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Original Article

Benzalkonium bromide induces caspase-dependent cell death mediated via the mitochondrial pathway in bladder carcinoma cells

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Abstract: Previously, we reported that benzalkonium bromide (BB) may be a novel agent for a single, immediate post-operative administration in bladder cancer. Here, we further investigate the anti-cancer effects of BB on bladder carcinoma cells and elucidate the underlying mechanisms. Human bladder carcinoma cell lines 5637 and T24 were used as the tumor model system, and normal bladder cell line SV-HUC-1 was used as a control in vitro. We found that BB could inhibit proliferation, arrest the cell cycle in the G0/G1 phase, and induce apoptosis in bladder carcinoma cells. However, these effects were weaker in SV-HUC-1 cells. An RT-PCR analysis showed that BB treatment led to decreased transcription of bcl-2 and increased transcription of bax, Cyt-c, caspase-3, and caspase-9. Moreover, the migration capacity of 5637 cells was inhibited by the BB treatments as well. These results together suggested that BB may induce caspase-dependent cell apoptosis that is mediated via the mitochondrial pathway in bladder carcinoma cells, and that it may be a promising drug for the intravesical treatment of bladder cancer.

Keywords: Bladder cancer, benzalkonium bromide, proliferation, apoptosis, mitochondrial

Introduction

Bladder cancer is the most common malignancy of the urinary tract, the 11th most commonly diagnosed cancer, and the 14th leading cause of cancer deaths worldwide [1]. Approximately 75% of patients with bladder cancer present with a non-muscle-invasive bladder cancer that is either confined to the mucosa (stage Ta, carcinoma in situ CIS) or the submucosa (stage T1) [2]. The European Organization for Research and Treatment of Cancer (EORTC) published the disease recurrence rates for non-muscle invasive bladder cancer (NMIBC), which ranged from 15 to 61% at one year, increasing to 31-78% at five years [3]. Intravesical therapy is widely used as an adjuvant measure to prevent recurrence after transurethral resection, but the overall effect is not satisfactory [4]. Decreasing the high recurrent rate of this disease, including finding new intravesical drugs, are still big challenges for urologists and oncologists.

Benzalkonium bromide is a quaternary ammonium bactericide and a cationic surfactant. This material has an effective bactericidal action and low toxicity, and is non-irritating to skin and tissue. It is widely used as surface disinfectant in hospitals [5]. Other quaternary ammonium compounds, such as benzalkonium chloride (BAC), exhibit significant activity against human tumor cells and normal cells [6, 7]. Benzalkonium salts such as benzalkonium bromide not only appear to be effective as disinfectants and spermicides but may also prove useful in the prevention and treatment of neoplasias and other diseases, particularly those linked to viruses and originating at the skin or mucosal surface [8]. Previously, we reported that benzalkonium bromide could reverse damage to the bladder mucosa and consequently be used as a novel intravesical medicine [9]. Though the mechanisms of benzalkonium bromide are unclear, researchers found that BAC caused mitochondrial injury [10] and activated

caspase-dependent and -independent pathways [11]. Therefore, we considered whether BB could induce the bladder cancer cell apoptosis as well.

Generally, mitochondrial damage could lead to the release of CytC and the activation of apaf-1, caspase-9, and caspase-3, one of the additional pathways involved in AIF. These two pathways participate in the BAC-induced death phenotype supported by Buron et al. [11]. In this study, we hypothesized that BB could activate mitochondrial apoptotic pathways and induce bladder cancer cell damage accordingly. This study focuses on the mechanisms of BB to induce the apoptosis of bladder cancer cells through a mitochondrial pathway.

Materials and methods

Reagents and antibodies

Benzalkonium bromide (5% solution in water) was purchased from Nanchang Baiyun Pharmaceutical Co. (China). Ham's F-12 medium, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) and Mitomycin C (MMC) powder were ordered from Sigma-Aldrich (St. Louis, USA). RPMI-1640 medium, fetal bovine serum, and penicillin-streptomycin were purchased from Gibco (Grand Island, NY, USA). An Annexin V-FITC apoptosis detection kit was purchased from Abcam (Cambridge, UK).

Cell lines and culture conditions

The human bladder urothelial cell carcinoma cell lines 5637 and T24 and the SV-HUC-A cell line were ordered from the American Type Culture Collection (ATCC). The cells were used fewer than 6 months after receipt or resuscitation. The T24 and 5637 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum and 1% of penicillin-streptomycin at 37°C, in humidified air containing 5% CO_2. The SV-HU-1 cells were cultured in Ham's F-12 medium supplemented with 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin at 37°C, in humidified air containing 5% of CO_2. The cells were seeded in 60 mm plastic tissue culture dishes, allowed to grow to 80% confluence and then treated with different concentrations of BB or MMC.

