

EXPERIMENTAL STUDY

Effect of berberine on activity and mRNA expression of N-acetyltransferase in human lung cancer cell line A549

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Supported by Xiamen Science and Technology Key Program Plan Grant (No. 3502Z20100006); Scientific Research Starting Foundation for New Teacher of Xiamen University (No. ZK1014)

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Accepted: September 23, 2013

prepared. The human lung cancer A549 cells were cultivated randomly in the wells of culture plate, and divided into the control group, and berberine 0.0008, 0.008, 0.08, 0.8 and 1.6 mM treatment groups, with 3 wells for each group. 24 h later, A549 cells in each group were collected respectively, the content of N-acetyltransferase was detected by Flow cytometry, and the mRNA expression of N-acetyltransferase was observed by reverse transcription polymerase chain reaction.

RESULTS: The N-acetyltransferase content in human lung cancer A549 cells decreased with the increasing of berberine concentration, significantly lower than that in the control group ($P < 0.05$ or $P < 0.001$); and the mRNA expression of N-acetyltransferase also decreased with the increasing of berberine concentration, significantly lower in Huanglian-su treatment groups ($P < 0.001$).

CONCLUSION: Berberine can inhibit the activity of N-acetyltransferase in human lung cancer cell line A549, and shows negative correlations of dose and time in a certain extent. The inhibited gene expression of N-acetyltransferase in human lung cancer A549 cell may probably represent one of the mechanisms for its antineoplastic effect.

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Key words: Berberine; Acetyltransferases; Cell line, tumor; Neoplasms

INTRODUCTION

Malignant tumor is one kind of pathological state of abnormal hyperplasia of the cells. According to the sta-

Abstract

OBJECTIVE: To study the effects of berberine on activity and mRNA expression of N-acetyltransferase in human lung cancer cell line A549.

METHODS: N-acetyltransferase antibodies were

tistics from Ministry of Health of China, malignant tumor has become the first factor in ten major death causes. Among them, mortality of lung cancer is the highest than that of the other cancers. The cancer-inducing factors predominantly come from the environmental agents, and about 80% cancers are caused by chemical carcinogens. Many chemical materials exist with stable structures in nature, and exposure in environmental chemical carcinogens is not sure to cause damage to the cell; but if catalyzed to active carcinogens by endosomatic enzyme, they may be combined with DNA to form DNA adduct, and induce gene mutation to generate cancer. N-acetyltransferase (NAT) is just one of the enzymes involved in catalyzing carcinogens.¹ Some research papers pointed out that NAT was related to the carcinogenicity of arylamine compound, if the NAT activity in human body increased, meanwhile, getting in touch with arylamine compound, the probability of cancer occurrence would increase.² Therefore, if the NAT activity can be decreased, and carcinogens have no way to be acetylated when they get into the body, the formation of DNA adduct may be reduced and unlikely cause mutation to form cancer.

Berberine, also called Umbellatine, is an alkaloid extracted originally in large amount from the commonly-used Chinese herb Huanglian (*Rhizoma Coptidis*); and this component is also contained in Huangbai (*Cortex Phellodendri Amurensis*). It is pointed out in *Ben Cao Bei Yao*³ that Huanglian (*Rhizoma Coptidis*) is extremely bitter and cold, and can purge fire and cool blood, and can purge lung-fire if used in combination with Huangqin (*Radix Scutellariae Baicalensis*), so it belongs to the heat-clearing and toxin-resolving medicinal herb. The early researches about berberine mostly lie in antagonizing microorganism; afterwards, except the antisepticise, berberine has many other effects to be confirmed, such as anti-inflammation, reducing blood sugar, lowering blood pressure, antagonizing arrhythmia, protecting the liver, and so on.⁴ It also has an anti-cancer effect, the mechanism lies in inhibiting the invasion and metastasis of cancer cells and reducing their proliferation induced by spontaneous and environmental stimulus.⁵ At present, the research papers show that berberine can inhibit many kinds of cancer cells, such as human leukemia cells, esophageal cancer cells, carcinoma cells of the large intestine, and so on.⁶⁻¹⁰ But, there are no reports about the actions of berberine on NAT in lung cancer. Therefore, the present research is designed is an attempt to study the effects of berberine on activity and gene expression of NAT in human lung cancer cell line A549 by means of the flow cytometry and reverse transcription polymerase chain reaction (RT-PCR), and to investigate whether berberine can restrain the activity of NAT to block the cancer-generating pathway so as to provide certain assistance for prevention and treatment of cancer.

