

# Luteolin inhibits multi-heavy metal mixture-induced HL7702 cell apoptosis through downregulation of ROS-activated mitochondrial pathway

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**Abstract.** With the rapid economic development in recent years, China is facing a great challenge due to heavy metal pollution. The heavy metals may enter the human body through ingestion of aqua products to cause great health risks. In the present study, the inhibitory effects of luteolin on the combined toxicity of multi-heavy metals (including zinc, manganese, lead, copper, cadmium, mercury, chromium and nickel) were investigated in HL7702 hepatocyte cells. An MTT assay demonstrated that 20  $\mu$ M luteolin significantly alleviated the multi-heavy metal mixture-induced cell death and morphological changes. Furthermore, 20  $\mu$ M luteolin significantly inhibited multi-heavy metal mixture-induced reactive oxygen species (ROS) generation, lipid peroxidation (malondialdehyde content) and caused a decrease in adenosine triphosphate levels in HL7702 cells. A JC-1 staining assay indicated that 20  $\mu$ M luteolin inhibited the mitochondrial membrane potential-reducing effect of the multi-heavy metal mixture. Apoptotic assays revealed that the multi-heavy metal mixture induced HL7702 cell apoptosis in a dose-dependent manner, which was significantly inhibited by 20  $\mu$ M luteolin. Western blot

analysis indicated that addition of luteolin to the multi-heavy metal mixture significantly alleviated cytochrome *c* release from the mitochondria into the cytosol. In addition, 20  $\mu$ M luteolin had a significant inhibitory effect on multi-heavy metal mixture-induced cleavage of caspase-9, caspase-3 and poly(adenosine diphosphate-ribose) polymerase-1 protein. Immunofluorescence staining demonstrated that addition of luteolin significantly alleviated caspase-3 cleavage induced by the multi-heavy metal mixture. The present results suggested luteolin exerts its inhibitory effects of on multi-heavy metal mixture induced cell apoptosis through downregulation of the ROS-activated mitochondrial pathway.

## Introduction

Rapid industrialization in China had certain drawbacks, and it is now facing a great challenge in heavy metal pollution. Processes of mining, smelting, industrial production, pesticide application as well as oil and other fuel combustion will inevitably result in widespread heavy metal pollution. China's main streams are suffering from varying degrees of heavy metal pollution (1-6). The various heavy metal elements in contaminated water may be accumulated in aquatic weeds, plankton and aquatic animals and finally enter the human body through various branches of the food chain (7).

Ningbo, an eastern coastal city in China, has >7.8 million inhabitants. In this area, the preferred daily diet mostly comprises aquatic products, including fish, shrimp, crab and shellfish. It is noteworthy that in recent years, the coastal waters and aqua products in the Ningbo area have been suffering from multi-heavy metal pollution, which mainly includes pollution with zinc, manganese, lead, copper, cadmium, mercury, chromium and nickel (8-10). Long-term consumption of aqua products may result in the accumulation of heavy metals and further human health hazards.

Multiple heavy metal elements may enter the human body simultaneously and the combined toxicity is complex due to their interaction. While the toxic molecular mechanisms of different heavy metals are not identical, they have certain

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effects in common. First, most heavy metal ions freely pass through the cell membrane and directly react with intracellular organelles and biological macromolecules, interfering with the intracellular calcium homeostasis and transport system (11). Furthermore, most heavy metals cause excessive generation of reactive oxygen species (ROS) and lead to organelle and DNA damage (12-15). These common points may be useful in searching for an effective antidote to antagonize the combined toxicity induced by multiple heavy metals.

Luteolin, a common flavonoid, occurs in broccoli, carrots, celery, cauliflower and peppers. Studies have confirmed that luteolin possesses important pharmacological properties, including antimicrobial, antiviral, anti-allergic, antioxidant, anti-inflammatory and anticancer effects (16-21). Other studies demonstrated that luteolin has an inhibitory effect on the toxicity induced by heavy metals. Zhou *et al* (22) suggested that luteolin protected SH-SY5Y cells against zinc-induced, ROS-mediated apoptosis. Liu *et al* (23) reported that luteolin regulated the redox imbalance, preserved mitochondrial function and depressed the caspase family-associated apoptosis induced by copper. Excessive ROS generation is associated with mitochondrial damage, while luteolin was reported to protect mitochondrial function through depression of ROS generation (24). In addition to zinc and copper, most other heavy metals also induce excessive ROS generation (25-30). Therefore, luteolin may be a potential effective antidote to prevent the combined toxicity induced by multiple heavy metals.

Therefore, the inhibitory effects of luteolin on the combined toxicity induced in HL7702 cells by a multi-heavy metal mixture, including eight common contamination metals identified in aqua products in the Ningbo area, were assessed and the underlying molecular mechanisms were investigated.