Cell viability assay

The viability of cells treated with BB was determined by the MTT assay. Briefly, the cells were seeded in 96-well microtiter plates (5×10^3 cells/well), with 200 µl of complete culture medium. After incubating overnight, the cells were treated with different concentrations of BB with three replicates for each concentration. The positive control contained 100 µg/ml MMC, and a negative control was also included. After culturing for 24, 48, or 72 h, the cell viability was determined by adding 20 µl MTT (5 mg/ml) to each well followed by incubation for 4 h. After careful removal of the medium, 100 µl DMSO was added to each well and shaken carefully. The absorbance was recorded on a microplate reader (Bio-Rad, USA) at a wavelength of 490 nm. The results represent the average of five parallel samples.

Cell apoptosis analysis

Flow Cytometry (FCM) apoptosis analysis. Cells were seeded at an initial concentration of 1×10^6/ml in a 6-well plate. After culturing for 24 h, the cells were treated with 2.5 and 5 µg/ml of BB. MMC (100 µg/ml) was used as a positive control and a negative control was also included. The cells were collected after 48 hours by trypsinization and washed twice with cold phosphate-buffered saline (PBS). Cells were suspended with 200 µl of binding buffer, and then 10 µl Annexin V-FITC, and 5 µl protein inhibitor (PI) were added before incubation in the dark for 30 min. A binding buffer (300 µl) was then added, and apoptosis was then immediately analyzed using FCM (model, FACSCalibur, USA) and ModfitLT3.0 software.

Cell cycle analysis

The T24, 5637, and SV-HUC-1 cells were treated with different concentrations of BB and different concentrations of MMC for 48 h. The cells were then harvested by trypsinization, washed twice in cold PBS, and fixed in 70% ethanol at 4°C overnight. After fixation, the cells were washed and resuspended in cold PBS and incubated in a solution of 10 mg/ml RNase and 1 mg/ml propidium iodide (PI) at 37°C in the dark for 30 min. Finally, the samples were analyzed by using FCM (model, FACSCalibur, USA). Data for the cell cycle analysis were analyzed using ModFit LTTM software (Verity Software House, Inc., Topsham, ME, USA) to determine the proportion of cells in the G0/G1, S and G2/M fractions of the cell cycle. The mean ± SD was calculated for the cell populations from triplicate data.