MATERIALS AND METHODS

Tumor cell line culture

The human lung cancer cell lines A-549 were provided by the National Health Research Institute (NHRI) cell bank (Taiwan, China). The A-549 cell lines were placed in 75 cm² culture flasks (Corning Inc., Corning, NY, USA) and grown at 37°C in humidified 5% CO₂ and 95% air in F12K nutrient mixture supplemented with 10% fetal bovine serum, penicillin-streptomycin (10000 U/mL penicillin and 10 mg/mL streptomycin) and 1% L-glutamine. Preparation of antibody essentials: one BALB/cJ 6-week-old female mouse was supplied by the Laboratory Animal Center of Medical College of National Taiwan University (NTU), and the mouse myeloma cells lines OKT 4 were provided by NHRI cell bank (Taiwan, China).

Experimental drugs and main reagents

Berberine (product lot No. B-3251) was bought from Sigma-Aldrich Inc. (St. Louis, MO, USA). F12K nutrient mixture (product lot No. 21127022), Penicillin-Streptomycin (product lot No. 15140122), L-glutamine (product lot No. 25030081) and fetal bovine serum (product lot No. 16000044) were bought from Gibco BRL Co., Ltd. (Grand Island, NY, USA). Carnitine acetyltransferase (product lot No. C-4899) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). RNeasy Mini Kit (product lot No. 74104) was bought from QIAGEN GmbH (Hilden, Germany). Pristine (product lot No. P-2870), Freund's Adjuvant (complete) (product lot No. F-5881), Freund's Adjuvant (incomplete) (product lot No. F-5506), phosphate buffered saline (PBS) (product lot No. P-5493), trypsin (product lot No. T-8003), formaldehyde solution (product lot No. F-8775), Methanol (product lot No. 34860), Triton X-100 (product lot No. T-8787), sodium citrate (product lot No. W-302600), NAT secondary antibody (product lot No. M-7023), bovine serum albumin (BSA) (product lot No. A-7906), sodium azide (product lot No. S-2002), ethidium bromide (product lot No. 46065), and propidium iodide (PI) (product lot No. 287075) were produced by Sigma-Aldrich Inc. (St. Louis, MO, USA).

Major apparatus

The following equipments were supplied as indicated: RCO3000T-9-V Cell-CO₂ Incubator (Thermo Scientific Revco, Thermo Fisher Scientific Inc., Waltham, MA, USA), LFI Horizontal Flow Workstation Floor & Table Top (Laminar Flow Inc., Taipei, China), Hettich EBA 12R Centrifuge (Andreas Hettich GmbH & Co., KG, Tuttlingen, Germany), Nikon Ellipse TE300 Inverted Fluorescence Microscope (Nikon, Tokyo, Japan), FACSCalibur YoC 2009 Flow Cytometry System (Becton Dickinson, San Jose, CA, USA), Gene Amp PCR System 2400 Instrument (Perkin Elmer, Foster City, CA, USA), Sub-Cell Model 96 Electrophoresis

Chamber (Bio-Rad, CA, USA), ChemiDoc XRS+ Gel Image Analysis System and Image Lab Software (Bio-Rad, CA, USA).

Flow cytometric assay for cell viability of A549 cells treated with or without Berberine

The A-549 cells were placed in 6-well plates at a density of 1×10^6 cells/well and were grown for 24 h. Different concentrations of berberine were then added to the cells for a final concentration of 0.0008, 0.008, 0.08, 0.8 and 1.6 mM, while only adding double distilled (D.D.) water for the controls and grown at 37°C, 5% CO₂ and 95% air for 6, 12, 24 and 48 h. The viability of A-549 cells was assayed by flow cytometry. Briefly, about 1×10^6 cells/well were fixed gently (drop by drop) by putting 70% ethanol (in PBS) in ice overnight and were then resuspended in PBS containing 40 mg/mL PI. After 30 min at 37°C, the cells were analyzed on a flow cytometry equipped with an argon ion laser at 488 nm wavelength. Then, the cell viability was determined.

