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Induction of G1 cell cycle arrest and apoptosis by berberine in bladder cancer cells

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ABSTRACT

Bladder cancer is the ninth most common type of cancer, and its surgery is always followed by chemotherapy to prevent recurrence. Berberine is non-toxic to normal cells but has anti-cancer effects in many cancer cell lines. This study was aimed to determine whether berberine inhibits the cell proliferation and induces cell cycle arrest and apoptosis in BIU-87 and T24 bladder cancer cell line. The superficial bladder cancer cell line BIU-87 and invasive T24 bladder cancer cells were treated with different concentrations of berberine. MTT assay was used to determine the effects of berberine on the viability of these cells. The cell cycle arrest was detected through propidium iodide (PI) staining. The induction of apoptosis was determined through Annexin V-conjugated Alexa Fluor 488 (Alexa488) staining. Berberine inhibited the viability of BIU-87 and T24 cells in a dose- and time-dependent manner. It also promoted cell cycle arrest at G0/G1 in a dose-dependent manner and induced apoptosis. We observed that H-Ras and c-fos mRNA and protein expressionswere dose-dependently and time-dependently decreased by berberine treatment. Also, we investigated the cleaved caspase-3 and caspase-9 protein expressions increased in a dose-dependent manner. Berberine inhibits the cell proliferation and induces cell cycle arrest and apoptosis in BIU-87, bladder cancer cell line and T24, invasive bladder cancer cell line. Berberine can inhibit the oncogentic H-Ras and c-fos in T24 cells, and can induce the activation of the caspase-3 and caspase-9 apoptosis. Therefore, berberine has the potential to be a novel chemotherapy drug to treat the bladder cancer by suppressing tumor growth.

1. Introduction

Bladder cancer is the ninth most common type of cancer in the world with approximately 356,000 new cases (274,000 males and 83,000 females) every year (Ploeg et al., 2009). In China, the age-standardized mortality rates of bladder cancer of male and female city dwellers are 3.54/100,000 and 1.19/100,000, respectively, in 2007. And in the countryside, the age standardized mortality rates are 1.92/100,000 and 0.52/100,000, respectively. In recent years, the occurrence of bladder cancer has gradually increased in some Chinese cities (Wei Zhang and Liu, 2005; Xu Guilan and Jijun, 1997). Seventy to eighty-five percent of the newly diagnosed cases are superficial tumors, which are mainly treated by the surgery of transurethral bladder tumor resection (TURBT). However, the rate of tumor recurrence is high following the surgery. A number of drugs like BCG and some chemotherapeutics including epirubicin, mitomycin (MMC) and hydroxycamptothecin (HCPT) are used to prevent the tumor recurrence after TURBT. However, these drugs usually produce severe complications, such as frequent micturition, urgent micturition, urination pain, hematuria, immune suppression, febrise, and lumbodynia (Shelley et al., 2007). Therefore, an effective drug with less complication is highly desired in bladder cancer therapy.

Berberine, a traditional Chinese anti-inflammatory medicine, has been proposed to have anti-cancer effects in many types of tumor cells, including the cell lines of Ewing tumor (Lanvers-Kaminsky et al., 2006), Anoikis-resistant MCF-7 and MDA-MB-231 breast cancer cells (Jantova et al., 2007), human esophageal cancer HeLa (Iizuka et al., 2000) and L1210 (Kettmann et al., 2004) cells, and prostate cancer cells (Agarwal, 2000). Although berberine is non-toxic in human (Jantova et al., 2003), it is cyto-toxic to B16 tumor cell line in vitro and can significantly reduce the tumor volume and weight in vivo (Letasiova et al., 2005).

Three oncogenic ras genes, are frequently mutated in cancers encode four highly related protein H-Ras, N-Ras and K-Ras (K4A and K4B), and H-Ras was endogenously expressed in T24 bladder cancer cell line (Taparowsky et al., 1982). H-Ras mutations associated with grades and stages of bladder cancer (Johne et al., 2003) and is detected in more than 35% of patients with urothelial carcinomas (Buyru et al., 2003). c-Fos, a downstream effector of H-Ras (Li et al., 2005), a member of the Fos family (c-fos, FosB and its smaller splice

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variants, Fra-1 and Fra-2), and plays a role in the progression of many carcinomas (Milde-Langosch, 2005). However, whether the H-Ras and c-fos suppressed in T24 cells treated by berberine is no report yet. So we intent to assess the expressions of the H-Ras and c-fos in T24 cells treated by berberine in order to detect the mechanism of the inhibition of berberine to the proliferation of the T24 cells.

In this study, we determined the effects of berberine on the superficial bladder cancer cell line BIU-87 and invasive bladder cancer cell line T24. We discovered that berberine inhibited G1 cell cycle progression and induced apoptosis of bladder cancer cells, indicating that berberine is a potent chemotherapeutics to bladder cancers.