## Materials and methods

**Materials and reagents.**  $(\text{CH}_3\text{COO})_2\text{Pb}\cdot 3\text{H}_2\text{O}$ ,  $\text{CdCl}_2\cdot 2.5\text{H}_2\text{O}$ ,  $\text{NiCl}_2\cdot 6\text{H}_2\text{O}$ ,  $\text{MnCl}_2\cdot 4\text{H}_2\text{O}$ ,  $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$ ,  $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$  and  $\text{K}_2\text{Cr}_2\text{O}_7$  (analytical grades, 99.0-99.8%) were all purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China).  $\text{CH}_3\text{ClHg}$  (analytical grade,  $\geq 99.0\%$ ) was purchased from Dr Ehrenstorfer GmbH (Augsburg, Germany). MTT was supplied by Amresco® (Solon, OH, USA). Luteolin (analytical grades,  $\geq 99.0\%$ ) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA) was from Sangon Biotech (Shanghai, China). The assay kits for the detection of lipid peroxidation (cat. no. S0131), ROS (cat. no. S0033), adenosine triphosphate (ATP; cat. no. S0026) and total protein were obtained from Beyotime Institute of Biotechnology (Shanghai, China). The mitochondrial membrane potential assay kit with JC-1 (cat. no. M8650) and the cell mitochondria isolation kit (cat. no. SM0020) were obtained from Solarbio Life Sciences (Beijing, China). The Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis kit (cat. no. V13241) was from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Antibodies B-cell lymphoma 2 (Bcl-2; cat. no. 2870), Bcl-2-associated X protein (Bax; cat. no. 2772), apoptotic protease activating factor 1 (Apaf1; cat. no. 8969), cleaved caspase-9 (cat. no. 7237), caspase-3 (cat. no. 9665), cleaved caspase-3 (Asp175; cat. no. 9661), cleaved PARP-1

Table I. Stock solution of the multi-heavy metal mixture prepared according to the proportions of daily consumption of each metal element through aqua products by an adult in the Ningbo area.

Heavy metal element	Concentration in stock solution (mg/l)	Consumption through aqua products by a 70 kg adult (mg/day)
Pb	1.042	0.012
Cd	2.085	0.024
Hg	0.174	0.002
Cu	48.820	0.562
Zn	167.047	1.923
Mn	27.016	0.311
Cr	6.428	0.074
Ni	4.691	0.054
Total	257.303	2.962

(Asp214; cat. no. 9544), used for western blot analysis in the present study were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Poly(adenosine diphosphate-ribose) polymerase-1 (PARP-1; cat. no. sc-1562), GAPDH (cat. no. sc-25778), cytochrome *c* (cat. no. sc-13561), pro-caspase-9 (cat. no. sc-7885) were all purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Goat anti-mouse IgG (cat. no. BA1050) and goat anti-rabbit IgG (cat. no. BA1054) were provided by the Boster Biological Technology (Wuhan, China). The HL7702 cell line was received from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China).

**Preparation of heavy metal mixture.** The multi-heavy metal mixture, which included copper, mercury, cadmium, zinc, lead, manganese, nickel and chromium was prepared according to the proportions of daily intake of each metal through aqua product consumption by an adult in the Ningbo area (Table I) (10,31). The sum of the concentrations of the eight heavy metal elements was used as the final concentration of the multi-heavy metal mixture.

**Cell culture.** HL7702 hepatocyte cells were maintained in RPMI-1640 medium (cat. no. SH30809.01; GE Healthcare Life Sciences, Little Chalfont, UK) containing 1% penicillin-streptomycin and 10% fetal bovine serum from Tianhang Biological Technology (Huzhou, China) under standard culture conditions (37°C; 95% humidified air and 5% CO<sub>2</sub>).

**MTT assay.** HL7702 cells were seeded into a 96-well plate at a density of 10,000 cells/well in 200  $\mu\text{l}$  medium and cultured for 48 h. The cells were then treated with multi-heavy metal mixture (concentrations of 0, 16.73, 19.30, 21.87, 24.44 or 27.01 mg/l) with or without luteolin (20  $\mu\text{M}$ ). After 12 h of incubation, 10  $\mu\text{l}$  MTT solution (3.5 mg/ml) mixed with 90  $\mu\text{l}$  phenol red-free culture medium was added into each well, followed by further incubation in the dark for 4 h. After the culture medium was discarded, 150  $\mu\text{l}$  dimethyl sulfoxide was added to each well. The plate was incubated on an incubator

