ORIGINAL ARTICLE

Establishing a methodology to study the influence of quercetin on the metabolism of the K562/ADM cell line using nuclear magnetic resonance technology

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The purpose of the present study was to establish an experimental method to study the effects of quercetin on the metabolism of the drug-resistant leukemia cell line K562/ADM using nuclear magnetic resonance (NMR) technology. Drug administration to the cells was performed according to proper experimental design and application. The cells were collected and inactivated by repeated freeze-thaw cycles using liquid nitrogen. Methanol (80%) was used to extract cell metabolites, and a sample concentrator (Termovap, ECOM, Czech Republic) was used to purify the samples. All samples were assessed using 500 MHz NMR (Bruker, Germany) with standard NOESY impulse sequence to obtain ^{1}H-NMR spectra of cell metabolites. The processing method used to assess cell metabolism established in the present study was convenient and led to minimal loss of cell metabolites. The ^{1}H-NMR spectra obtained using NMR technology reliably reflected changes in cell metabolism.

Key Words: *K562/ADM; Metabolism; Methodology; Quercetin*

Metabonomics was initially defined by Nicholson et al (1) in 1999 and subsequently developed into a discipline that quantitatively describes the endogenous metabolites of an organism and its changes (2). Metabonomics research is based on biological fluids, cell extractions, cell culture fluid and tissues, and often use HPLC, GC, MS, NMR, IR and other technologies for detection (3). Quercetin is a natural polyhydroxy flavonoid that commonly exists in fruit, vegetables and other natural plants. It has general pharmacological action, such as antitumour, antioxidant, anti-inflammatory and antithrombotic properties (5,6), and can reverse the multiple drug resistance of leukemia cells (7,8). Metabolites, which are the material foundation of discovering and identifying biomarkers, play an important role in studying cell metabolism. However, to obtain integral metabolites and comprehensive metabolite profiling analyses, optimal extraction methods are needed. The present study established a convenient methodology for isolating metabolites based on researching the influence of quercetin on the metabolism of the drug-resistant leukemia cell line K562/ADM.

Material, instrumentation and supplier summary

SW-CJ-IFD clean bench (Sujing Group, Suzhou Antai Air Technology Co, Ltd); SC-04 low speed centrifuge (Anhui Zhongke Zhongjia Science Instrument Co, Ltd); TS100 inverted fluorescence microscope (Nikon, edipse); constant temperature carbon dioxide incubator (Thermo Scientific); SB5200D ultrasonic cleaner (Ningbo Xinzhi Biological Polytron Technologies Inc); ACCULAB one over ten-thousand analytical balance (Beijing Sartorius Instrument System Co, Ltd); LS-50HJ vertical pressure steam sterilizer (Jiangyin Binjiang Medical Equipment Co, Ltd); 500 MHz NMR (Bruker, Germany); cell culture flask (America, Corning); dimethyl sulfoxide (Beijing PuBoXin biotechnology co, Ltd); modified RPMI-1640 culture medium (Gibco); Sijiqing fetal bovine serum (Zhejiang Tianhang Biological Technology Co, Ltd); phosphate-buffered saline (Thermo Fisher biochemical products Beijing Co, Ltd, Beijing, China); penicillin and streptomycin solution (100×, Beyotime Institute of Biotechnology); D_2O, 3-(trimethylsilyl)propionic acid sodium salt (Shenzhen, Merrill Chemical Technology Co, Ltd); quercetin API (lot number 2013080601, content ≥97.0%, Guangzhou Aichun Pharmaceutical Science and Technology Co, Ltd); K562/ADM cell line (Nanjing Kaiji Biological Technology Development Co, Ltd).

METHODS

Cell culture

Cells (K562/ADM) were removed from liquid nitrogen storage and quickly placed in a water bath at 37°C for thawing, and subsequently transferred to a centrifuge tube for centrifuging at 1000 rpm for 3 min. The supernatant was removed, and 10% fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin RPMI culture solution was added to the resuspended cells. K562/ADM cells were transferred to a cell culture flask and mixed using a pipette, then incubated at 37°C with 5% CO_2 at a density of 5×10^4 cells/mL to 7×10^4 cells/mL. Recovered cells were transferred into centrifuge tube and centrifuged at 1000 rpm for 5 min. Following removal of the supernatent, the cells were resuspended in fresh medium and transferred into a culture flask followed by incubation at 37°C with 5% CO_2.