2. Materials and methods

2.1. Reagents

RPMI 1640, fetal bovine serum (FBS), 100 mg/ml pencillin–streptomycin, ACSCalibur, Annexin V-conjugated Alexa Fluor 488 (Alexa488) were purchased from Invitrogen, Carlsbad, CA, USA. Five milligrams per milliliter of MTT, dimethylsulfoxide (200 ml), propidium iodide, 100 mg/ml RNAase A, and berberine were purchased from Sigma, St. Louis, MO, USA. Berberine was prepared by being dissolved in DMSO (maximum concentration, 0.1%). DMSO was applied as control.

2.2. Cells, culture conditions and treatments

Human bladder cancer BIU-87 and T24 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The BIU-87 and T24 cells were cultured as monolayers with RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS) and 100 mg/ml pencillin–streptomycin (Invitrogen, Carlsbad, CA, USA) in an incubator with humidified atmosphere of 95% air and 5% CO2 at 37 °C.

In all treatments, berberine was initially dissolved in a small amount of DMSO and made up to the maximum final concentration of 0.1% (v/v) in the complete cell culture medium. The sub-confluent cells (50–60%) were treated with either varying concentrations of berberine, or vehicle alone (0.2% ethanol in media) as control.

2.3. MTT assay for cell viability

BIU-87 and T24 cells were plated in 96-well culture plates with 10^4 cells/well. After overnight incubation, the cells were treated with varying concentrations of berberine (0, 1, 5, 10, 25, 50, 75, or 100 μg/ml) for 24, 48, and 72 h. The cells were treated with 5 mg/ml MTT and the absorbance was recorded at 540 nm with a reference at 650 nm. The effects of berberine on cell viability were assessed by the relative percentage of viable cells that was normalized to that of the vehicle-treated control cells, which were arbitrarily assigned 100% viability.

2.4. Cell cycle assay

Bladder cancer BIU-87 and T24 cells (50–60%) were treated with varying concentrations of berberine in complete medium for 48 h before being harvested, washed with cold phosphate-buffered saline (PBS), and processed for cell cycle analysis. About 10^5 cells were resuspended in 3 ml cold PBS to which 1 ml cold ethanol for 1 h incubation at 4 °C. The cells were centrifuged at 1100 rpm. for 5 min, and the pellet was washed with cold PBS, re-suspended in 500 μL PBS, and incubated with 32 μL RNase (20 μg/ml final concentration) for 30 min. The cells were incubated with propidium iodide (50 mg/ml) on ice for 1 h in the dark. The cell cycle distribution was analyzed using FACS Calibur instrument (BD Biosciences, San Jose, CA, USA).

2.5. Cell apoptosis assay

Cells were fixed in 70% ethanol for 1 h, washed with PBS, and treated with 20 μg/ml RNAase A (Sigma, St. Louis, MO) for 1 h at 37 °C. Cells were then stained with 50 mg/ml propidium iodide (Sigma) as previously described (Kim et al., 2009). For Annexin V-conjugated Alexa Fluor 488 (Alexa488) staining, cells were washed with PBS, treated with diluted trypsin-EDTA solution, centrifuged, washed twice with cold PBS, and re-suspended in binding buffer (10 mM Hepes pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$). A 100 μL aliquot of suspension (10^5 cells) was incubated with 5 μL of Alexa488 and 5 μL of PI for 15 min at room temperature in dark. Binding buffer (400 μL) was added to each mixture, and the samples were analyzed by flow cytometry within 1 h. All experiments were performed in triplicates.

2.6. RNA extraction, reverse transcription, and PCR

Total RNA was extracted using the Trizol® (Invitrogen, Carlsbad, CA) after the T24 cells were treated with 0, 5,10,25,50 μg/ml berberine for 48 h, or 25 μg/ml berberine for 24 and 48 h, according to the manufacturers' instruction. cDNA was synthesized using random primers (oligo dt) and MMLV reverse transcriptase (TOYOBO). The PCR for GAPDH, H-Ras and c-fos mRNA were performed by using the following primer pairs derived from the primer bank, H-Ras, the sense primer was ACAACACCAAGT CTTTTGAGGAC, the antisense primer was GCCTGCCGAGATTCCACAG; and the sense primer of c-fos was CCACCCGAAC AGTCTCTCCT, the antisense was AGAAGCCCAAAAGC-CATAGGT; GAPDH: forward Primer CAACTGGTCGTGGACAACCAT, Reverse Primer was GCACGGACACTCACAATGTTC.

A total 32 PCR cycles at 94 °C for 30 s, 58 °C for 30 s, and 68 °C for 45 s for H-Ras and c-fos, GAPDH expressions were used as a control for RNA loading and reverse transcription efficiency and amplified with its specific primers using 28 cycles. PCR products were resolved in 1.5% agarose gels, stained with ethidium bromide, and visualized in UV light.