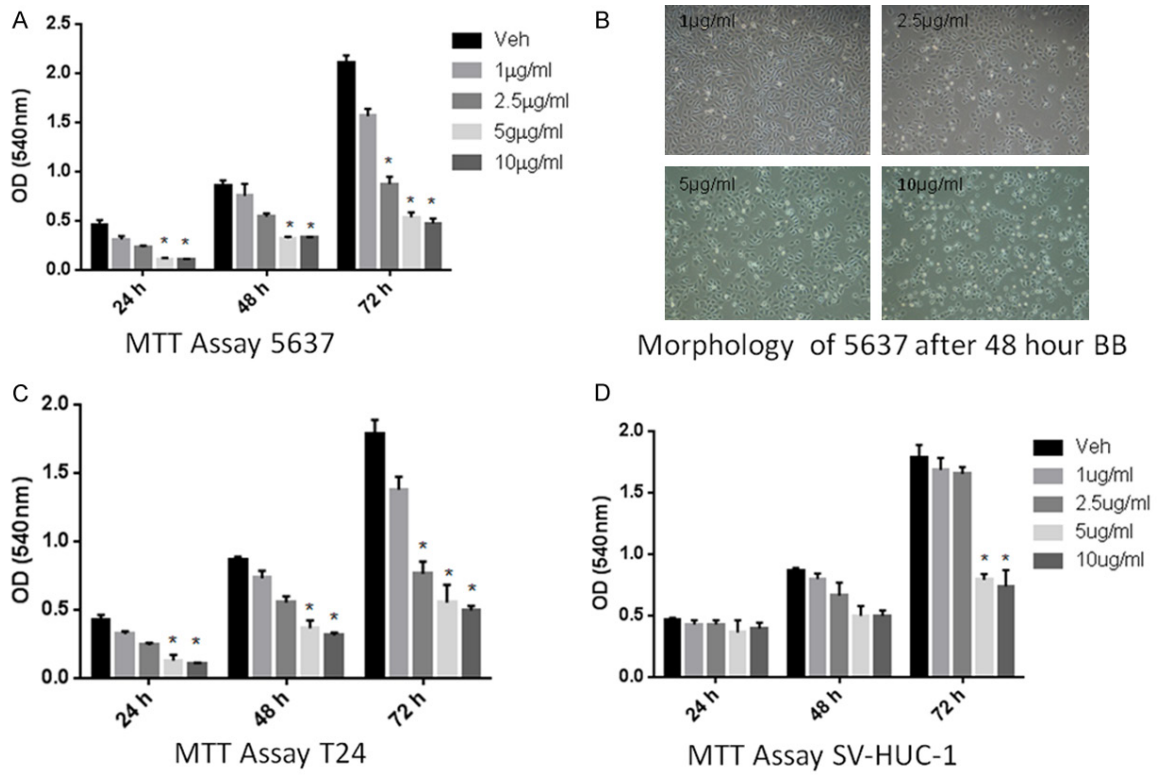

Figure 1. The inhibitory effect of BB on 5637 and SV-HUC-1 cells detected by an MTT assay. 5637 cells (A), T24 cells (C), and SV-HUC-1 (D) cells were treated with different concentrations of BB (1, 2.5, 5, 10 μg/ml) at three time points (24, 48 and 72 h). (B) Representative images of the cell morphology of 5637 after 48 hours of BB treatment. All data were from three independent experiments. *P < 0.05 vs. control.

Real-time PCR analysis

The mRNA expressions of bcl-2, bax, Cyt-c, Apaf-1, caspase-3, caspase-9, PARP-1, AIF, and Endo G were performed by RT-PCR. For the bcl-2, bax, cyt-c, apaf-1, caspase-3, caspase-9, parp-1, AIF, Endo G genes and Gapdh gene, the cDNA products were then amplified by PCR using the primers as shown in Supplementary Table 1. Real-time PCR reactions were performed with the iQ5 Q-PCR system (Bio-Rad). The cycling conditions were as follows: 95°C for 120 s followed by 40 cycles of 95°C for 15 s, 59°C for 20 s, and 72°C for 20 s. After PCR, a melting curve analysis was performed to demonstrate the specificity of the PCR product, which was displayed as a single peak (data not shown). Every sample was analyzed in triplicate. Cycle threshold (Ct) values were normalized to GAPDH, and comparative quantification of target mRNA was done by the ΔΔCt method. The relative expression ratio (R) of a target gene was expressed for the sample vs. the control in comparison to the GAPDH gene. The value R was calculated based on the following equation: $R = 2^{-\Delta\Delta Ct}$, where Ct represents the cycle number at which the fluorescence signal is first significantly different from background and ΔΔCt is (Cttarget - CtGAPDH) treatment - (Cttarget - CtGAPDH) control.

Statistical analysis

Data in all experiments are demonstrated as the means ± SD. Statistical difference was calculated using a one-way or two-way ANOVA and independent *t*-test of sample pairs with Graph Pad 5 software, P < 0.05 was considered statistically significant.

Results

BB inhibits the proliferation of bladder cancer cell lines

To determine whether BB is a potential bladder cancer treatment agent, its effects on the growth of bladder cancer cell lines were studied, including 5637 and T24 bladder tumor cell lines. As shown in **Figure 1A**, BB treatment