NAT primary antibody preparation

The BALB/c mouse was used as the supplier of antibodies, and was first injected with 0.5 mL of pristine into the peritoneal cavity; 10-15 days later, 100 µg of antigen (NAT), mixed intensively with an equal volume of Complete Freund's adjuvant through the empty needle linked with three-direction joints, was inoculated to the back of mouse by subcutaneous injection as the first immunization. Adjuvant was changed into Incomplete Freund's adjuvant, and the operation was repeated again after 10-15 days as the second immunization, then repeated once again after another 10-15 days as the third immunization. And myeloma cells ($0.5-1 \times 10^6$) in PBS were then injected into the mouse by intraperitoneal injection after 10-15 days, and ascites fluids of the mouse that normally generated after one week was taken by suction and centrifugated for 5 min by 2000 rpm. The clear supernatant liquid was collected as the polyclonal NAT primary antibodies for further experiments.

Detecting the content of NAT in A549 cells

The A549 cells were inoculated into the culture dishes and cultivated in 1×10^6 /well, with 2 mL F12K nutrient mixture added. The first well added with 20 µL D. D. Water was served as the control group. 20 µL of berberine in 5 different concentrations (0.08, 0.8, 8, 80 and 160 mM) were respectively added into the second to sixth well. And the cells were incubated for 24 h at 37°C in 5% CO₂ incubator. After the nutrient mixture was removed and washed with PBS, a little Trypsin was added to blow the cells by micropipette. The cells were collected in 15 mL centrifuge tube and centrifugated at 4°C for 5 min by 1500 rpm, and the clear supernatant liquid was out welled. 5 mL of PBS was added and the tubal wall was clapped to let the cells in suspension,

then centrifugated at 4°C for 5 min by 1500 rpm. 100-200 µL PBS was reserved, and the cells were suspended by the clapping tubal wall, then shifted into 96-well culture dish and centrifugated at 4°C for 4 min by 1500 rpm. 100 µL of 1% formaldehyde was added after the clear supernatant liquid was removed; 5 min later, 100 µL of 99% Methanol was added for 30 min reaction and centrifugated at 4°C for 4 min by 1500 rpm, and washed 3 times by 100 µL of PBS [including 0.1% bovine serum albumin (BSA)] after the clear supernatant liquid was removed. 100 µL of 0.1% Triton X-100 and 0.1% sodium citrate were added in PBS, then reacted on ice for 45 min, centrifugated at 4°C for 4 min by 1500 rpm, washed 3 times by 100 µL of PBS (including 0.1% BSA) after the clear supernatant liquid was removed. 100 µL of primary anti-mouse NAT antibody (diluted 50 times by PBS including 0.1% BSA and 0.1% sodium azide) was added and reacted on ice for 2 h, and then centrifugated at 4°C for 4 min by 1500 rpm, and washed 3 times with the above-mentioned methods. 100 µL of 1% NAT secondary antibody (FITC-conjugated goat anti-mouse IgG antibody) was added and reacted away from light for 30 min, and then centrifugated at 4°C for 4 min by 1500 rpm, and washed 3 times with the above-mentioned methods. 100 µL of 0.1% BSA was added in PBS, then the content of NAT in the cells was analyzed by flow cytometry. This experiment was repeated 3 times.

Detecting the gene expression of NAT in A549 cells by RT-PCR method

Like the procedures for detecting the content of NAT in A549 cells, after adding a little Trypsin to blow the cells by micropipette, the total RNA was drawn out by the RNA Kit, and 500 ng of RNA was added into the 0.5mL microcentrifuge tube, then added RNA free water until the volume dose was 11.5 µL, added 1 µL of oligo-dT and reacted at 70°C for 10 min, added 7.5 µL of solution [2 µL of 100 mM dithiothreitol (DTT), 1 µL of 10 mM deoxy-ribonucleoside triphosphate (dNTP), 4 µL of 5×buffer and 0.5 µL of RT] and reacted at 37°C for 1 h to obtain the complementary deoxyribonucleic acid (cDNA). 1 µL of cDNA was added into 24 µL of solution [19.05 µL of H₂O, 2.5 µL of 10× buffer, 0.75 µL of MgCl₂, 0.5 µL of dNTP, 0.5 µL of NAT or β-actin primer 1 (Table 1), 0.5 µL of NAT or β-actin primer 2 (Table 1) and 0.2 µL of Taq DNA polymerase]. Polymerase chain reaction (PCR) was performed under the following conditions: preheating at 94°C for 30 min, degeneration at 94°C for 45 s, renaturation at 55°C for 30 s, and elongation at 72°C for 90 s, for a total of 35 cycles; and eventually reacted at 72°C for 10 min, then preserved the PCR end products at 4°C. DNA dye was added into the sample in a ratio of 1 : 5. The amplified products of each sample were electrophoresed in 1.5% agarose gel containing ethidium bromide dye. DNA marker (DL2000) was used as the standard molecular weight.