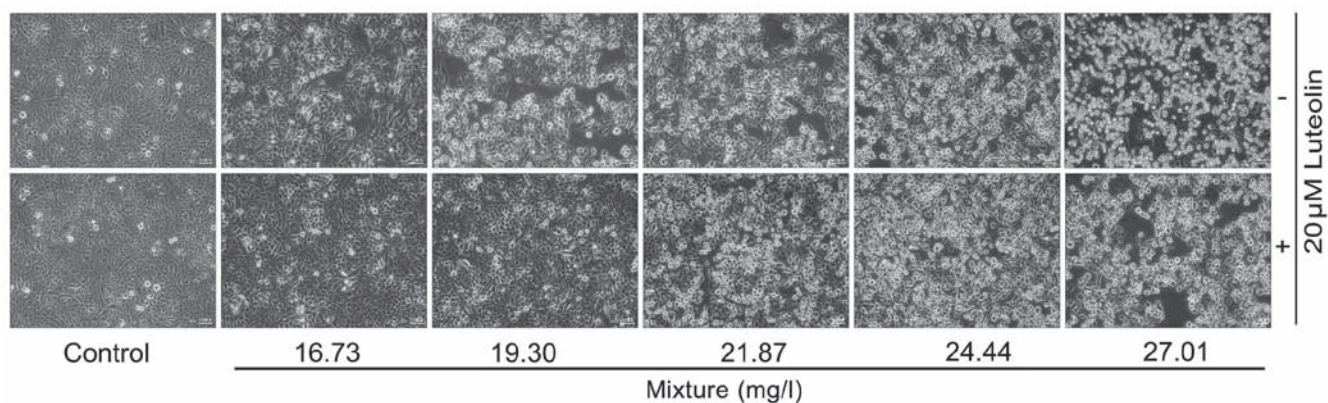


Figure 1. Morphological changes of HL7702 hepatocyte cells after treatment with multi-heavy metal mixture alone or in combination with luteolin. Magnification, x40. Mixture, multi-heavy metal mixture.

shaker at room temperature for 15 min. The optical density was measured at a wavelength of 492 nm using a microplate reader.

**Lipid peroxidation detection.** HL7702 cells were seeded into a 6-well plate at a density of 250,000 cells/well in 2.5 ml medium and cultured for 48 h. The cells were then treated with multi-heavy metal mixture (0, 16.73, 19.30, 21.87, 24.44 or 27.01 mg/l) with or without luteolin (20  $\mu$ M). After 12 h of incubation, the cells were harvested and then lysed by adding 120  $\mu$ l Nonidet P-40 lysis buffer to each well. The lysate was centrifuged at 1,200 x g for 15 min at 4°C. The protein concentration was determined using the bicinchoninic acid method. Supernatant (100  $\mu$ l) was mixed with 200  $\mu$ l malondialdehyde (MDA) detection buffer and then boiled for 15 min. After cooling to room temperature, the mixture was centrifuged at 1,000 x g for 15 min. The supernatant was then transferred into a 96-well plate (200  $\mu$ l/well) and the amount of lipid peroxidation was detected at a wavelength of 532 nm by a microplate reader. The MDA levels were normalized to the protein concentration.

**ROS detection.** Intracellular ROS levels were determined using a ROS detection kit. HL7702 cells were treated with multi-heavy metal mixture with or without luteolin as for the MTT assay in a black 96-well plate. The cells were then gently washed 2 times with serum-free medium. The cells were then incubated with 200  $\mu$ l serum-free medium containing 10  $\mu$ M H<sub>2</sub>DCFDA at 37°C for 35 min. The fluorescence distribution of each well was detected by a fluorospectrophotometer (excitation wavelength, 488 nm; emission wavelength, 535 nm).

**ATP detection.** HL7702 cells were treated with multi-heavy metal mixture and luteolin as for the lipid peroxidation assay. Subsequently, they were gently washed 2 times with 4°C sterile PBS and lysed by adding 200  $\mu$ l Nonidet P-40 lysis buffer. The lysate was centrifuged at 12,000 x g for 5 min at 4°C. A 20- $\mu$ l aliquot of the supernatant was transferred into a dedicated centrifuge tube containing 100  $\mu$ l ATP detection buffer at room temperature. ATP standard solutions (0, 0.5, 1, 5, 10, 50 and 100  $\mu$ M) were used to obtain a standard curve. ATP levels were detected by measurement of the relative light unit value with a luminometer and were normalized to the protein concentration.

**JC-1 staining assay.** HL7702 cells were treated with multi-heavy metal mixture with or without luteolin as for the MTT assay in a black 96-well plate. Subsequently, 100  $\mu$ l phenol red-free culture medium mixed with 100  $\mu$ l JC-1 staining solution was added into each well and further incubated in the dark at 37°C for 20 min. After gently washing the cells 2 times with 200  $\mu$ l JC-1 staining wash buffer (4°C), 200  $\mu$ l phenol red-free culture medium was added into each well. The fluorescence of each well was observed under a fluorescence microscope and images were captured.

**Apoptosis detection.** Apoptosis was detected using the Alexa Fluor<sup>®</sup> 488 Annexin V/Dead Cell Apoptosis kit. HL7702 cells were treated with multi-heavy metal mixture and luteolin as for the lipid peroxidation assay. The cells were then harvested by EDTA-free trypsinization provided by Thermo Fisher Scientific, Inc. Following centrifugation at 67 x g for 5 min at room temperature, the cells were resuspended in 500  $\mu$ l Annexin binding buffer containing 1  $\mu$ l propidium iodide dye and 5  $\mu$ l Alexa Fluor<sup>®</sup> 488-conjugated Annexin V. The single-cell suspension was further incubated in the dark for 30 min and apoptosis was then monitored by flow cytometry.

**Western blot analysis.** HL7702 cells were seeded into 20x100 mm culture dishes at a density of 1,200,000 cells/dish in 10 ml medium and cultured for 48 h. The cells were then treated as described above and then gently washed 2 times with PBS at 4°C.

For whole-cell protein preparation, 500  $\mu$ l Nonidet P-40 lysis buffer containing 10  $\mu$ M phenylmethane sulfonyl fluoride was added into each dish to lyse cells on ice for 10 min. The lysate was filled into Eppendorf tubes and centrifuged at 15,000 x g for 3 min at 4°C. Of the supernatant, 350  $\mu$ l was used for whole-cell protein detection.