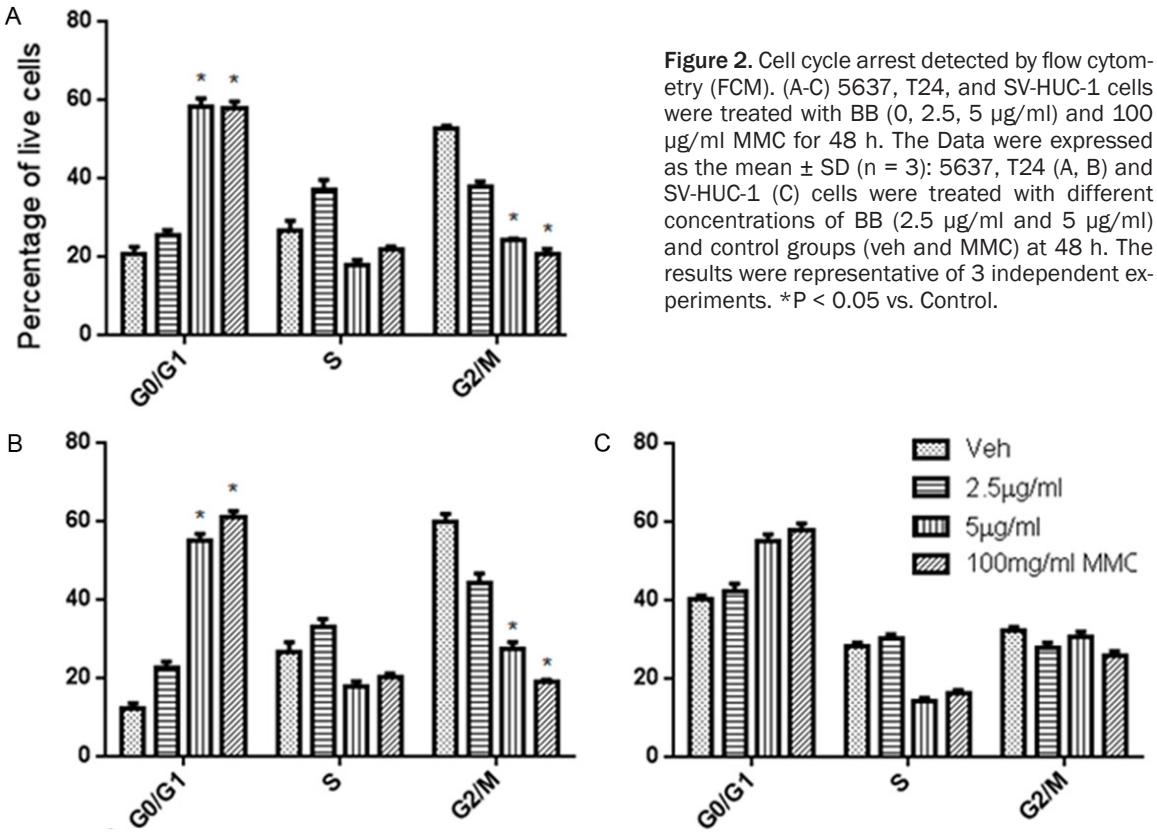

Figure 2. Cell cycle arrest detected by flow cytometry (FCM). (A-C) 5637, T24, and SV-HUC-1 cells were treated with BB (0, 2.5, 5 μg/ml) and 100 μg/ml MMC for 48 h. The Data were expressed as the mean ± SD (n = 3): 5637, T24 (A, B) and SV-HUC-1 (C) cells were treated with different concentrations of BB (2.5 μg/ml and 5 μg/ml) and control groups (veh and MMC) at 48 h. The results were representative of 3 independent experiments. *P < 0.05 vs. Control.

inhibited the growth of 5637 cells in a dose- and a time-dependent manner. Much stronger cell growth inhibition was observed at the 10 μg/ml dose of BB treatment at 72 h; however, cell growth was minimally affected by the 1 μg/ml dose of BB compared to the control group. Furthermore, we tested another bladder tumor cell line, T24, with the same interventions as with the 5637 cells. Similar results were observed as shown in **Figure 1C**, which reflected the stable inhibitory effect of BB on bladder tumor cells. Moreover, in order to detect the influence of BB on normal bladder cells, the same experiment was conducted using SV-HUC-1 cells, which is a normal bladder cell line. As shown in **Figure 1D**, low concentrations of BB (1.25 and 2.5 μg/ml) barely suppressed the viability of SV-HUC-1 cells compared to the control group; however, the inhibitory effect increased significantly when the concentration was upregulated to 5 or 10 μg/ml. Consequently, the concentration of BB was set at 2.5 and 5 μg/ml in the following experiments.

BB arrests bladder cancer cells in the G0/G1 phase of the cell cycle

To investigate the impacts of BB on cell cycle progression, cell cycle distribution was evaluated by flow cytometry. The results showed that BB significantly arrested cell cycle progression in 5637 (**Figure 2A**) and T24 (**Figure 2B**) cells by increasing the percentage of cells in the G0/G1 phase. Compared with the untreated control, the percentage of 5637 cells in the G0/G1 phase was increased from 20.67% to 58.33% after 48 h of treatment with 5 μg/ml of BB (**Figure 2A**). A significant increase in G0/G1 phase cells was also observed when the 5637 cells were treated with 100 mg/ml MMC after 48 hours; however, a low concentration of BB (2.5 μg/ml) has much less ability to arrest 5637 cells in the G0/G1 phase than the first two interventions. Similar results were observed in the T24 cells (**Figure 2B**). However, in SV-HUC-1 cells (**Figure 2C**), although 5 μg/ml of BB and 100 mg/ml of MMC treatment increased the arrest of cells in the G1/G0 phase, the increases were much less than those observed in the 5637 and T24 bladder tumor cells.