2.7. Western blot

Cells were plated onto 50 ml culture flasks with 0, 5,10,25,50 μg/ml berberine for 48 h, or 25 μg/ml berberine for 24 and 48 h, then were harvested and washed with PBS. Cells were lysed with RIPA buffer with protease inhibitor. After centrifugation at 12,000 rpm, the supernatant was used to determine protein concentration with the BCA assay (BEYOTIME). Protein (50 μg) samples were separated by SDS-PAGE (8% or 12%) and transferred onto polyvinylidene difluoride (PVDF) membranes using a semi-dry transfer. Membranes were blocked using Tris-buffered saline with 5% nonfat milk and incubated with antibodies of H-Ras and c-fos (Bioworld), β-actin (Santa Cruz), procaspase-3 and cleaved caspase-3, procaspase-9 and cleaved caspase-9 (Abcam). After washing membranes with TBST, the membranes were incubated with respective secondary antibodies labeled with horseradish peroxidase. To insure equal protein loading, each membrane was probed with the GAPDH antibody. Membranes were developed with the chemiluminescence kit (Amersham Pharmacia, Piscataway, NJ, USA) and protein levels were detected using chemiluminescence reader LAS 4000 mini series (Fuji Film, Tokyo, Japan).

2.8. Statistical analyses

All experiments were repeated at least three times, and all data were compiled from a minimum of triplicate experiments. Data used for statistical analysis are expressed as the mean ± standard error. The results from treated and untreated control cells were analyzed by Student's *t*-test and a P-value of less than 0.05 was considered as statistically significant.

3. Results

3.1. Berberine inhibits the growth of BIU-87 and T24 cells

Both cells were exposed to different concentrations of berberine for 24, 48, and 72 h. As shown in Figs. 1 and 2, the cell growth of BIU-87 and T24 cells was significantly decreased in an obvious dose- and time-dependent manner. The viability of BIU-87 cells and T24 cells was reduced to 22.7% and 27.7% in 72 h after berberine treatment (100 μg/ml), respectively. We chose the appropriate concentrations of berberine (10 μg/ml and 25 μg/ml) according to the results of MTT assays for following apoptosis assays and Western blot assays. The inhibitory effects of berberine treatments were greater on BIU-87 cells than on T24 cells.

3.2. Berberine inhibit the expressions of the H-Ras and c-fos in the T24 cells in different concentrations and times

A gradual reduction in both H-Ras and c-fos expressions was observed in the T24 cells with the prolongation of the treated hours and the increase of the concentrations of berberine, the results of RT-PCR as shown in Fig. 5a and b, and the results of western blot in Fig. 6a and b. These results indicate that the inhibition of the berberine to the H-Ras and c-fos are dose-dependently and time-dependently. And the most effective treatment time is the 48 h.

3.3. Berberine induces G0/G1 arrest in BIU-87 and T24 cells

Fluorescence-activated cell sorter (FACS) analysis was performed to determine which phase of cell cycle berberine functions on to inhibit the cell proliferation, As shown in Fig. 3, significantly more cells were in G0/G1 phase after 48 h of berberine treatment in a dose-dependent manner, comparing to the untreated cells.

3.4. Berberine induces apoptosis in BIU-87 and T24 cells

G0/G1 arrest usually results in apoptosis. We went to test whether berberine treatment induced apoptosis in BIU-87 and T24 cells, which may cause the observed loss of viability. The percentages of apoptotic cells after treatments with 10 μg/ml and 25 μg/ml berberine were assessed with the Annexin V-conjugated Alexa Fluor 488 Apoptotic Detection Kit. The late and early apoptotic cells are shown in the upper right (UR) and lower right (LR) quadrants of the FACS histogram in Fig. 4. Forty-eight hours of berberine treatment induced both late and early apoptosis in a dose-dependent manner in BIU-87 and T24 cells (Fig. 4a–c). The percentages of total apoptotic cells after berberine treatments were as follows: 0.04% (vehicle control), 7.76% (10 μg/ml, $P<0.05$), 19.61% (25 μg/ml, $P<0.01$). The percentages of total apoptotic cells of BIU-87 cells after the berberine treatments were as follows: 0.06% (vehicle control), 11.42% (10 μg/ml, $P<0.05$), 54.40% (25 μg/ml, $P<0.01$). These data suggest that induction of apoptosis in BIU-87 and T24 cells after berberine treatment could be a major mechanism of berberine-caused inhibition of cell viability (Fig. 4A–C).

3.5. Berberine induces activation of caspase-3 and caspase-9 in T24 cells

To determine whether caspase-3 plays a role in berberine mediated apoptosis of T24 cells, we assessed the activated caspase-3 protein level of the T24 cells treated by the berberine for 48 h in 10 μg/ml and 25 μg/ml concentration as shown in Fig. 7. Western blot results show that the protein level of the procaspase-3 has no difference in different concentrations, however, the level of cleaved caspase-3 increased from untreated (0 μg/ml) to 10 μg/ml and 25 μg/ml. We also assessed the level of procaspase-9 and cleaved caspase-9. As shown in Fig. 8, the cleaved caspase-9 increased in a concentration-dependent manner. These findings suggest that the mechanism of T24 cell apoptosis induced by berberine is involved in activation of caspase-3 and caspase-9.

