Original Article

MCI extraction from Turkish galls played protective roles against X-ray-induced damage in AHH-1 cells

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Abstract: Objective: To investigate the protective effects of MCI extract from Turkish galls against apoptosis induced by X-ray radiation in the AHH-1. Methods: The cells were divided into: control group; X-ray radiation group; MCI group, in which the confluent cells were preincubated with 5 μg/ml MCI for 2 h followed by radiation. For the radiation, cells preincubated with MCI were exposed to X-ray beams with a dose of 8 Gy in total. Cell viability, apoptosis and intracellular alteration of redox were monitored by MTT and flow cytometry. Results: Compared with radiation group, the number of cells arrested at the G0/G1 phase was significantly reduced in MCI group \((P < 0.05)\). X-ray radiation induces remarkable apoptosis in AHH-1, which was reversed by MCI. Compared with the radiation group, the generation of intracellular reactive oxygen species (ROS) was abrogated by pre-incubation with MCI \((P < 0.05)\). In addition, the up-regulation of procaspase-3 induced by radiation was reversed by MCI. Radiation could induce up-regulation of Bax and down-regulation of Bcl-2; however, it is reversed completely after administration of MCI. Further, the enhanced expression of ERK and JNK induced by radiation was reversed by MCI. Conclusions: MCI extract from Turkish galls played protective effects on the X-ray induced damage through enhancing the scavenging activity of ROS, decreasing Bax/Bcl-2 ratio and the down-regulating the activity of procaspase-3, as well as modulating the mitogen-activated protein kinase (MAPK) signaling pathways.

Keywords: Turkish galls, radiation, apoptosis, MAPK signal pathway

Introduction

Radiotherapy has been commonly used in treating cancer with the capacity to kill cancer cells through ionizing radiation [1]. However, its effectiveness is usually limited by the potential injuries to normal tissue by ionizing radiation. Currently, extensive studies have been carried out to develop radiation-protective agents [2, 3]. Unfortunately, few agents have been approved by FDA for clinical practice. For example, WR-2721 (amifostine) was approved in clinical application by FDA in 1996, which contributed to the protection of normal tissue exposed to radiation [4]. Nevertheless, several side effects affected its efficiency in application. Recently, more and more attention have been paid to the natural drugs as these drugs are easily derived with low economical cost and potential anti-radiation effects combined with few side effects.

Turkish galls, the extract and fractions of Quercus lusitanic var. infectoria galls (Oliv.) as larvicidal agents, have been reported to show various biological properties, such as anti-fungal effects, anti-tumor activities [5]. Accumulating evidences reveal that several active components from Quercus lusitanic var. infectoria galls have been isolated, among which the most common type of component is Turkish gallotannin [6]. To date, the most acknowledged properties of Turkish gallotannin are focused on the oxidative stability as it could bind with the free radicals directly and involve in the removal of active oxygen and free radicals [7, 8]. On this basis, we speculate that Turkish gallotannin may play an important role in the anti-radiation, which may be further used in clinical practice.

In this study, radiation injury models were induced using human lymphoblast cells, designated AHH-1, through exposure to X-ray with a
Anti-apoptotic effects of MCI extract from Turkish galls in X-ray induced AHH-1 injury

On this basis, we investigate the potential protective roles of MCI extract from Turkish galls, and identify the potential mechanisms involved in this process.

Materials and methods

Materials

Turkish galls were purchased from the Hospital of Xinjiang Traditional Uyghur Medicine, and were identified by Professor Junping Hu from the Xinjiang Medical University. The reference substance was purchased from National Institutes for Food and Drug Control (Beijing, China) with an approval No. of 110831-200302.

Extraction of MCI

Dry herbs (5 kg) were extracted with ethanol as previously described [9]. The mixture was concentrated, and the residue was suspended in water, followed by extraction with ethyl acetate and ethylether. The ethyl acetate fraction was isolated by MCI chromatograph system (Sigma Aldrich, St. Louis, MO, USA). The structure of MCI was identified by HPLC with a chosen wave length of 760 nm. The purity of MCI was no less than 99%. Reference specimen was used as control.

Cell culture of AHH-1

Human AHH-1 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin at 37°C in an atmosphere of 5% CO₂. The medium were replaced every 24 h. The cells were passaged every 7 days until a confluence of 80%.

Experimental design

The cells in the exponential phase were divided into: normal control; irradiation group, which were subject to irradiation; and gallic acid group, which were subject to 50 μg/ml gallic acid for 2 h, followed by irradiation. Irradiation was performed using a Varian 2300 C/D linear accelerator, and the total irradiation dose was 8.0 Gy. The irradiation field was 25 cm×25 cm, which was 100 cm away from the radiation source.