Cell passage

Cell passage was performed when cell density reached ≥80%. Cells were transferred to a centrifuge tube at 1000 rpm for 5 min and the supernatent was discarded. Next, the cells were resuspended and washed with phosphate-buffered saline (PBS) solution one to three times. Cells were resuspended in fresh culture medium and transferred into a culture flask at a density of 5×10^4 cells/mL to 7×10^4 cells/mL, then incubated at 37°C with 5% CO_2.

Cell cryopreservation

Cell cultures that exhibited good growth were collected in the logarithmic growth phase, centrifuged and counted before cryopreservation . Cell density needed to be controlled at 5×10^5 cells/mL to 6×10^5 cells/mL.

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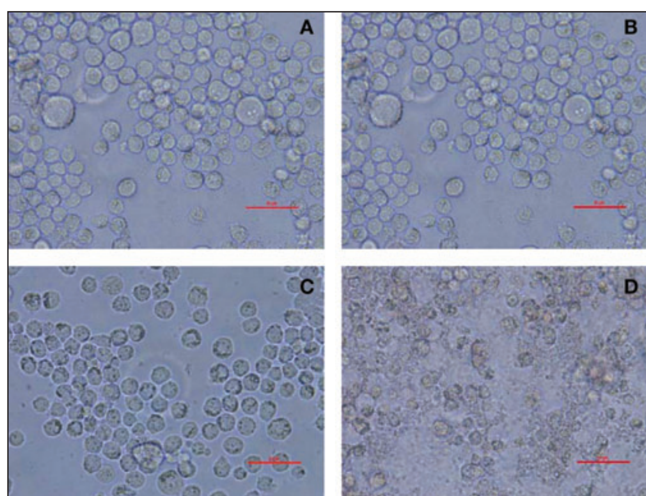

Figure 1) *Morphology of K562/ADM cells (original magnification ×400).* **A** *Blank control group.* **B** *Low-dose group.* **C** *Medium-dose group.* **D** *High-dose group*

Next, cells were placed in cell freezing medium (1:9 ratio of DMSO:FBS) and blended; the solution was then stored at 4°C, −20°C, −80°C for 10 min, 30 min and 16 h to 18 h, respectively. All samples were ultimately placed in liquid nitrogen.

Cell experiment

K562/ADM cells in the logarithmic growth phase were collected, counted and transferred to a 75 cm^2 cell culture flask at a density of 1×10^5 cells/mL. Samples were divided into four groups according to the final concentration of quercetin used in the experiments: blank control (culture solution with 1:1000 DMSO); low dose (10 µmol/L); medium dose (20 µmol/L); and high dose (40 µmol/L). All of the above were incubated at 37°C with 5% CO_2.

Cell collection and processing

After 72 h in culture, the cells were centrifuged at 1000 rpm for 5 min and then collected after washing with PBS three times followed by flash freezing in liquid nitrogen. The cells underwent three freeze-thaw cycles before NMR treatment. Two millilitres of 80% methanol was added to the samples and blended, and ultrasonication (3 s on/2 s off) was performed while the samples were on an ice bath for 10 min, followed by centrifugation at 12,000 rpm for 15 min. The supernatant was collected and 1 mL 80% methanol was added to the residue. This treatment was repeated twice. The supernatants were then combined and dried using a nitrogen blow down evaporator.

Collection and processing of cell culture fluid

Cells were centrifuged after culture for 72 h and the supernatant was collected, of which 3 mL was obtained for filtering using a 0.22 µm filter (Waters, USA). Subsequently, centrifugation was performed at 12,000 rpm for 20 min followed by storage in liquid nitrogen. Solvent was removed under vacuum and the residue was freeze-dried to obtain freeze-dried powder before NMR treatment.

NMR sample analysis

Sample preparation: The cell extracts were dissolved in 6 mL 0.01% TMSP/D_2O solution and centrifuged at 15,000 rpm for 20 min; 0.5 mL of supernatant was placed in an NMR tube and kept cold for analysis.

NMR detection

All ^{1}H-NMR spectra were generated using 500 MHz NMR (Bruker, Germany) with a 5 mm probe. A standard NOESY impulse sequence was used to obtain spectra of the cell extracts. In this pulse sequence, wait time (RD, 2 s) and mixing time (tm 0.1 s) was conducted water peak suppression. Related parameters were set as follows: SWH, 12 kHz; sampling time, 1.36 s; TD, 32768; NS, 64. All FID signals of ^{1}H-NMR were multiplied by an exponential window function whose broadening factor was 0.5 Hz before Fourier transform.