4. Discussion

In this study, we determined the effects of berberine treatments on the growth, cell cycle arrest and apoptosis of bladder cancer cell lines, BIU-87 and T24. BIU-87 cell line established from human superficial bladder transitional cell carcinoma (BTCC) by Lizhang Yu in 1989 in China (Shelley et al., 2007), and T24 cell line was established from human invasional BTCC by Bubenik J established (Lanvers-Kaminsky et al., 2006). These two cells represent two most widely used BTCC. Therefore, we chose these two cell lines to explore the effects of berberine on bladder cancer cells.

We hypothesized that berberine inhibits the growth of two cell lines and promotes cell cycle arrest and apoptosis. Our results well support this hypothesis and we found that berberine treatment inhibited the proliferation of BIU-87 and T24 bladder cancer cells in a time- and dose-dependent manner (Figs. 1 and 2). We also found that berberine treatment had greater inhibitory effects on BIU-87 cells than on T24 cells. More specifically, 72 h after the berberine treatment at 100 μg/ml, the percentages of viable cells of BIU-87 and T24 were only 22.7% and 27.7%, respectively.

To the best of our knowledge, this study is the first to report the effects of berberine on bladder cancer cells' cell cycle arrest and apoptosis. We found that berberine caused G0/G1 arrest dose-dependently and the percentage of T24 cells arrested in G0/G1 phase was lower than that of BIU-87 cells. Similar effects were observed in other cancer cells (Iizuka et al., 2000; Letasiova et al.,

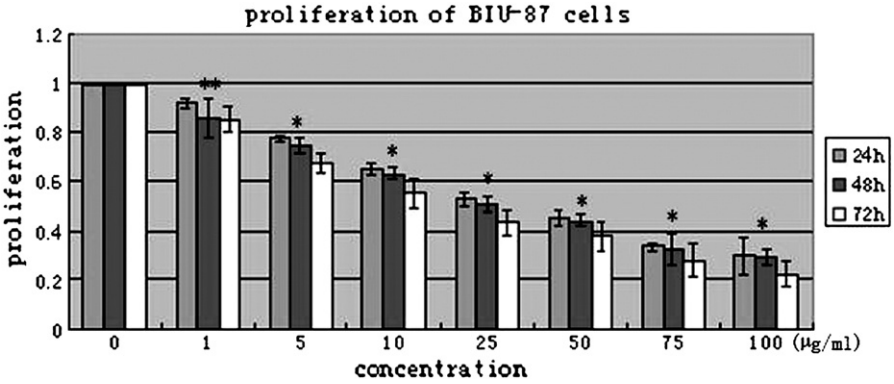

Fig. 1. Cell viability of BIU-87 bladder cancer cells treated with berberine. BIU-87 cells were treated with distilled water as control and with increasing concentrations of berberine for 24, 48 and 72 h. The data represent the means of at least three independent experiments and the corresponding standard error. *: $P<0.01$; **: $P<0.05$.

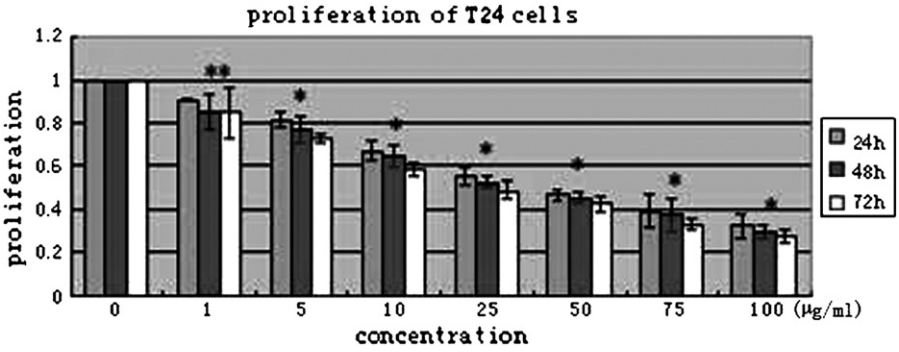

Fig. 2. Cell viability of T24 bladder cancer cells treated with increasing concentrations of berberine for 24, 48 and 72 h. The data represent the means of at least three independent experiments and the corresponding standard error. *: $P<0.01$; **: $P<0.05$.

2005), although some studies also suggested that berberine treatment might cause G2 arrest (Jantova et al., 2003, 2007).

Our flow cytometry apoptosis analysis of BIU-87 and T24 cells revealed the presence of late or early apoptosis following berberine treatments (Fig. 4). Furthermore, more berberine-treated BIU-87 entered apoptosis than T24 cells with the same treatments. The results indicate that berberine induces apoptosis in both superficial and invasive bladder cancer cells, while the superficial ones showed stronger induction.