To prepare cytosolic protein, a cell mitochondria isolation kit was used according to manufacturer's instructions and 80  $\mu$ l supernatant was used for the protein detection.

Protein concentrations were determined by the BCA method. Supernatant was mixed with 5X loading buffer (4:1 in volume) and boiled for 5 min.

Polyacrylamide stacking (6%) gels and resolving (10%) gels were used to separate proteins of different molecular weights. Then the proteins were transferred onto PVDF membranes,



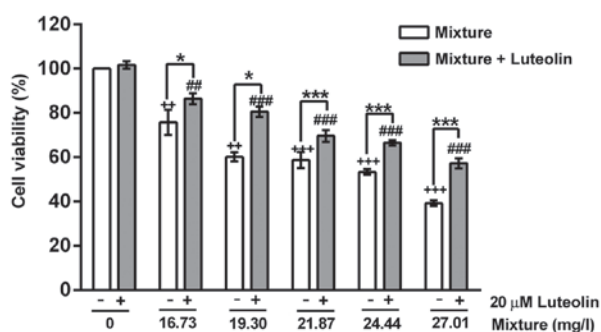


Figure 2. Viability of cells after treatment with multi-heavy metal mixture alone or in combination with luteolin. \* $P < 0.05$ , \*\*\* $P < 0.001$ , mixture alone compared with mixture + luteolin; \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , compared with control - (without any treatment); ## $P < 0.01$ , ### $P < 0.001$ , compared with control + 20  $\mu\text{M}$  luteolin. Mixture, multi-heavy metal mixture.

blocked in 5% skimmed milk for 3 h at room temperature and incubated for 12 h at 4°C with primary antibodies. After that, membranes were probed with secondary antibodies at room temperature for 1.5 h. Immunoblotting was performed for Bcl-2, Bax, Apaf1, cytochrome *c*, pro-caspase-9, cleaved caspase-9, caspase-3, cleaved caspase-3 (Asp175), PARP-1, cleaved PARP-1 (Asp214) and GAPDH at 1:1,000 dilution. The Tanon imaging processing system (Tanon Science and Technology Co., Ltd., Shanghai, China) was used for processing and evaluation of the western blots by ECL solution.

**Immunofluorescence staining for cleaved caspase-3.** HL7702 cells ( $n=50,000$ ) were seeded onto a glass coverslip and maintained under standard culture conditions for 48 h. The cells were treated with multi-heavy metal mixture and luteolin as described above. The glass coverslips were successively dipped in PBS (5 min, 2 times), 4% paraformaldehyde (10 min), PBS containing Tween-20 (PBST; 5 min, 2 times), 0.1% Triton X-100 (10 min), PBST (5 min, 2 times), 1% BSA-TBST blocking buffer (2 h), 1% BSA-TBST solution containing cleaved caspase-3 rabbit antibody (2 h; 1:1,000 dilution at room temperature), PBST (10 min, 2 times), 1% BSA-TBST solution containing goat-anti-rabbit antibody marked with Alexa Fluor® 488 (2 h; 1:2,000 dilution at room temperature) and PBST (10 min, 2 times). The cell nuclei were stained with 10  $\mu\text{M}$  DAPI. The immunofluorescence staining of cleaved caspase-3 was captured by a fluorescence microscope.

**Statistical analysis.** Each experiment was performed three or more times. Differences within groups were analyzed using one-way analysis of variance and a post hoc least significant difference test with SPSS 13.0 (SPSS, Inc., Chicago, IL, USA).  $P \leq 0.05$  was considered to indicate a statistically significant difference.

## Results

**Cell viability and morphological changes.** Treatment with multi-heavy metal mixture alone significantly reduced the cell viability and induced cell morphological changes in a dose-dependent manner, while addition of 20  $\mu\text{M}$  luteolin significantly inhibited these toxic effects induced by the multi-heavy metal mixture (Figs. 1 and 2).