BB increases apoptosis of bladder cancer cells

In order to assess whether BB also induces the apoptotic death of bladder tumor cells, we treated the 5637 cells with BB under similar conditions as above, and then analyzed the cells by flow cytometry following Annexin V and

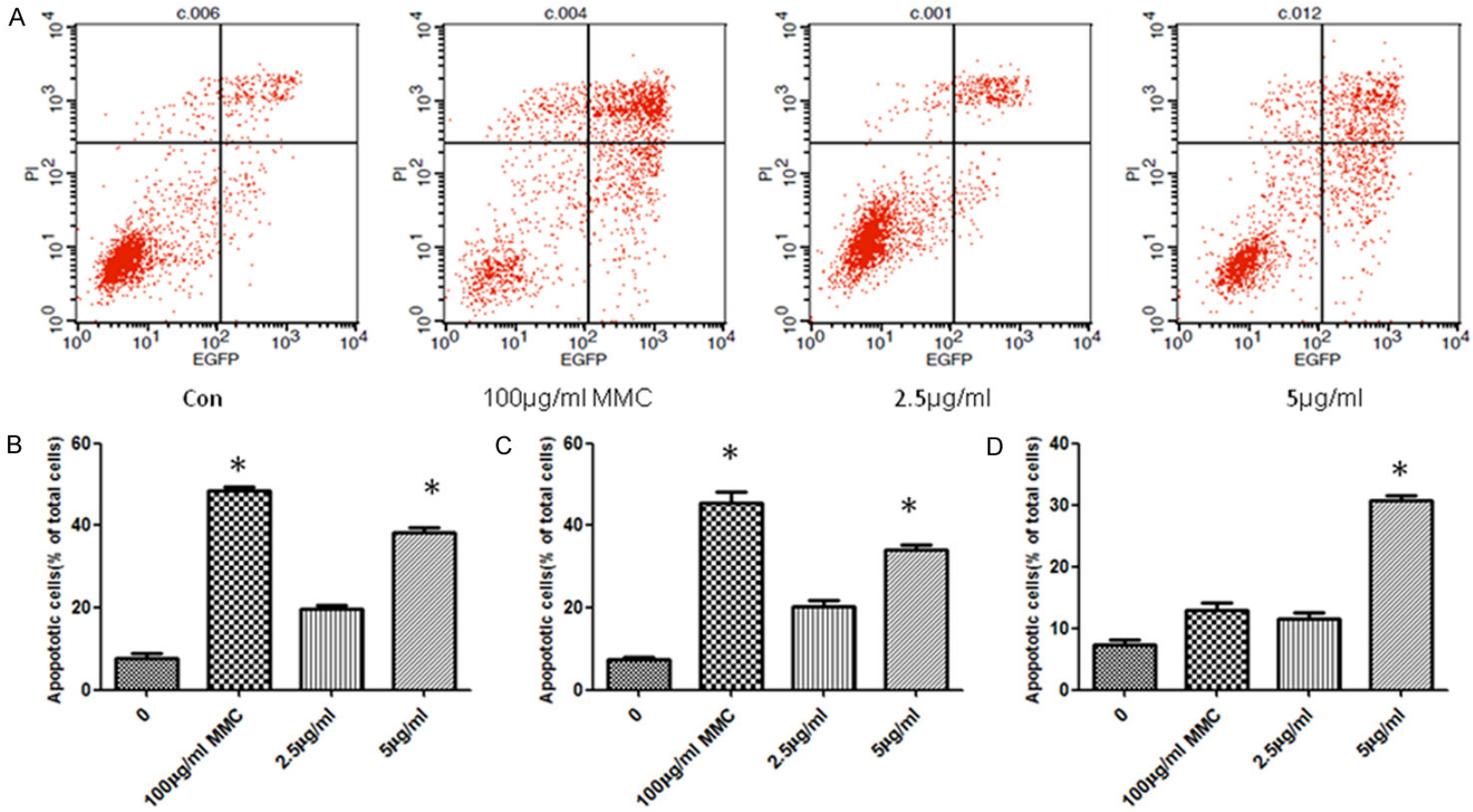

Figure 3. BB induced apoptosis was detected by flow cytometry (FCM). (B, C) 5637 cells and T24 cells were treated with different concentrations of BB (2.5 μg/ml; d, 5 μg/ml), MMC (100 μg/ml) and control group at 48 h. (D) SV-HUC-1 cells were treated with different concentrations of BB (2.5 μg/ml, 5 μg/ml) and the control group at 48 h. The apoptotic peak observed in the FCM analysis is shown (A). The percentage of apoptotic cells is presented as the mean ± SD (n = 3); The results shown are representative data from 3 independent experiments. *P < 0.05 vs. Control.