Table 1. Effects of MCI extract on proliferation of AHH-1 cells

<table>
<thead>
<tr>
<th>Group</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>1.304 ± 0.053</td>
<td>1.258 ± 0.148</td>
<td>1.080 ± 0.049</td>
</tr>
<tr>
<td>Irradiation group</td>
<td>0.902 ± 0.049*</td>
<td>0.710 ± 0.124*</td>
<td>0.252 ± 0.094*</td>
</tr>
<tr>
<td>Gallic acid group</td>
<td>0.511 ± 0.075**</td>
<td>0.308 ± 0.027**</td>
<td>0.130 ± 0.032**</td>
</tr>
</tbody>
</table>

The data were OD values obtained at 570 nm. *P < 0.05, compared with control group; †P < 0.05, compared with irradiation group.

Figure 1. Effect of MCI extract on X-ray-induced apoptosis in AHH-1 cells. Flow cytometry was performed to observe the vitality of cells in the control group, radiation group, MCI extract group, respectively. Data are the means ± standard deviation (SD). **P < 0.01, vs control; ††P < 0.01, vs irradiation group.
phosphate buffered saline (PBS) twice. After centrifugation at 1000 rpm for 5 min, the pellet was collected and was suspended in MTT solution, followed by addition of DMSO. The fluorescence intensity was determined using microreader with a wavelength of the 570 nm.

**Cell arrest determination**

Cell cycle analysis was carried out with FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). The cells were randomly divided into: MCI extract group (5 µg/ml), negative control group, radiation group. Subsequently, the cells were washed with PBS (pH 7.4) and fixed with 70% ice-cold ethanol at 4°C overnight. Afterwards, the cells were stained with 20 µg/ml PI solution for 15 min at room temperature. The percentage of cells in G1, S and G2/M phase of the cell cycle was determined as previously described [10]. Data acquisition and analysis were performed by CellQuest Software.

**Production of ROS in cells**

The generation of ROS was determined using the DCFH-DA method using a flow cytometry according to the previous method. Briefly, AHH1 cells were collected and washed using PBS.

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**Figure 2.** Effect of MCI extract on X-ray-induced cell cycle arrest in AHH-1 cells. The cell cycle distribution was calculated in the control group, irradiation group, and MCI extract group by CellQuest software. Data are the means ± SD of three independent experiments. *P < 0.01, vs control; **P < 0.01, vs control.
Anti-apoptotic effects of MCI extract from Turkish galls in X-ray induced AHH-1 injury

After centrifugation at 1,000 rpm for 8 min, the pellet was resuspended in 10 μmol/L DCFH-DA, and incubated at 37°C for 30 min. The free DCFH-DA probe was removed by washing with serum-free DMEM medium for 3 times. Subsequently, the mixture was centrifuged at 1000 rpm for 8 min, followed by resuspending the pellet using PBS. The generation of ROS was displayed by the fluorescence intensity as revealed on the flow cytometry.

**Western blot analysis**

Protein extract from the AHH-1 cells was performed using 0.5 ml RIPA lysis buffer, followed by centrifugation at 13,200 rpm for 4°C for 15 min. Proteins (100 μg) were separated by electrophoresis on a 10% SDS-PAGE gel and transferred to a PVDF membrane. The concentration of protein was determined using BCA commercial kit purchased from Beyotime Co., Ltd (Shanghai, China). Afterwards, the membrane was blocked with 5% (w/v) non-fat dry milk and incubated with primary antibodies against Bcl-2 (1:200), Bax (1:200), ERK (1:200), JNK (1:200), p-ERK (1:200), p-JNK (1:200), and β-actin (1:3000, Fude Biological Technology, Hangzhou, China) overnight at 4°C. Then the mixture was incubated with horseradish peroxidase-conjugated specific secondary antibodies (1:5000, Fude Biological Technology, Hangzhou, China) for 1 h at room temperature. After washing with PBS, the bound primary antibody was visualized with the Enhanced Chemiluminescence System from Amersharm (Piscataway, NJ, USA) and exposed to film. The same membrane was probed for β-actin (Boster Corporation, Wuhan, China) for loading control. The relative density of PAR to β-actin was analyzed with the AlphaEaseFC software (Genetic Technologies, Inc. Miami, FL, USA).

**Statistical analysis**

All the data are presented as mean ± standard deviation. SPSS 13.0 software was used for the data analysis. *P* < 0.05 was considered statistically significant.

**Results**

**MCI extract contributed to elevated survival of AHH-1 after radiation**

The apoptosis rate of the cells in each group was summarized in Table 1 and Figure 1. Three types of cells were observed according to the Annexin V-FITC/PI staining, including living cells, cells underwent apoptosis or necrosis, respectively. Remarkable apoptosis was induced in radiation group compared with the control group at 24 h, 48 h and 72 h, respectively. In addition, MCI extract could significantly decrease the apoptosis compared with the radiation group at the 24 h, 48 h, and 72 h, respectively. All these indicated that MCI extract contributed to the elevation of survival rates of AHH-1 after radiation.