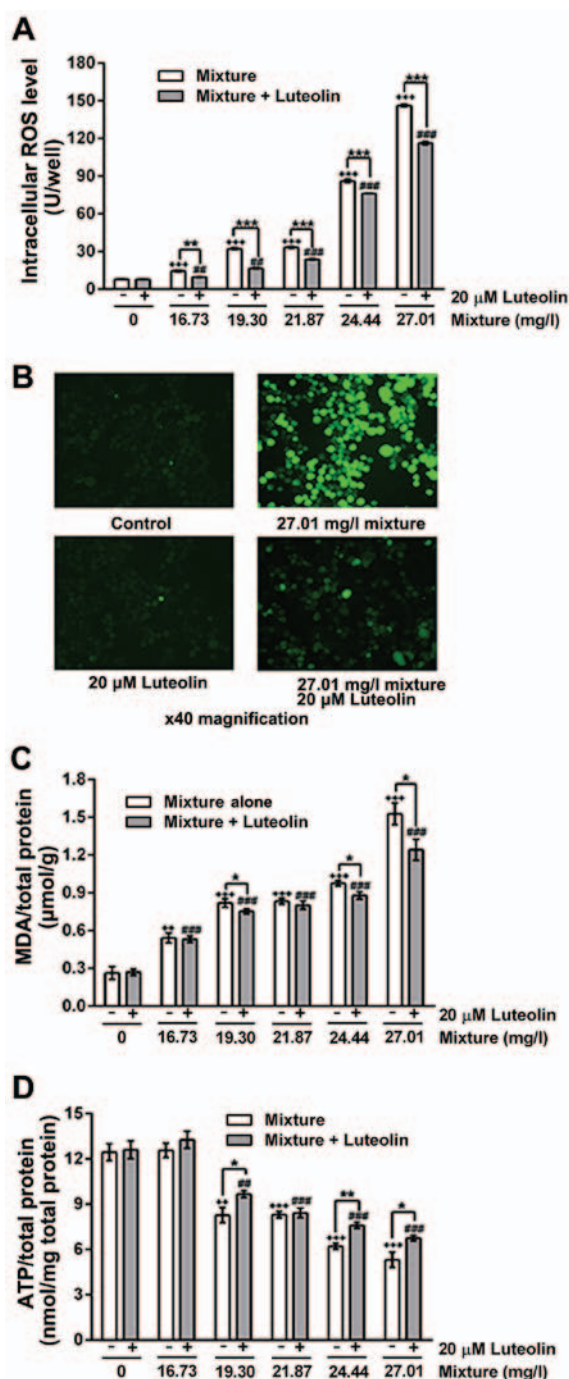


Figure 3. (A) ROS levels, (B) immunofluorescence images of ROS (magnification, x40), (C) MDA content and (D) ATP levels of cells treated with multi-heavy metal mixture alone or in combination with luteolin. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , mixture alone compared with mixture + luteolin; \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , compared with control - (without any treatment); ## $P < 0.01$ , ### $P < 0.001$ , compared with control + 20  $\mu\text{M}$  luteolin. Mixture, multi-heavy metal mixture; ROS, reactive oxygen species; MDA malondialdehyde; ATP, adenosine triphosphate.

**ROS, lipid peroxidation and ATP level changes.** The multi-heavy metal mixture induced intracellular ROS generation (Fig. 3A and B) and lipid peroxidation (Fig. 3C) in a dose-dependent manner, and decreased the intracellular ATP content (Fig. 3D). Addition of 20  $\mu\text{M}$  luteolin significantly inhibited the multi-heavy metal mixture-induced ROS generation and lipid peroxidation as well as the decrease of intracellular ATP.

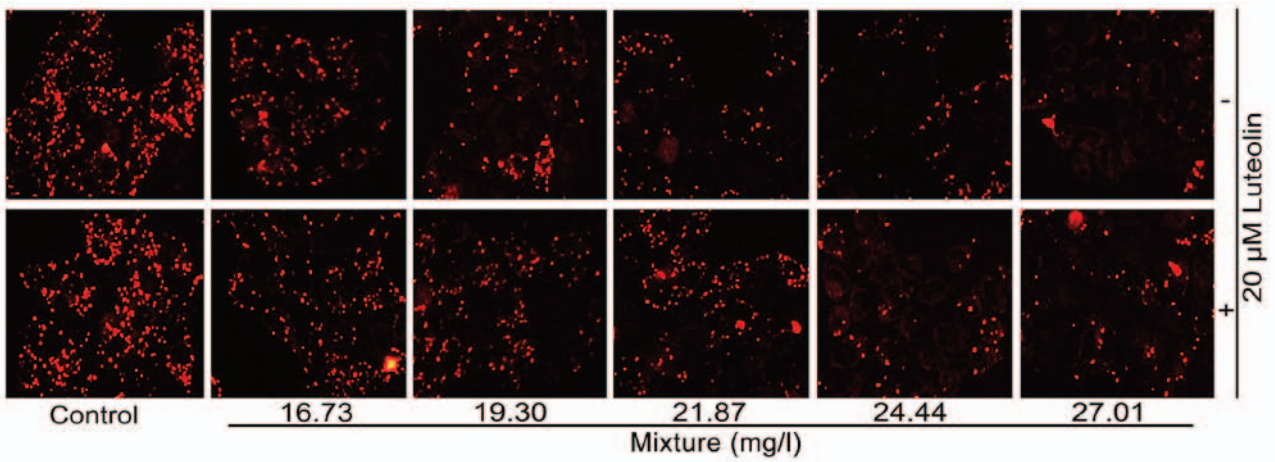


Figure 4. Mitochondrial membrane potential changes after cells were treated with multi-heavy metal mixture alone or in combination with luteolin. Mitochondria with high potential could be stained and vice versa. Magnification, x400. Mixture, multi-heavy metal mixture.