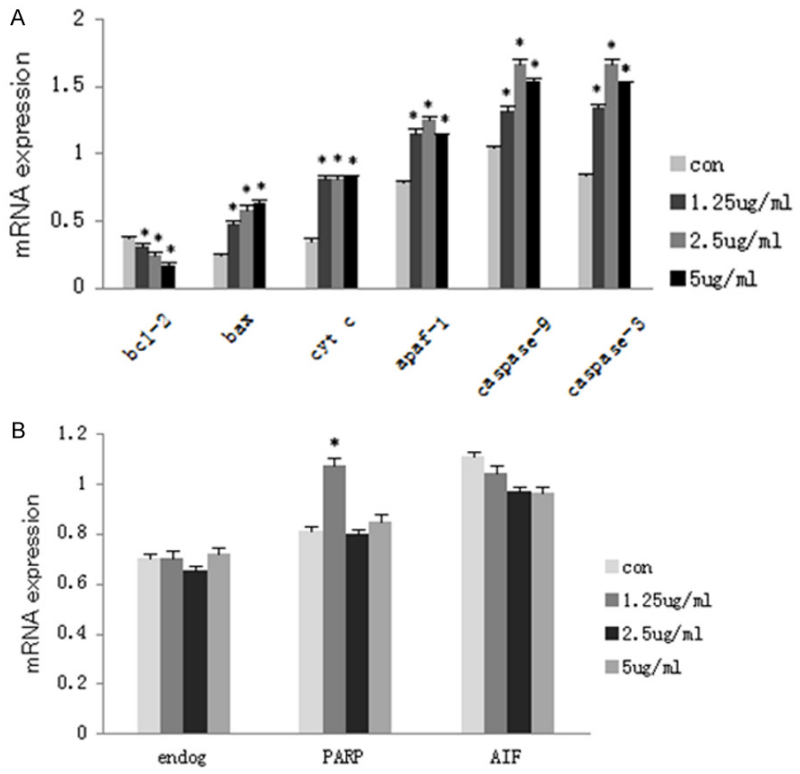

Figure 4. The intensity of bcl-2, bax, cyt-c, apaf-1, caspase-9, caspase-3, and endog, PARP AIF mRNA bands were determined and normalized with actin's intensity by using the UVP imager. The results shown are representative data from 3 independent experiments. *P < 0.05 vs. control.

PI staining. As shown in **Figure 3B**, the 5 μg/ml concentration of BB effectively induced a 64.89% apoptotic cell population in the 5637 cells, and similar results were observed when the 5637 cells were treated with 100 μg/ml MMC. A similar trend was observed in the T24 cells as well (**Figure 3C**). Nevertheless, BB could also induce apoptosis in the SV-HUC-1 cells, but the effect was much less than what was seen in the 5637 and T24 cells (**Figure 3D**).

BB-induced apoptosis via the regulation of caspase and Bcl-2 family members in bladder cancer cells

The relative mRNA expressions are shown in **Figure 4A**. Obviously, our RT-PCR analysis showed that the transcription expression of bcl-2 gradually decreased after BB treatment for 48 h in the 5637 cells, while the transcription expression of bax, Cyt-c, Apaf-1, caspase-3, and caspase-9 increased. Moreover, the transcription expression of endog, PARP, and AIF had no obvious change compared to the control (**Figure 4B**). This suggests that BB may induce the caspase-dependent cell death of 5637 cells mediated via the mitochondrial pathway.

BB inhibits bladder cancer cell migration

To investigate whether BB affects the migration capacity of 5637 cells, transwell experiments were conducted under different treatments (**Figure 5**). The results showed that BB and MMC significantly decreased the migration of 5637 cells compared to the control group.