The gels with amplified fragments were observed and photographed through a UV transilluminator. Gel electrophoretic bands were imported into the ChemiDoc XRS + Gel Image Analysis System. The β -actin was used as an internal reference, the expression intensity was analyzed by the ImageLab Software, and the results were expressed as the absorbance ratio to the reference β -actin. For the upstream and downstream of the primers, see the following Table 1.¹¹

Statistical analysis

The data are expressed as mean \pm standard deviation (SD). Statistical analysis of the data was performed using the IBM SPSS statistics 19.0 software (SPSS Inc. Chicago, IL, USA). The LSD-*t* was used when the comparisons were made among the groups. The results were considered to be statistically significant when $P < 0.05$.

RESULTS

Effect of berberine on the content of NAT in A549 cells

After the A549 cell lines were intervened by Berberine and the intra-cellular NAT was dyed by antibody, it was found by flow cytometry that the content of NAT decreased with the increasing of drug concentration. In the 24 h results, the percentage of NAT content in A549 cells was $97.28\% \pm 0.93\%$ in the control group; after intervention by berberine with increasing concentration of 0.0008, 0.008, 0.08, 0.8 and 1.6 mM, the percentages were $98.71\% \pm 0.26\%$, $99.49\% \pm 0.18\%$, $98.88\% \pm 0.25\%$, $68.04\% \pm 2.02\%$, $49.06\% \pm 0.84\%$ respectively. The contents of NAT in 0.0008 and 0.008 mM groups were higher than that in the control group ($P < 0.05$); but the contents of NAT in 0.8 mM and 1.6 mM groups were lower than that in the control group ($P < 0.001$).

Effect of berberine on gene expression of NAT in A549 cells

After intervention by berberine in different concentrations of 0.0008, 0.008, 0.08, 0.8 and 1.6 mM for 24 h, the genes of NAT in A549 cell lines were amplified through PCR with 35 cycles. It was found by Ultraviolet (UV) that the mRNA expression of NAT decreased

with the increasing of berberine concentration. The decreased multiples of mRNA expression of NAT in A549 cell lines in 0.0008, 0.008, 0.08, 0.8 and 1.6 mM groups were 0.99 ± 0.01 , 0.76 ± 0.02 , 0.74 ± 0.03 , 0.26 ± 0.05 and 0.10 ± 0.04 respectively (Figure 3). Compared with the control group, the mRNA expression levels of NAT in 0.008, 0.08, 0.8 and 1.6 mM groups were significantly lower ($P < 0.001$). After the A549 cell lines were intervened by the berberine in 0.8 mM concentration through time course observation with a 6 h interval, the effect of action time of berberine on the cells was that the mRNA expression of NAT decreased following the time-lapse. Action time of 0 h of berberine on the cells was used as the control group. The decreased multiples of mRNA expression of NAT at the 6, 12, 18 and 24 h were 0.60 ± 0.03 , 0.63 ± 0.03 , 0.45 ± 0.05 and 0.19 ± 0.04 respectively (Figure 2). The mRNA expression levels of NAT in the 6, 12, 18 and 24 h groups were quite obviously lower than that of the control group (all $P < 0.001$).

Effect of berberine on A549 cells viability

After the A549 cells had been treated with or without different concentrations of berberine for different time-periods, the percentage of viable cells was determined by flow cytometry. The data showed that berberine decreased the percentage of viable cells in a dose- and time-dependent manner (Figure 3).