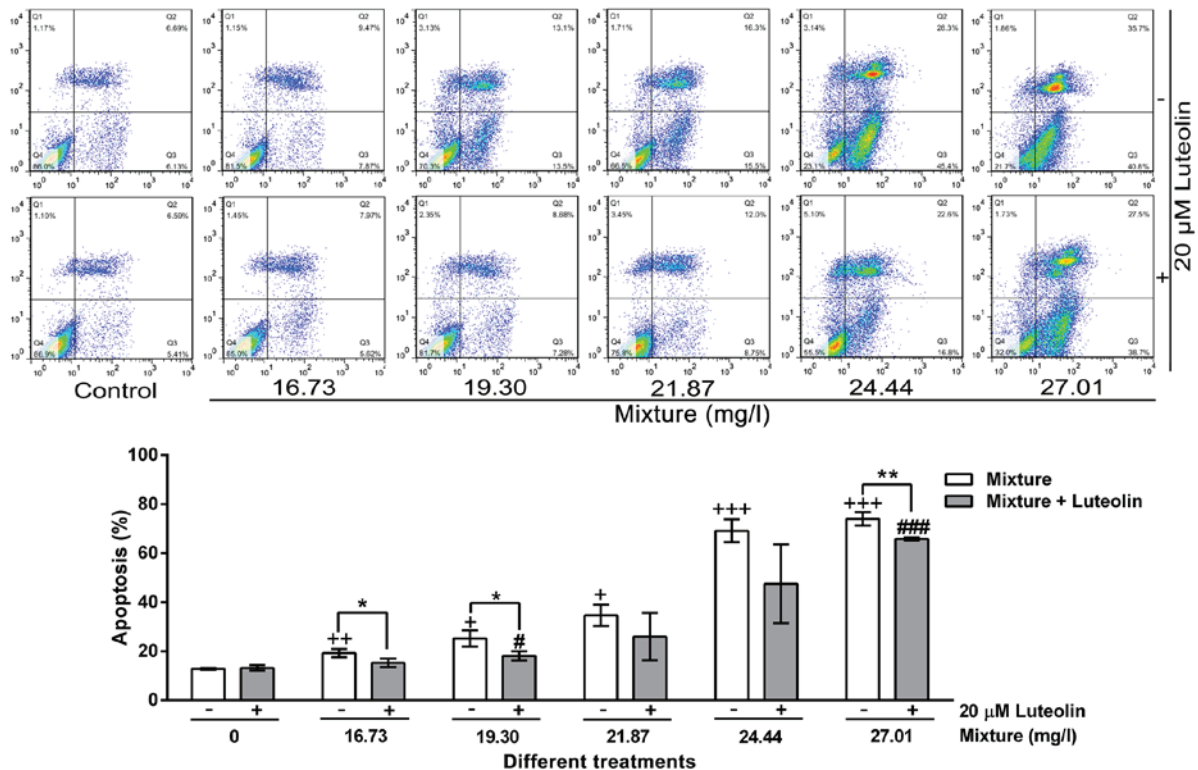


Figure 5. Apoptosis analysis after HL7702 cells were treated with multi-heavy metal mixture alone or in combination with luteolin. Cells were analyzed by flow cytometry after double-staining with Alexa Fluor® 488 Annexin V (x-axis) and propidium iodide (y-axis). \*P<0.05, \*\*P<0.01, mixture alone compared with mixture + luteolin; +P<0.05, ++P<0.01, +++P<0.001, compared with control - (without any treatment); #P<0.05, ###P<0.001, compared with control + 20 μM luteolin. Mixture, multi-heavy metal mixture; Q, quadrant.

**Mitochondrial membrane potential changes.** With the increase in the multi-heavy metal mixture concentration, the mitochondrial membrane potential decreased gradually, while 20 μM luteolin attenuated these changes (Fig. 4).

**Apoptotic analysis.** Multi-heavy metal mixture induced HL7702 cell apoptosis in a dose-dependent manner, while 20 μM luteolin inhibited this effect (Fig. 5).

**Effects on mitochondrial apoptosis-associated signaling proteins.** Addition of 20 μM luteolin inhibited the multi-heavy

metal mixture-induced increase of the Bax/Bcl-2 ratio in the cells. Furthermore, 20 μM luteolin significantly inhibited the multi-heavy metal mixture-induced increases of cleaved caspase-9, cleaved caspase-3 and cleaved PARP-1 protein. In addition, 20 μM luteolin significantly alleviated the multi-heavy metal mixture-induced cytochrome *c* release from the mitochondria into the cytosol (Fig. 6).

**Immunofluorescence staining results in cleaved caspase-3.** Immunofluorescence staining revealed that at higher doses, multi-heavy metal mixture treatment alone induced significant