Discussion

The goals of the present study were to demonstrate the in vitro anticancer effects of benzalkonium bromide on human bladder cancer cells and to elucidate the underlying mechanisms. In the pre-experiments, we found that high concentrations can directly cause cell necrosis (≥ 1000 μg/ml) (data not shown). Our results revealed that low concentrations of benzalkonium bromide had anti-cancer effects on bladder carcinoma cells, including inhibiting proliferation and inducing apoptosis and cell cycle arrest in a time and dose dependent manner. The Inhibitory effect of 5 μg/ml BB was similar to 100 μg/ml mitomycin, actually. 5 μg/ml BB and 100 μg/ml MMC had a similar G0/G1 cell cycle arrest after administration for 24 h, and the arrest effect of BB became stronger after 48 h. Our findings are original and have not been reported in the English literature, and it is the first study to show that BB can induce apoptosis in bladder cancer cells.

Clouzeau et al. confirmed that BAC involves two major apoptotic pathways, a caspase-dependent one, characterized by cytochrome c release from the mitochondria to the cytoplasm and the presence of active caspase-3, and a caspase-independent one that uses AIF binding to DNA and leading to chromaten conden-

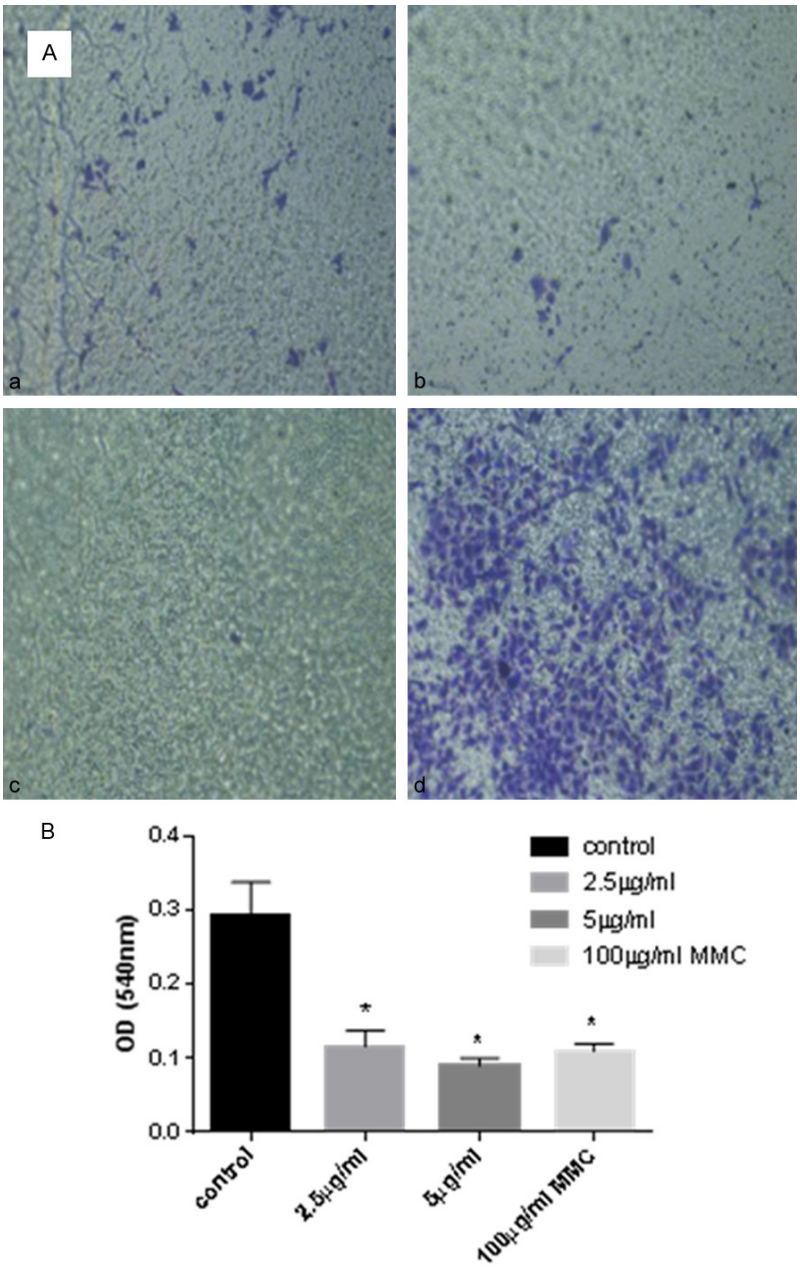

Figure 5. BB induced decreasing cell migration was detected by the transwell experiments. A: 5637 cells were treated with different concentrations of BB (a, 2.5 μg/ml; b, 5 μg/ml), MMC (c, 100 μg/ml) and the control group (d, control) at 48 h. B: The OD of 540 nm were expressed as the mean ± SD (n = 3). The results shown are representative data from 3 independent experiments. *P < 0.05 vs. control.