DISCUSSION

For the past few years, cancer has become the main cause for death in human diseases. How to prevent and treat cancer is a current important topic. Prevention and treatment for cancer include two aspects: the one is aiming at the tumor suppressor gene for controlling the cancer cell growth and apoptosis; and the other one is stressed on the cancer-generating pathway. The formation of cancer can be divided into three stages: (a) initiation phase: variation appears in genes of the cell related to the extraneous material; (b) promotion phase: present with cell phenotype changes; and (c) progression phase: proliferation and pervasion of the cancer cell, and with invasion to the other important tissues and organs.¹² The cancer-inducing factors mainly come from the environment, which includes: (a) cig-

Table 1 The PCR primers of β -actin and NAT

Name of primer	Sequence of primer	Amplified product fragment length (bp)
β -actin	Upstream (primer 1)	5'-GCTCGTTCGTCGACAACGGCTC-3'
	Downstream (primer 2)	5'-CAAACATGATCTGGGTTCATC TTCTC-3'
NAT	Upstream (primer 1)	5'-CACCCGGATCCGGGATCATGGACATTGAAGC-3'
	Downstream (primer 2)	5'-GGTCCTCGAGTCAATCACCATGTTTGGGCAC-3'

Notes: NAT: N-acetyltransferase; PCR: polymerase chain reaction. β -actin: genebank accession number NM-001101; NAT: genebank accession number X17059.

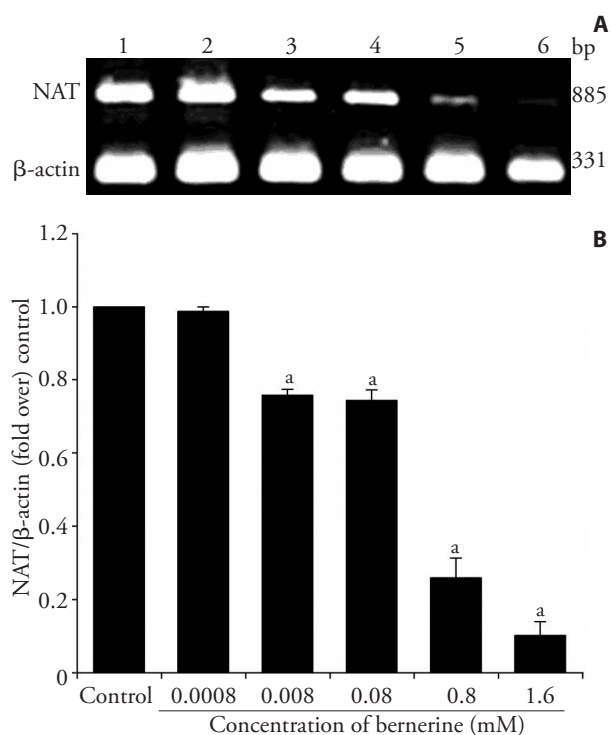


Figure 1 Effect of different concentration of berberine on gene expression of NAT in A549 cells

A: gel electrophoresis of RT-PCR products with primers for NAT in different dose groups. 1: control group; 2: 0.0008 mM group; 3: 0.008 mM group; 4: 0.08 mM group; 5: 0.8 mM group; 6: 1.6 mM group. B: histograms of gel electrophoresis of RT-PCR products with primers for NAT, and with corresponding β-actin RT-PCR products as the controls in human lung cancer cell line A549 after treatment with different concentrations of berberine for 24 h. Compared with the control group, ^a $P < 0.001$. NAT: N-acetyltransferase; RT-PCR: reverse transcription polymerase chain reaction.

arete: about 90% lung cancers are related with smoking;¹³ (b) alcohol: it is related with the occurrence of hepatic cirrhosis and liver cancer;¹⁴ (c) radiation: some radioactive substances can cause liver cancer or leukemia;¹⁵ (d) natural toxins: aflatoxin can induce live cancer;¹⁶ (e) chemical materials: about 80% cancers are induced by chemical carcinogens.¹⁷ The chemical carcinogens includes: (a) polycyclic aromatic hydrocarbons: this kind of compounds commonly existing in natural environment, like coal, tobacco and barbecue, are assured of the first inducing cause for cancer. Yamagiwa and Ichikawa have confirmed that oncogens existing in oil of cypress may induce skin cancer in rabbits; and the oncogen is banzo (α) pyrene, confirmed by Ernest Kenaway.¹⁸ (b) nitroso compounds: may be contained in bloated pickles, if eating them for a long time, esophageal cancer and gastric cancer may be induced.¹⁹ (c) organic compound and inorganic compound, including acetamide, chloroform, dioxin, beryllium, cadmium, arsenic etc, may cause cancer. (d) arylamines: they may cause cancer through feeding,²⁰ for example, 2-amino-fluorene (2-AF) can be changed into 2-acetylaminofluorene (2-AAF) after metabolism through NAT, later into strong active ultimate carcinogens through other enzyme metabolism, and finally combined with DNA to