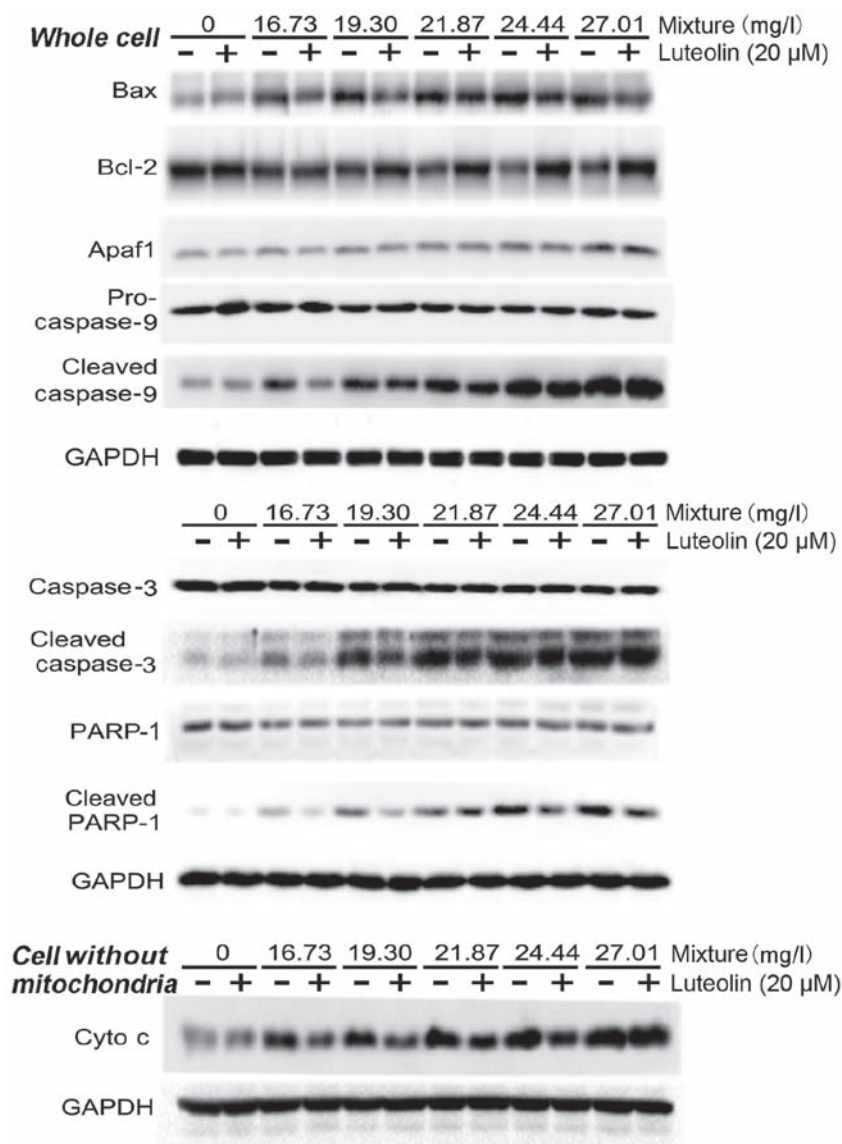


Figure 6. Mitochondrial apoptosis-associated signaling protein expression after HL7702 cells were treated with multi-heavy metal mixture alone or combined with luteolin. Mixture, multi-heavy metal mixture; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; Cyto c, cytochrome c; PARP-1, poly(adenosine diphosphate-ribose) polymerase-1; Apaf1, apoptotic protease activating factor 1.

caspase-3 cleavage (green color) in HL7702 cells, and addition of 20  $\mu$ M luteolin inhibited this effect (Fig. 7).

## Discussion

The liver is an important multifunctional organ performing detoxification and metabolism of xenobiotics (32), substance synthesis and metabolic balancing of nutrients. In a previous study by our group, a MTT assay was used to screen various antioxidant chemicals for their protective effects and identified that 20  $\mu$ M luteolin significantly inhibited the cytotoxic effects if multi-heavy metal mixture in HL7702 cells. Therefore, HL7702 hepatocytes were used in the present study to investigate the combined toxicity of multi-heavy metal mixture and the inhibitory effects of luteolin as well as the underlying molecular mechanisms.

Normally, ROS generation and quenching are in a dynamic balance state due to the intracellular antioxidant system. Certain harmful extracellular factors may break this balance,

resulting in excessive ROS generation beyond the cell scavenging ability to then induce organelle damage, abnormal expression of proteins or eventually cell death (33-35). Studies have demonstrated that most heavy metal ions cause excessive intracellular ROS generation (12,22,36,37). The present results indicated that the multi-heavy metal mixture induced intracellular ROS generation in a dose-dependent manner, while the antioxidant luteolin had a significant quenching effect on this ROS generation. Lipid peroxidation is another indicator of cell damage from oxidative stress. Excessive ROS released from the mitochondria into the cytosol may induce cellular lipid peroxidation (38). The cellular MDA content is widely used as an index of lipid peroxidation levels. In the present study, luteolin was demonstrated to significantly prevent multi-heavy metal mixture-induced lipid peroxidation.

As an important energy molecule, ATP participates in most intracellular biogenic activities. The intracellular ATP levels decline once cells undergo apoptosis, necrosis or encounter adverse factors. The ATP detection results of the



