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Original article

Coelomic fluid of the earthworm *Eisenia fetida* induces apoptosis of HeLa cells in vitro

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Abstract

Earthworm, Dilong in Chinese, is a traditional Chinese medicine used to treat many diseases including tumors. To investigate whether the coelomic fluid of the earthworm *Eisenia fetida* inhibits viability and induces apoptosis of HeLa cells, MTT assay, AO/EB fluorescent staining method and DNA agarose electrophoresis were applied. The cytotoxicity of coelomic fluid on HeLa cell showed a concentration dependent manner after 48 h treatment as detected by the MTT assay. Coelomic fluid (1 mg/ml) exhibited toxic effects on HeLa cells with an inhibition rate of 84.22%, leading to cell lysis. The inhibition rates in 0.1 mg/ml and 0.01 mg/ml treatments were 10.24% and 2.99%, respectively. Morphological changes of typical apoptosis were observed by AO/EB staining in 0.1 mg/ml and 0.01 mg/ml concentration with apoptotic rates of 79.1% and 22.2%, while the cells were necrotic in 1 mg/ml concentration as indicated by red stained cells. The agarose gel electrophoresis of DNA revealed a smear pattern at the concentrations of 0.1 mg/ml and 0.01 mg/ml of coelomic fluid. At the concentration of 0.1 mg/ml, the coelomic fluid induced apoptosis in HeLa cells in a time dependent manner. This work suggests that some of the coelomic fluid components might be useful for pharmaceutical applications in the treatment of cancer, but our knowledge is still very limited. Testing this hypothesis will require intense controlled investigations.

Keywords: Earthworm; Coelomic fluid; HeLa cell; Apoptotic

1. Introduction

Earthworm, Dilong, is a cold, slightly salty traditional Chinese natural product. Li Shizhen illustrated 40 diseases in the book *Bencao Gangmu*, in which earthworms act as a main component. In clinics, earthworm can be used to treat chronic bronchitis, bronchial asthma, psychosis, digestive tract ulcer, peptic ulcer, epidemic parotitis, herpes zoster, urticaria, burn, scald, bladder calculi, urinating obstacle, and cancer [1–3]. Recently more and more researches focus on the main component investigation with antitumor activity from different parts of the earthworm. Several components with antitumor activity were found from the whole body tissue of earthworm including G-90, a mixture of different molecules with molecular weights of 33 kDa, 40 kDa, 42 kDa, 45 kDa and

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60 kDa [4–7]. Coelomocytes from the earthworm *Eisenia fetida* spontaneously killed tumor cells when cultivated for up to 3 h and mixed with K562 [8].

Earthworm coelomic fluid contains biologically active molecules and leukocytes that participate in phagocytosis, encapsulation and granulomas. Several proteins and peptides with antitumor and antibacterial activities have been found in coelomic fluid [9–13]. This research investigates the antitumor activity of coelomic fluid of the earthworm *E. fetida* and analyzes the mechanisms of the antitumor activity of coelomic fluid using in vitro different methods. These results and other similar ones that use natural products from animals [14–16] might help to settle a basic question for the pharmacological development of earthworm coelomic fluid in the future as an effective ancient medicine.

2. Materials and methods

2.1. Earthworms and preparation of coelomic fluid

Earthworms, *E. fetida*, were collected from a commercial company Tianjin Jia Liming Earthworm Farm Co. and fed with composted cow manure in a plastic container with a moisture of 60–70% and a temperature of 18–25 °C. The coelomic fluid of earthworm was collected following the method described earlier [17]. After centrifuged under 1000 rpm at 4 °C for 10 min, the supernatant was collected and freeze dried.

2.2. Reagents

DMEM is a product of HeClone, L-glutamine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RNase and proteinase K, ethidium bromide (EB) and acridine orange (AO) were purchased from Sigma Chemical Co. Fetal bovine serum (FBS) was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. Other reagents were all chemical grade products.

2.3. HeLa cells

HeLa cells were obtained from the Veterinary College of China Agricultural University and maintained in DMEM medium as described before [18].

2.4. MTT assay of cytotoxicity activity of coelomic fluid

The cells were seeded onto 96-well flat bottom microtiter plates (NUNC, Roskilde, Denmark) at a concentration of 1.5×10^4 cells/well. When the cells grow to monolayers the DMEM medium was then discarded and treated with 200 μl of 0 mg/ml, 1 mg/ml, 0.1 mg/ml and 0.01 mg/ml of coelomic fluid in DMEM medium. Each concentration was set up in three replications and each experiment was repeated three times. Forty-eight hours later, 20 μl MTT with a concentration of 4 mg/ml was added to each well. After the cells were incubated for an additional 4 h at 37 °C, supernatants were carefully removed and 100 μl dimethylsulfoxide (DMSO) was added. After insoluble crystals were completely dissolved, absorbance at 570 nm was obtained to determine the inhibition rate of coelomic fluid against HeLa cells. Inhibition rate = [(1 − mean OD of treated group/mean OD of untreated control) × 100%] [19].

2.5. Apoptosis assay

Log phase cells were seeded onto 24-well micro-titer plate at a concentration of 1.5×10^4 cells/well. After the cells grow to monolayers then the DMEM medium was discarded and the cells were treated with 200 μl of 0 mg/ml, 1 mg/ml, 0.1 mg/ml and 0.01 mg/ml of coelomic fluid dissolved in DMEM medium. Each concentration was set up in six replications and each experiment was repeated three times. Forty-eight hours later the cells were collected in order to run on AO/EB assays and DNA agarose gel electrophoresis assays.

2.5.1. AO/EB double staining assays

Four micro-litres of AO/EB (AO: 100 μg/ml; EB: 100 μg/ml in PBS) dye mixture was mixed with 100 μl treated and untreated cells of 1×10^6 cells/ml, and then 10 μl of cells were added onto the glass slides to test by fluorescence microscopy (Olympus, Japan) using epi-illuminisation and a filter combination suitable for observing fluorescein immediately with a magnification of ×100. For quantification, three different fields were counted under the microscope and at least 300 cells were enumerated in each field, and the number

Table 1
Inhibition rate of coelomic fluid on HeLa cells calculated after MTT staining

Concentration of coelomic fluid (mg/ml)	1	0.1	0.01
Inhibition rate (%)	84.22 ± 0.86 (<0.05%)	10.24 ± 1.03 (<0.05%)	2.99 ± 0.95

Cytotoxicity effect of coelomic fluid on HeLa cell was investigated in three different concentrations by MTT staining method. Groups (1 mg/ml and 0.1 mg/ml) were different from 0.01 mg/ml significantly with *P*-values less than 0.05.