**MCI extract contributed to the relieve of G0/G1 phase arrest**

A large number of cells were arrested at the G0/G1 phase in the radiation group. In addition, significant decrease was observed in the DNA content of S phase in the radiation group compared with that of the control group (Figure 2). However, after interference of MCI extract, significant improvement was revealed in the
Anti-apoptotic effects of MCI extract from Turkish galls in X-ray induced AHH-1 injury

Figure 4. Effect of MCI extract on the expression of apoptosis-related proteins using Western blot analysis. A. Levels of apoptosis-targeted proteins, including procaspase-3, Bax and Bcl-2 in the control group, irradiation group and MCI extract group. B. Levels of apoptosis-targeted proteins, including ERK, p-ERK, JNK and p-JNK were examined using Western blot. The quantification of procaspase-3, Bax and Bcl-2 proteins was normalized to β-actin.
cell arrest of G0/G1 phase (P < 0.05). In addition, the DNA content of S phase was remarkably increased after MCI extract interference (P < 0.05). Compared with the radiation group, remarkable increase was revealed in the modulation of G2/M phase in MCI extract group. Taken together, it confirmed that MCI extract contributed to the repair of radiation injury through modulating the arresting of G0/G1 phase.

**MCI extract inhibited the generation of ROS after radiation**

After radiation, the generation of ROS in the radiation group was about 73-fold higher than that of control group. However, after interference of MCI, the generation of ROS was remarkably decreased compared with the radiation group (P < 0.01, Figure 3).

**MCI extract down-regulated the expression of apoptosis-related genes**

To investigate the effects of MCI extract on the expression of apoptosis related proteins, Western blot analysis was performed to determine the expression of procaspase-3, Bcl-2 and Bax, respectively. Compared with the control group down-regulation of procaspase-3 was induced by radiation (Figure 4A). In the MCI extract group, the radiation-induced down-regulation of procaspase-3 was reversed completely. For the expression of Bax, remarkable up-regulation was noticed after radiation, while in the MCI group, the up-regulation of Bax was attenuated. For the expression of Bcl-2, MCI extract could reverse the down-regulation of Bcl-2 induced by radiation. These results indicated that MCI extract may inhibit apoptosis through modulating the apoptosis-related genes.

**MCI extract inhibited the phosphorylation of ERK and JNK**

In this study, we determined the phosphorylation of proteins involved in the MAPK signaling pathway including ERK (p-ERK) and JNK (p-JNK). Western blotting analysis indicated significant increase was noticed in the phosphorylation of ERK and JNK after radiation compared with the control group (Figure 4B). Nevertheless, the phosphorylation of ERK and JNK was remarkably attenuated after treating with MCI extract.

**Discussion**

Extensive studies have been carried out to determine the effects of natural drugs on the anti-radiation fields [11, 12]. Recently, the radiation effects of these drugs have been mainly associated with the removal of free radicals, protection of immune system, and modulating of hematopoietic system and cytokines. In this study, we investigated the protective effects of MCI extract on radiation injury models induced using X-ray with a dose of 8 Gy.

Radiation induced DNA injury has been considered as the major cause for cellular apoptosis. For the cells undergoing double strand breaking, excessive accumulation of ROS has been identified, which is considered to be closely related to oxidative stress. According to our results, a large number of ROS was generated in the radiation group compared with the control. For the AH1-1 cells preincubated with MCI extract, a significant decrease was observed in the generation of intracellular ROS. This confirmed that MCI extract could induce protective effects on AH1-1 cells subject to radiation. For the mechanism, we speculated it might be highly related with the scavenging effects on oxygen radicals.

The susceptibility of cells to death signals is depended, in part, on the ratio between pro-apoptotic and anti-apoptotic Bax/Bcl-2 proteins [13, 14]. Bcl-2 contributed to the prevention of cytochrome c release and caspase activation, while Bax promoted the release of cytochrome c into the cytosol from mitochondria and activates caspase 3 [15]. For the cells preincubated with MCI extract before radiation, the ratio of Bax/Bcl-2 decreased compared with the cells underwent radiation, demonstrating it functioned in the apoptosis through inhibiting the X-ray mediated Bax/Bcl-2 imbalance. Meanwhile, MCI extract could reverse the decreased expression of procaspase-3 induced by X-ray radiation. These results indicated that MCI extract could inhibit X-ray induced apoptosis in AH1-1.

It has been well acknowledged that MAPK signaling pathway plays pivotal roles in the transmission of extracellular signals to nucleus [16]. Generally, the activation of ERK contributed to cell proliferation, while activation of JNK and/or p38MAPK played important roles in the regulation of cell death [17]. To date, no studies have
Anti-apoptotic effects of MCI extract from Turkish galls in X-ray induced AHH-1 injury

been conducted to investigate the mechanism of MCI extract involved in the inhibition of cellular apoptosis. In our study, the expression of p-JNK was up-regulated in the MCI extract group compared with the radiation group, which finally may lead to cell apoptosis. In addition, the increased expression of p-ERK may contribute to the cellular proliferation. Taken together, all these facts may contribute to the protective effects of MCI extract on the inhibition of radiation induced apoptosis in AHH-1.

In summary, our study indicated MCI extract protects the AHH-1 cells from radiation induced apoptosis by scavenging the intracellular ROS, decreasing Bax/Bcl-2 ratio and the down-regulating the activity of pro-caspase-3, as well as modulating the MAPK signaling pathways.

Acknowledgements

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Disclosure of conflict of interest

None.

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