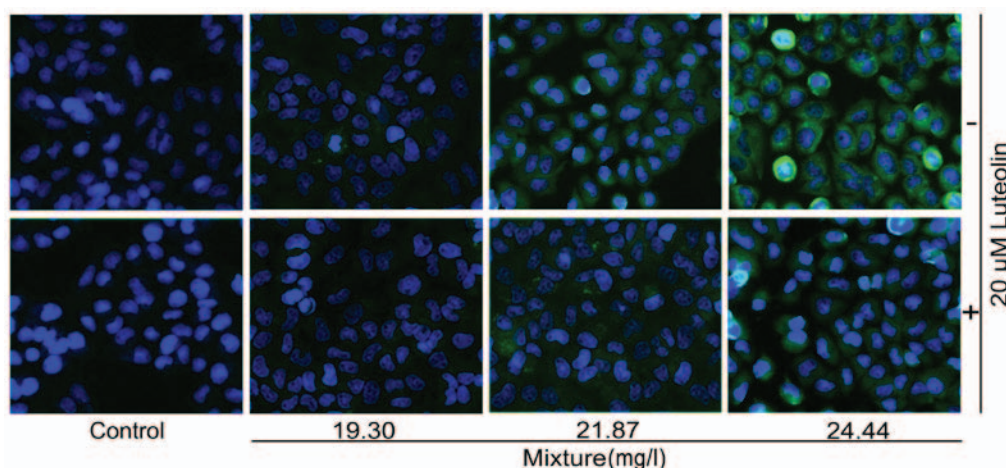


Figure 7. Cleaved caspase-3 staining (green) of HL7702 cells treated with multi-heavy metal mixture alone or combined with luteolin. Cell nuclei were stained with DAPI (blue). Magnification, x400. Mixture, multi-heavy metal mixture.

present study suggested that 20  $\mu\text{M}$  luteolin significantly inhibited the multi-heavy metal mixture-induced effect on decreasing ATP levels. Excessive ROS and decreased ATP levels indicate impaired mitochondrial function (39-42). Mitochondrial JC-1 staining may be used to detect changes in the mitochondrial membrane potential. Under normal conditions, the JC-1 monomer aggregates in the mitochondrial matrix to form polymer J-aggregates (red color). Once the mitochondrial membrane potential decreases, JC-1 monomer cannot aggregate in the mitochondrial matrix, which results in less or no polymer J-aggregate formation (less or no red color). With increasing multi-heavy metal mixture concentration, the mitochondrial membrane potential gradually decreased, while 20  $\mu\text{M}$  luteolin significantly attenuated this change.

The simultaneous decrease of intracellular ATP levels and mitochondrial membrane potential often accompanies cell apoptosis. Apoptosis is an initiative action to implement programmed cell death (43), which is strictly controlled by multiple genes, including the protein families of Bcl-2 and caspases, as well as the c-myc oncogene and P53 tumor suppressor gene (44-46). The death receptor pathways, including membrane receptor, cytochrome *c* and caspase pathways, may be activated by a series of physiological and pathological signals (47,48). In the present study, apoptosis was monitored by flow cytometry. The results indicated that the multi-heavy metal mixture induced HL7702 cell apoptosis in a dose-dependent manner, which was significantly inhibited by 20  $\mu\text{M}$  luteolin.

Bcl-2 and Bax are proteins belonging to the Bcl-2 family and control the mitochondrial membrane permeability to regulate the release of cytochrome *c* (45). Bax upregulates the permeability of the mitochondrial membrane accompanied by the release of cytochrome *c* from the mitochondria into the cytosol, while Bcl-2 has the opposite role (45,49). Cytochrome *c* in the cytosol activates caspase family proteins and forms the cytochrome *c*/Apaf1/caspase-9 apoptosome, which then leads to apoptosis (50). In the present study, mitochondrial apoptosis pathway-associated signal protein expression was detected by western blot analysis. The results suggest that treatment with the multi-heavy metal mixture led to a significant upregulation of the Bax/Bcl-2 ratio, as well as the levels of Apaf1, and cleavage

of caspase-9, caspase-3 and PARP-1. The immunofluorescence staining results in the intact HL7702 cells confirmed that the mitochondrial apoptosis pathway was activated (positive staining for cleaved caspase-3). Furthermore, these results also suggested that 20  $\mu\text{M}$  luteolin attenuated multi-heavy metal mixture-induced changes in signaling proteins of mitochondrial apoptosis pathways.

Therefore, the potential underlying molecular mechanisms of multi-heavy metal mixture-induced cytotoxicity may be summarized as follows: At first, the heavy metal ions enter the cells and induce intracellular ROS generation and mitochondrial damage. Subsequently, the permeability of the mitochondrial membrane is upregulated by the Bax protein, which leads to mitochondrial cytochrome *c* release from the mitochondria into the cytosol and subsequent formation of the apoptosome. The apoptosome initiates the cascades of caspase-3 and PARP-1 cleavage, and eventually cell apoptosis. Luteolin inhibited multi-heavy metal mixture-induced apoptosis by quenching the excessive ROS and further by blocking the oxidative stress-mediated mitochondrial apoptosis pathway.

In conclusion, the present study demonstrated that the multi-heavy metal mixture containing eight common metals prepared according to the proportions in which daily intake of each metal occurs through aqua product consumption by an adult in the Ningbo area induced oxidative stress injury and mitochondrial damage in HL7702 cells. Luteolin protected HL7702 cells from multi-heavy metal mixture-induced toxicity through downregulation of the ROS-mediated mitochondrial apoptosis pathway. Luteolin may be beneficial to prevent the multi-heavy metal pollution-induced health hazards arising from long-term aqua product consumption. However, the inhibitory effect of luteolin on the combined toxicity of multi-heavy metals was only evaluated by *in vitro* experiments in the present study. A further *in vivo* study will be required to verify the above *in vitro* experimental results.

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