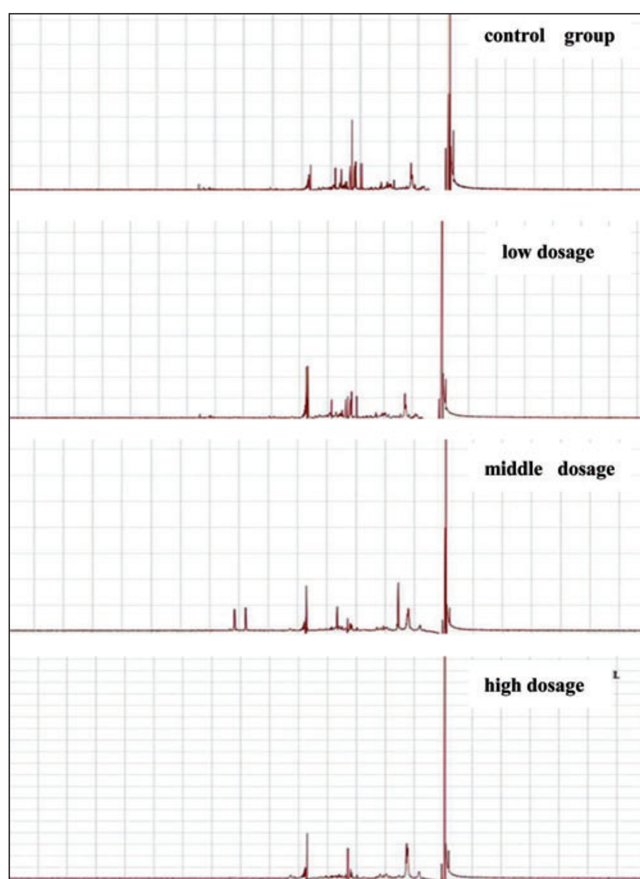

Figure 2) *^{1}H-Nuclear magnetic resonance spectra*

RESULTS

Morphological observation of K562/ADM cells

Cellular morphology of the K562/ADM cells after treatment with quercetin for 72 h was assessed under reverse microscopy (original magnification ×400) (Figure 1).

As shown in Figure 1, the cellular shape of the blank control group (1:1000 DMSO) is high-blooded without shrinkage or cracking. No significant difference was observed compared with pretherapy. However, in the low-dose group (10 µmol/L quercetin), a small number of cells had ruptured and exhibited lower activity. In the high-dose group (40 µmol/L quercetin), the phenomenon of cell shrinkage and rupture was obvious. Thus, it was educible that quercetin could inhibit the growth of K562/ADM in a dose-dependent manner.

NMR assessment of cell extracts

NMR assessment was conducted as described, and the results obtained underwent Fourier transform to obtain ^{1}H-NMR spectra, which were then conducted phase calibration and baseline adjustment. Chemical displacement of all spectra was calibrated with TMSP (δ=0.00). TMSP-marked peaks were obvious in the four ^{1}H-NMR spectra. There was apparent characteristic peak and high peak value, as shown in Figure 2.

DISCUSSION

Drug-resistant K562/ADM cells were cultured in 1000 ng/mL adriamycin medium to maintain drug resistance (9). To avoid the influence of adriamycin on the results, K562/ADM cells should be cultured in a normal culture medium without adriamycin for two

weeks before the experiment. Two weeks later, K562/ADM cells should be placed into culture solution containing adriamycin so as to maintain drug resistance.

The process of cell collection and processing was implemented after treatment with quercetin for 72 h. The present study vacuum freeze-dried the cell extracts by repeated freezing and thawing the cell extract three times to inactivate the cells, then an 80% methanol solution was added to extract the intracellular metabolites; the collected metabolite solutions were subsequently freeze dried to powder form. However, this method required a lyophilizer to remove organic solvent, which required specialized equipment and not easy to conduct. Therefore, a nitrogen blow down evaporator was ultimately chosen for drying the samples.

There have been many detection technologies used in metabonomics research includiung HPLC, MS and GC. The present study chose NMR for the following advantages (10-12): Pre-treatment of samples was easy and the required detection amount was small. Detection could be performed as soon as D_2O was added for field lock and special treatment of the samples was unnecessary. The structure and original properties of the samples were not destroyed; detection time is short (approximately 5 min to 10 min); and sample purification was not necessary. Meanwhile, various two-dimensional spectroscopy could be used for the determination and structural identification of unknown metabolites.

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