H-Ras is a membrane-bound guanosine triphosphate and guanosine diphosphate-binding (G) protein that serves as a molecular switch, conveting signals from the cell membrane to the nucleus, and ras can lead to G1 progression through increasing the levels of cyclin D1, which promote the progression of cells through the G1 checkpoint and into S phase (Adjei, 2001). Shambhumathe et al. introduced concogenic H-Ras into bladder J82 cancer cells and expression of H-Ras promoted J82 cells to acquire tumorigenic ability (Choudhary and Wang, 2007). H-Ras is associated with the proliferation and cell cycle of the cancer cells. Since the T24 cell line was the only cell line known to overexpress oncogenic H-Ras, we investigated the expressions of the H-Ras and c-fos in T24 cells treated with berberine. And in our results, a gradual reduction in both H-Ras and c-fos expressions was observed in the T24 cells in dose-dependent and time-dependent manner. It suggests that the inhibition of the proliferation of T24 cells treated with berberine through the H-Ras and c-fos pathway.

To further understand berberine's effects on bladder cancers, we will explore the signaling pathways in the induction of apoptosis by berberine treatment. It has been previously reported that berberine-induced apoptosis is initiated by reactive oxygen species, which activates the JNK/p38 MAPK and FasL pathways in human prostate cancer cells and colonic carcinoma cells (Hsu et al., 2000, 2007; Huang et al., 2006; Meeran et al., 2008; Roublevskaia et al., 2000). NF-kB signaling pathway that activates p53 was also suggested in berberine induced apoptosis (Ho et al., 2009; Lee et al., 2007; Liu et al., 2009;

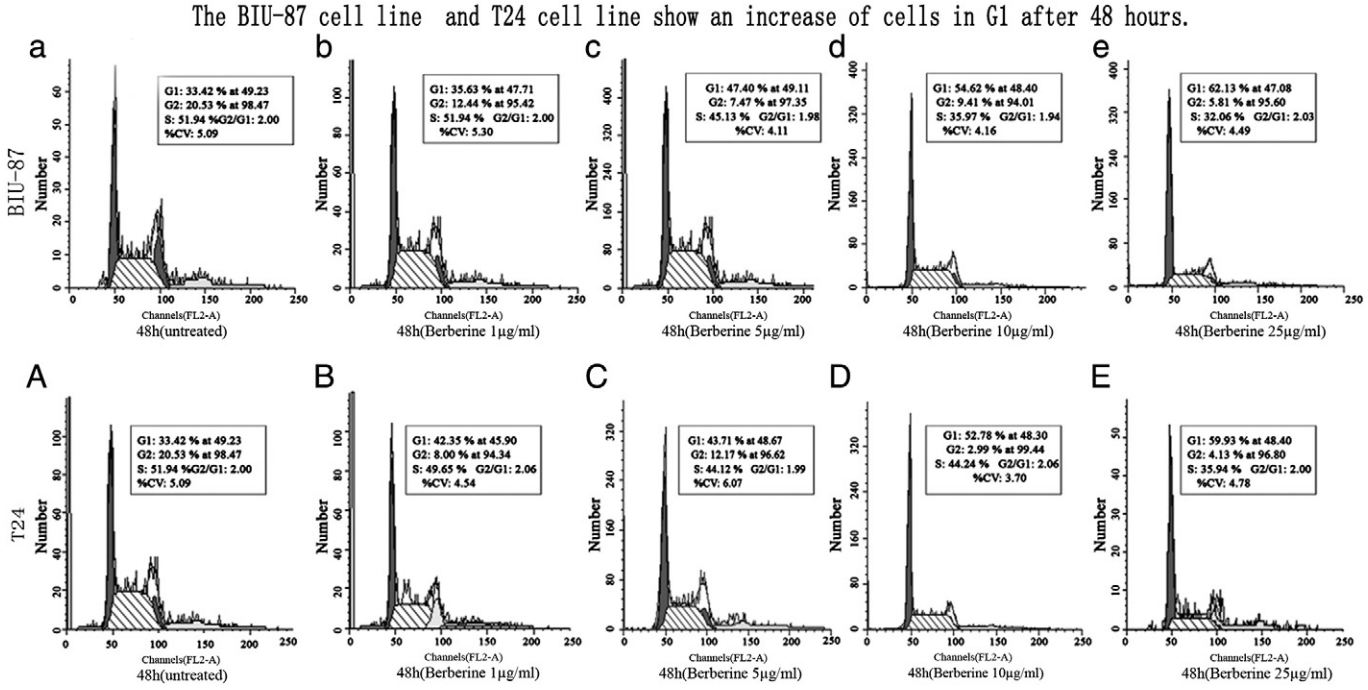

Fig. 3. Cell cycle analysis of BIU-87 and T24 bladder cancer cells treated with increasing concentrations of berberine. The percentages of cells at each stage of the cell cycle were analyzed by FACS after PI staining. a) BIU-87 cells were treated with distilled water as control; b–e) BIU-87 cells were treated with 1 μg/ml, 5 μg/ml, 10 μg/ml, and 25 μg/ml of berberine. A) T24 cells were treated with distilled water as control; B–E) T24 cells were treated with 1 μg/ml, 5 μg/ml, 10 μg/ml, and 25 μg/ml of berberine. The BIU-87 cell line and T24 cell line show an increase of cells in G1 after 48 h. The data shown were from a representative experiment of three independent experiments and the corresponding standard error.

