotic cells include protein synthesis and protein folding to form the correct three-dimensional conformation [11]. Malfunctions of endoplasmic reticulum induced by various factors, such as ones that regulate protein folding, post-translational modification, lipid and steroid synthesis, gene expression, cellular metabolism and calcium signaling, can lead to UPR (unfolded proteins response), resulting in ERS [12]. As a result, the endoplasmic reticulum launches various coping mechanisms to alleviate the damage, allowing the cell to adapt to the environmental stress. On the other hand, if the recovery of cellular adaptation is not achieved prolonged ERS triggers apoptosis [13]. ERS-induced apoptosis took part in the pathogenesis and development of disease, such as heart disease [14], cerebral ischemia [15], diabetes [16], atherosclerosis [17] and cancer [18]. The hepatocyte contains abundant endoplasmic reticulum. ERS in the hepatocyte, especially ERS-mediated hepatocyte injury and apoptosis, might be an important contributor to hepatic diseases [19,20].

In our study, we established tunicamycin-induced ERS model in L02 cells, observed the protective effect of the EWAs and explored the underlying mechanisms in ERS-induced injured hepatocyte.

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That can provide references for the biological activity study of the EWAs in vivo.

2. Materials and methods

2.1. Preparation of the EWAs

In our previous work, the EWAs was obtained by fresh frozen *Eisenia foetida* and stored at −20 °C. The EWAs was yellowish-white floc and easily soluble in water. The EWAs was consisted of polypeptides (75%), polysaccharides (17%), vitamin D and inorganic salts (such as calcium and phosphorus). The EWAs was dissolved in DMEM (Dulbecco's Minimum Essential Medium) supplemented with 10% (v/v) FBS (fetal bovine serum).

2.2. Cell culture

L02 cells were from the Key Laboratory of Pharmacology and Medical Molecular Biology (Luoyang, China). Cells were cultured in DMEM containing 10% (v/v) FBS, supplemented with antibiotics (100 U/mL penicillin G and streptomycin sulfate), and incubated at 37 °C in a humidified chamber supplemented with 5% CO_2.

2.3. Establishment of ERS model in L02 cells

MTT assay was used to test the best condition of tunicamycin treatment in L02 cells. L02 cells in exponential growth phase were harvested, plated at 6×10^3 cells per well in 96-well cell culture plates. L02 cells were treated with tunicamycin (20, 40, 60, 80 or 100 μg/mL in DMEM supplemented with 10% FBS) for 12, 24 or 48 h set as model groups. 4 h before the end of culture, 15 μL MTT (5 mg/mL, Sigma, USA) was added into each well. Cell culture supernatant was discarded and cells were solubilized in 150 μL DMSO per well. Absorbance (A) was measured at 490 nm using an ELX800 Universal Microplate Reader (Bio-Tek Instruments). The survival rate was calculated as follows: survival rate (%) = $(A_{model}/A_{control}) \times 100\%$.

Western blotting analysis was used to detect protein expressions of GRP78 and CHOP. L02 cells in exponential growth phase were assigned to control group (treated with normal culture medium) and model group (treated with 60 μg/mL tunicamycin for 24 h). Subsequently, cells were lysed in ice-cold lysis buffer supplemented with 1 mM PMSF. The supernatant was saved after the lysates were centrifuged (12,000*g*, 30 min, 4 °C), and the protein concentration was measured by bicinchoninic acid protein assay kit (Solarbio). The supernatant protein from each sample was subjected to SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). Separated proteins were transferred to a PVDF membrane (0.45 mm, Millipore Co., Ltd.). The PVDF membrane was blocked with 5% skim milk in TBST, then incubated with different primary antibodies as follows: anti-β-actin (1:400 dilution, ZSGB-BIO, Beijing, China), anti-GRP78 (1:1000 dilution, Abcam, Cambridge, UK) and anti-CHOP (1:500 dilution, Proteintech, Wuhan, China) overnight at 4 °C. After being washed with TBST buffer for three times (every time 15 min), further incubation with the corresponding HRP (horse reddish peroxidase)-conjugated secondary antibody (1:1000 dilution, ZSGB-BIO, Beijing, China) for 1 h at 37 °C was conducted, and then washed with TBST buffer for another three times. The antibody–antigen complexes were detected using the DAB (3,3′-diaminobenzidine) chromogen and H_2O_2 substrate system. Optical density of the bands was quantified by Gel-Pro Analyzer 4 software. To calculate the protein signal, we subtracted background, normalized the value to β-actin. The data of each group were presented as a percentage of the control.

2.4. The proliferation-promoting effect of the EWAs in ERS-induced injured L02 cells

MTT assay was used to test the proliferation-promoting effect of the EWAs in ERS-induced injured L02 cells. L02 cells in exponential growth phase were harvested and plated at 6×10^3 cells per well in 96-well cell culture plates. After treatment with tunicamycin (60 μg/mL) for 24 h, cells were exposed to the EWAs (0.1, 0.2, 0.4, 0.8, 1.2 or 1.6 mg/mL) for 24 h. In addition, normal L02 cells were treated with the EWAs (1.6 mg/mL) for 24 h to test the toxicity of the EWAs in vitro. The survival rate was calculated as follows: survival rate (%) = $(A_{EWAs}/A_{control}) \times 100\%$.

2.5. The anti-apoptosis effect of the EWAs in ERS-induced injured L02 cells

TUNEL staining was carried out to observe the anti-apoptosis effect of the EWAs in ERS-induced injured L02 cells. Briefly, L02 cells were assigned to four groups: control group (treated with normal culture medium), model group (treated with 60 μg/mL tunicamycin for 24 h + normal culture medium for 24 h), EWAsL (treated with 60 μg/mL tunicamycin for 24 h + 0.4 mg/mL EWAs for 24 h), EWAsH (treated with 60 μg/mL tunicamycin for 24 h + 1.2 mg/mL EWAs for 24 h). Subsequently, the cells were fixed in 4% Paraformaldehyde solution. According to the manufacturer's instructions (Roche Inc., Basel, Switzerland), TUNEL staining was performed to detect apoptosis. Then cells were incubated with converter-POD buffer. DAB substrate was used for color detection. TUNEL-positive apoptotic cells exhibited brown nuclear staining, which were visualized and counted with microscopy on 100 magnifications. TUNEL-positive cells were

Table 1
The primers, product length and the annealing temperature of PERK, eIF2a, ATF4, CHOP and β-actin.

Gene	Primers Sequence		Product length	Annealing temperature
PERK	Forward	5′ TTGTCGCCAATGGGATAG 3′	291 bp	50 °C
	Reverse	5′ CAGTCAGCAACCGAAACC 3′		
eIF2a	Forward	5′ TCAGGGTGGTAAAGTATGT 3′	426 bp	50 °C
	Reverse	5′ GGATTGACTATGGTGGGT 3′		
ATF4	Forward	5′ CGATTCCAGCAAAGCACC 3′	219 bp	52.3 °C
	Reverse	5′ CATCCACAGCCAGCCATT 3′		
CHOP	Forward	5′ CACTCTTGACCCTGCTTC 3′	307 bp	54.3 °C
	Reverse	5′ AGTCGCCTCTACTTCCCT 3′		
β-actin	Forward	5′ CTCCATCCTGGCCTCGCTGT 3′	268 bp	55 °C
	Reverse	5′ GCTGTCACCTTCACCGTTCC 3′		