sation and cell death [12]. Our study showed that BB treatment decreased the viability of 5637 and T24 cells and induced caspase-dependent apoptotic signaling pathways as indicated by an increase in caspase transcriptional activity, such as the Cyt-c, apaf-1, caspase-9, and caspase-3 gene levels. Here, apoptosis induced by BB and BAC was found to have comparable mechanisms to the caspase-dependent pathway. But the expressions of PARP-1, AIF, and the Endo G gene had no obvious regularity indicating that the BB induced apoptosis pathway may not be associated with the caspase-independent pathway.

These results support the hypothesis that BB may be a novel agent for use in a single, immediate post-operative administration in bladder cancer treatment [9]. Benzalkonium salts are clinically and hygienically used for the control of bacterial growth [13]. Benzalkonium salts consist of a group of positively charged surface-active alkylamine biocides with the general formula alkyldimethyl benzylammonium chloride or bromide. They interact with guanine nucleotide triphosphate-binding proteins (G proteins), thereby affecting signal transduction in a variety of cell types and processes [14]. Therefore, benzalkonium salts not only appear to be effective as disinfectants and spermicides but may also prove useful in the prevention and treatment of neoplasias and other disease, particularly those linked to viruses and originating on the skin or mucosal surface [8]. BB, which is similar to benzalkonium salt as a derivative of benzalkonium, in our study indicated that a certain concentration range of BB can inhibit the growth of bladder cancer cells. Patients with non-muscle invasive bladder suffered a high recurrence (1 year: 15-61%; 5 years: 31-78%) [3]. Decreasing the high rate of recurrence of this kind of bladder tumor is always a big challenge for urologists. One immediate post-operative admi-

nistration of chemotherapy should be given to all patients after TUR of presumably non-muscle invasive bladder cancer, but, even though it reduces the rate of recurrence, it still has a limited role [3]. Considering the mechanism of recurrence of bladder cancer, clonal planting and field cancerization are involved, if a drug for two kinds of mechanisms has a great role, it will improve the curative effect and reduce the recurrence rate. On the other hand, BB could directly undermine the bladder mucosa, inducing the apoptosis of tumor cells, which seems to be the ideal drug but which needs to be confirmed further. Furthermore, further animal models should be applied to confirm these results.

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Disclosure of conflict of interest

None.

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Supplemental Table 1. The prime of bcl-2, bax, cyt-c, apaf-1, caspase-3, caspase-9, parp-1, AIF, Endo G genes and Gapdh

Gene	Sequence	(bp)	Product length
Bcl-2	Forward primer	GTGGAGGAGCTCTTCAGGGA	304
	Reverse primer	AGGCACCCAGGGTGATGCAA	
Bax	Forward primer	GGCCCACCAGCTCTGAGCAGA	477
	Reverse primer	GCCACGTGGGCGTCCCAAAGT	
Cyt-c	Forward primer	TGTGCCAGCGACTAAAAAGA	103
	Reverse primer	CCTCCCTTTTCAACGGTGT	
Apaf-1	Forward primer	TGGCATTCCTGTTGTCTCTTCTT	179
	Reverse primer	ATCCTGGTTCACCTTTCAATTTG	
Caspase-3	Forward primer	GGAAGCGAATCAATGGACTCTG	170
	Reverse primer	CTGAATGTTTCCCTGAGGTTTGC	
Caspase-9	Forward primer	GCCAACCCTAGAAAACCTTACC	123
	Reverse primer	GCACCGACATCACCAAATCCT	
Parp-1	Forward primer	TGATTGAGAAACTCGGGGG	132
	Reverse primer	ACACAACTCGGATGTTGGCT	
AIF	Forward prime	TCCAGCCACCTTCTTTCTATGTCT	236
	Reverse primer	AAGCGTTGTTCTACTCTTCACCTC	
Endo-G	Forward primer	GAACAACCTGGAGAAATATAGCCG	176
	Reverse primer	GGATCAGCACCTTGAAGAAGTG	
Gapdh	Forward primer	GGGTGTGAACCATGAGAAGTATG	145
	Reverse primer	GATGGCATGGACTGTGGTCAT	