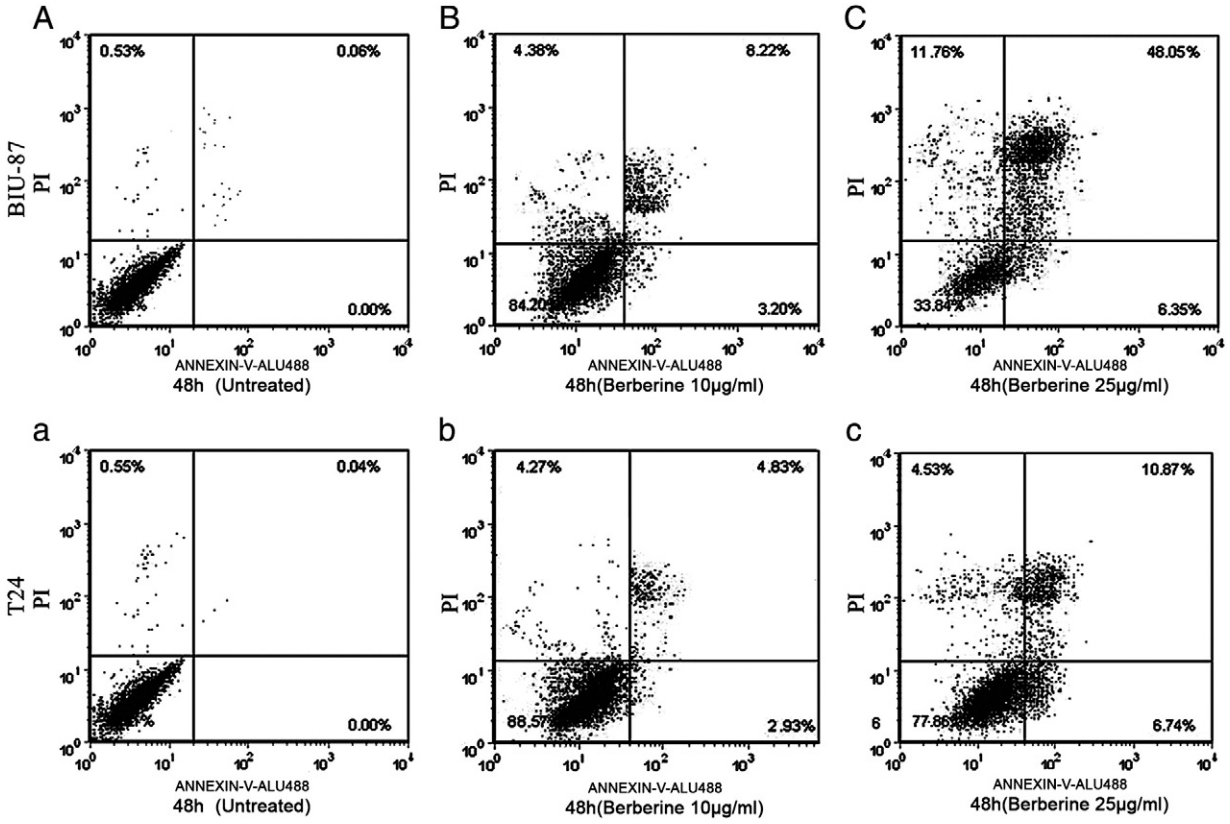

Fig. 4. The percentages of apoptosis of BIU-87 cells and T24 cells after berberine treatments. Berberine treatments in (A–C) BIU-87 cells and (a–c) T24 cells for 48 h. All cells were stained with Alexa488 in a buffer containing PI. The percentage of surviving cells is shown in the lower left quadrant; the percentages of early and late stages apoptosis cells are shown in the upper right and lower right quadrants, respectively. Data from a representative experiment (from a total of three) are shown.