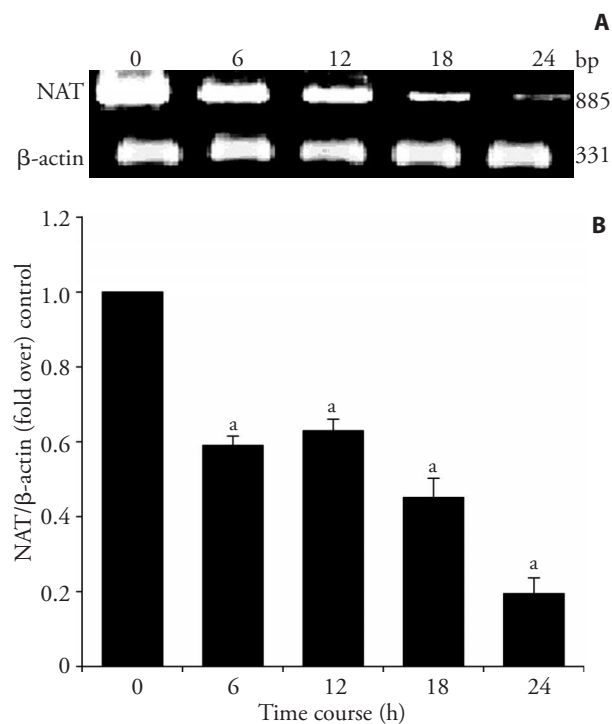


Figure 2 Effect of action time of berberine on gene expression of NAT in A549 cells

A: gel electrophoresis of RT-PCR products with primers for NAT in different time groups; B: histograms of gel electrophoresis of RT-PCR products with primers for NAT, and with corresponding β-actin RT-PCR products in human lung cancer cell line A549 after treatment with 0.8 mM berberine for time course. Compared with the control group, ^a $P < 0.001$. NAT: N-acetyltransferase; RT-PCR: reverse transcription polymerase chain reaction.

cause cancer.²¹ According to the speed of the arylamine acetylated by NAT, the individuals can be distinguished as quick acetylator and slow acetylator.²² Epidemiological data showed that the quick acetylator may be easy to cause carcinoma of the colon and rectum,²³ while the slow acetylator may be easy to induce bladder cancer.²⁴ NAT, 34Kda in molecular weight, mainly plays a role in metabolizing the external medicine or compound for endogenous biochemistry synthesis or inducing the activity; and it is also the first checkpoint to metabolize chemical carcinogen. If there is no NAT, strong active carcinogen may be formed. So, in compliance with the theory of cancer-forming pathway, progress will be made if some kinds of medicines can inhibit the activity of NAT and block the pathway.

This study is aiming at the cancer cell forming pathway. When chemical carcinogens like 2-AF get into the body, the acetyl of acetylcoenzyme A (AcCoA) may be transferred into the N-group of 2-AF by NAT in cytoplasm and form 2-AAF, then metabolized to N-hydroxy-acetylaminofluorene (N-OH-AAF) by Cytochrome P-450, and combined further with DNA to form the DNA adduct, thus causing the errors and mutation when the cells are duplicating DNA, and inducing cancer.² The mutation of DNA adduct has a narrow spectrum²⁵, so the process of cancer cell formation through NAT pathway may be concluded as follows:

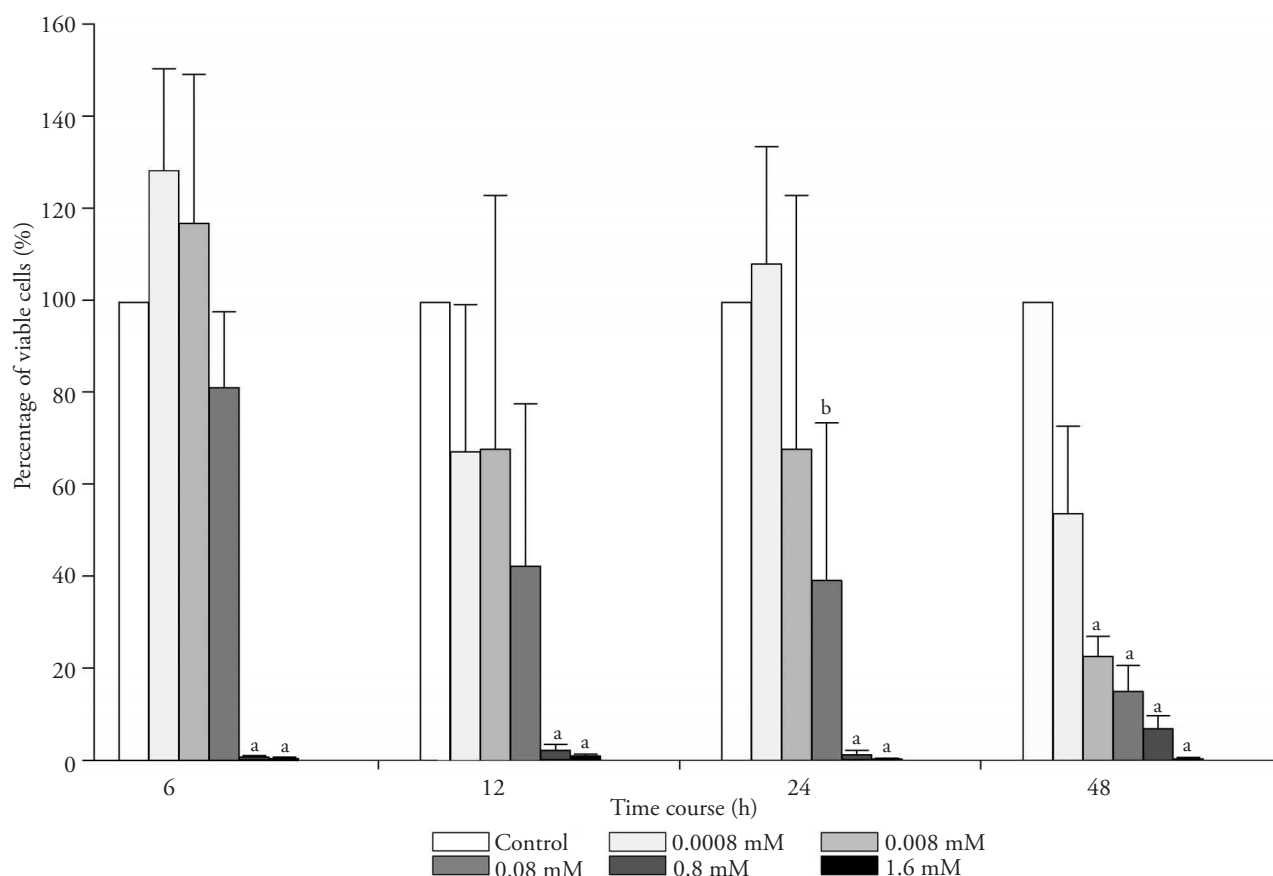


Figure 3 Flow cytometric determination of viable cell number of human lung cancer A549 after exposure to berberine for 6, 12, 24 and 48 h

For significant difference between the control group and treatment group ($n=3$), ^a $P<0.001$, ^b $P<0.05$.

2-AF
 ↓ NAT
 2-AAF
 ↓ Cytochrome P-450
 N-OH-AAF
 ↓
 N-Oac-AF
 ↓
 Nucleic acid adduct
 ↓
 DNA replication and mutation
 ↓
 Cancer cell

The main purpose of the present study is to determine whether NAT activity in lung cancer cells can be reduced or not. We used NAT antibody to make dyeing on A549 cancer cells intervened by berberine. The results showed that the intra-cellular NAT content presented a dose-dependent inhibited tendency following the increasing of concentration of berberine, and the mRNA expression of NAT also showed a similar tendency. The NAT activity was restrained by berberine, so we can draw an preliminary conclusion that berberine may reduce the probability of forming cancer caused by chemical materials acetylated to high affinity adducts that speed DNA mutation when the cells are exposed in environmental chemical materials. But after all, A549 is the cell lines of cancerization; although berberine can lower their NAT activity, how berberine takes effect on NAT in human body is not yet sure.

And for this, further researches should be done to clarify the prophylactic effect of berberine. The effect of viability decreased at A549 cell line will be explored by us in another ongoing study on cancer cell growth. berberine is the major alkaloid extracted from the Chinese herb Huanglian (*Rhizoma Coptidis*). The findings about berberine from the present study can help us in research on NAT activity of normal cells, and in study of the pathway for cancer prevention and treatment.

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