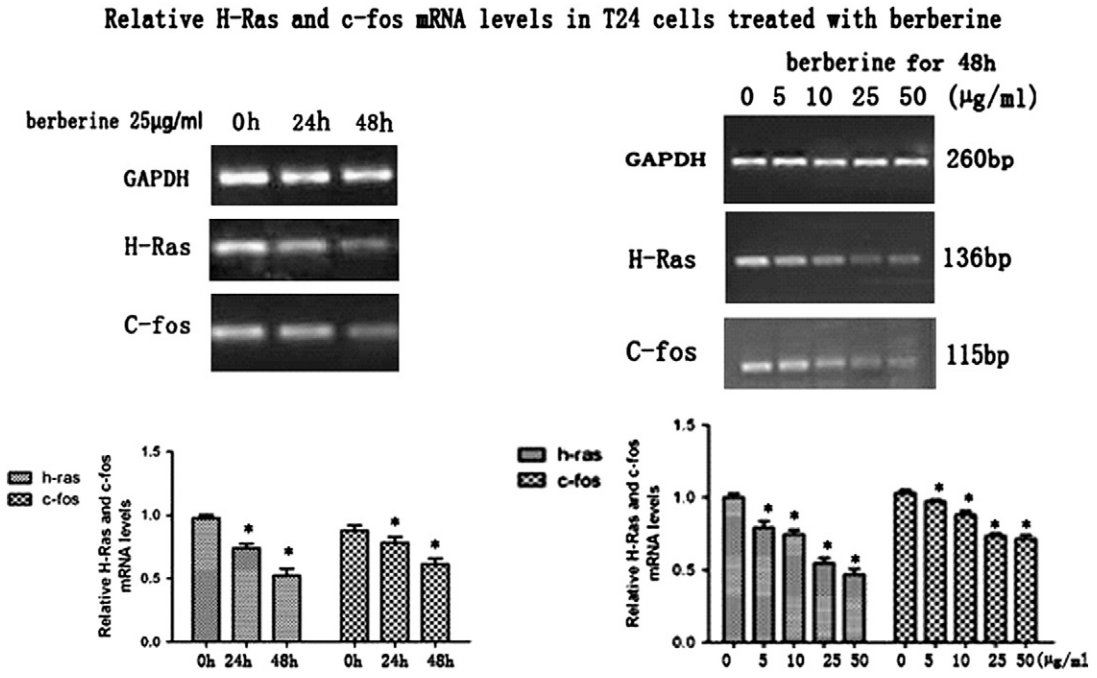

Fig. 5. H-Ras and c-fos mRNA levels were examined in T24 cells treated with berberine in different concentrations and times using RT-PCR. (a) A gradual reduction in both H-Ras and c-fos expressions was observed in the T24 cells treated with 25 μg/ml berberine for 12, 24 and 48 h. (b) A significantly decrease in both H-Ras and c-fos expressions was observed in the T24 cells treated with berberine in dose-dependent manner for 48 h. The data shown were from a representative experiment of three independent experiments and the corresponding standard error. *: $P<0.01$.

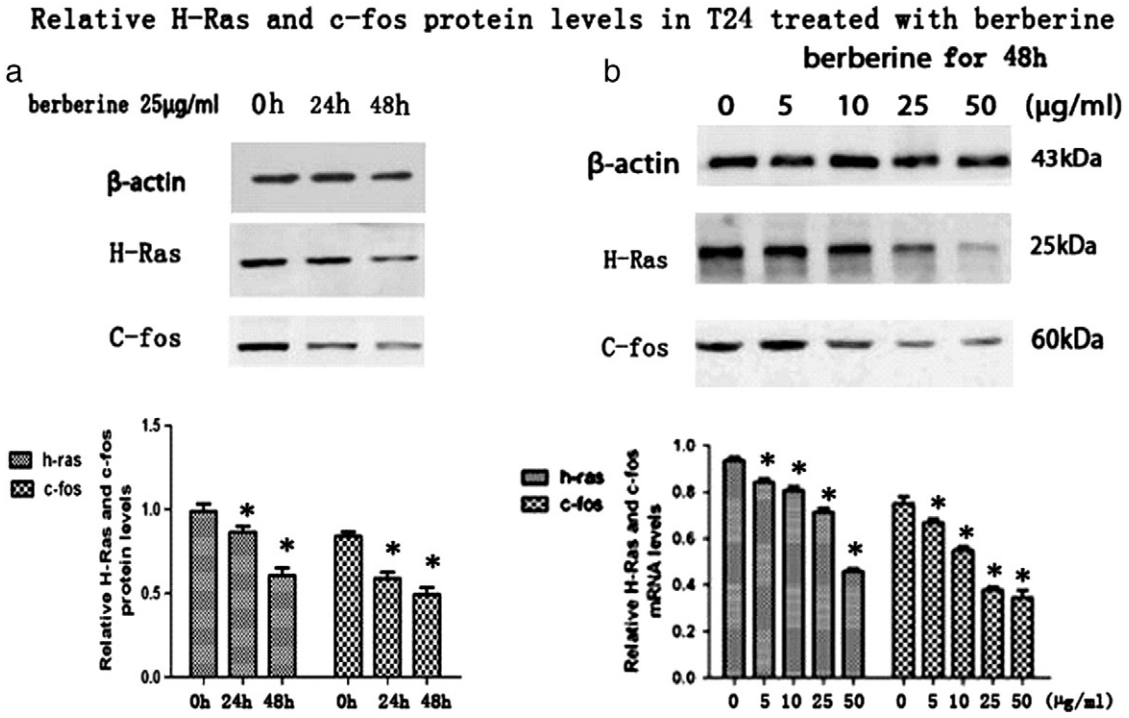

Fig. 6. Relative H-Ras and c-fos protein levels in T24 cells treated with berberine in different concentrations and times using Western blotting. (a) After treated with 25 μg/ml berberine for 24 h and 48 h, the protein was extracted and detected the expressions of the H-Ras and c-fos using Western blotting; the protein levels of the H-Ras and c-fos decreased in time-dependent manner. (b) T24 cells were treated with berberine for 48 h in different concentrations of 0, 5, 10, 25, 50 μg/ml. A gradual reduction in both H-Ras and c-fos expressions was observed in T24 cells in dose-dependent manner. The data represent the means of at least three independent experiments and the corresponding standard error. *: $P<0.01$.

Muralimanoharan et al., 2009; Pandey et al., 2008). Berberine induces caspase-3 dependent apoptosis and caspase-9 dependent apoptosis in human prostate carcinoma cells (Mantena et al., 2006), the human promonocytic U937 cells (Jantova et al., 2007) and human hepatoma cell line SMMC7721 (Wang et al., 2007). We assessed the effect of he berberine on the activation of caspase-3 and caspase-9, and the increased expression of the proteins of cleaved caspase-3 and cleaved caspase-9 in T24 cells in dose-dependent manner. We suggest that berberine induces apoptosis of T24 cells through a caspase pathway. More detailed studies should reveal the pathways that are critical for the induction of apoptosis by berberine.

In summary, our study indicates that berberine inhibits the proliferation of BIU-87 and T24 bladder cancer cells by inhibiting the expressions of the oncogenic H-Ras and c-fos and inducing G0/G1 cell cycle arrest and

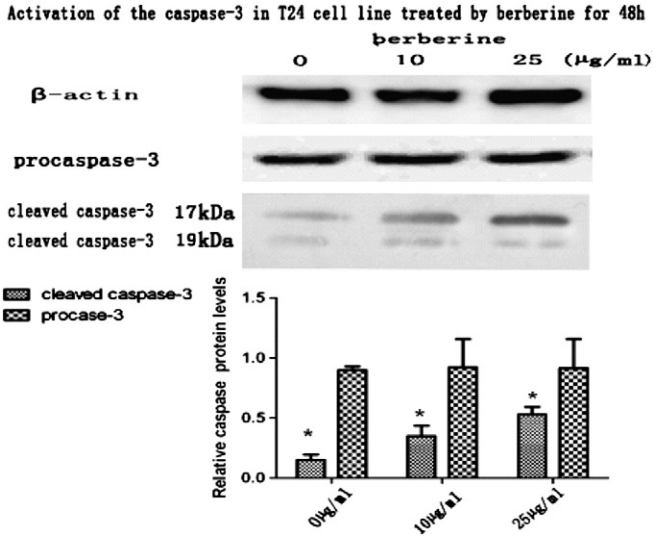

Fig. 7. Berberine induced apoptosis of T24 cells through activating caspase-3. T24 cells were treated with 10 μg/ml or 25 μg/ml berberine for 48 h. Results of western blot showed procaspase-3 protein level have no significant change compared with untreated cells. And the protein level of cleaved caspase-3 significant increased with the increase of the concentration. The data represent the means of at least three independent experiments and the corresponding standard error. *: $P<0.01$.

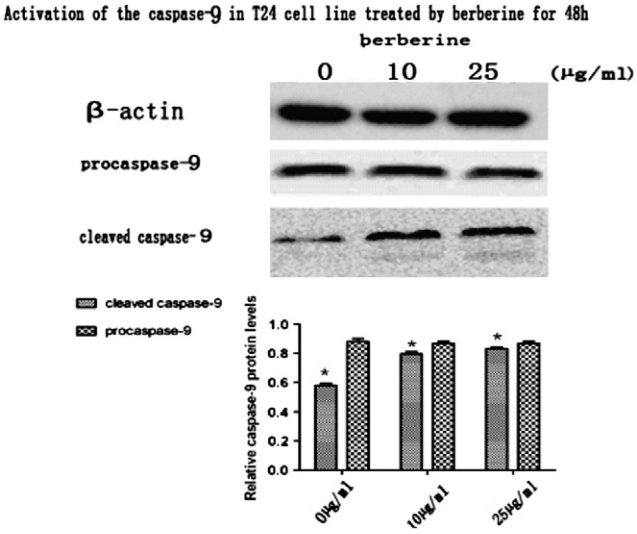

Fig. 8. The activation of the caspase-9 in T24 cells was assessed by western blot. Cells were treated with berberine in 10 μg/ml and 25 μg/ml for 48 h. Western blot showed the protein level of cleaved caspase-9 elevated with the increase of the concentration compared with untreated cells. The data represent the means of at least three independent experiments and the corresponding standard error. *: $P<0.01$.

caspase-3 dependent and caspase-9 dependent apoptosis. It has the therapeutic potential for bladder cancer treatments by suppressing tumor growth.

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