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Original Research Article

Berberine hydrochloride inhibits bladder cancer cells and induces apoptosis by inhibiting the PI3k/Akt signal route

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Abstract

Purpose: *To study the effect of berberine hydrochloride on G2/M phase of the cell cycle, and the associated mechanism of action.*
Methods: *Human bladder cancer cell line UMUC3 was cultured with graded concentrations of berberine hydrochloride (50, 100, and 200 μM) for 24 h. Cell cycle distribution and apoptosis of UMUC3 cells were analyzed by flow cytometry. Levels of PI3K/Akt signal route-associated proteins in UMUC3 cells were determined using immunoblotting.*
Results: *There were higher numbers of G2/M phase cells in each berberine hydrochloride dose group than in the control cells, and it increased with increase in berberine dose. The population of G0/G1 phase cells in each berberine dose group was significantly and dose-reliantly lower than control value. Increase in berberine dose resulted in increase in p21 protein expression, while protein expressions of CDK1, cycling B1, and CDC25C were reduced ($p < 0.05$). Apoptosis level was significantly higher in each dose group and was accentuated with increase in berberine dose ($p < 0.05$). Immunoblot results showed that with increase in dose, there was up-regulation in protein expressions of Bax, and c-caspases-9/3, while the protein expressions of bcl-2, caspases-3/9, and PARP were reduced. Western blot assay data showed that expressions of PI3K p85 and p-Akt proteins decreased, while protein expression level of PTEN increased with increase in berberine dose. In contrast, Akt protein levels were comparable in all groups.*
Conclusion: *Berberine hydrochloride induces arrest of bladder cancer cells at G2/M phase and accelerates their apoptosis via a mechanism related to inhibition of PI3k/Akt pathway. Thus, berberine hydrochloride has potentials for use as a drug for the management of bladder cancer.*

Keywords: *Berberine hydrochloride, PI3kakt pathway, Bladder cancer cells, G2M phase arrest, Apoptosis*

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INTRODUCTION

Bladder cancer is a malignancy associated with the bladder mucosa, and the most common cancer in the urinary system [1]. In China, the incidence of bladder cancer in men ranks seventh among systemic malignant tumors, while that in women ranks more than tenth. The incidence of bladder cancer increases with age, with a high incidence in the age range of 50 - 70

years [2]. Bladder cancer is treated with surgery, and reducing the recurrence and metastasis of bladder cancer is of great significance for prolonging the lives of patients [3].

The PI3k/Akt signal pathway enhances cell survival, regulates cell cycle, inhibits apoptosis, and promotes tumor angiogenesis, invasion, and metastasis [4]. Berberine has several pharmacological properties such as antioxidant, anti-inflammatory, cholesterol-lowering, anti-diabetic, anti-obesity, and antibacterial activities [5]. In addition, its anticancer effect and mechanism of action have been widely studied. The results obtained in these studies suggest that berberine may become a drug for the prevention and treatment of breast, lung, gastric, liver, colorectal, ovarian, cervical, and prostate cancer [6,7]. However, not much work has been done on its impact on bladder carcinoma. The present research investigated the effect of berberine hydrochloride on G2/M phase of cell cycle and apoptotic changes in bladder carcinoma cell line UMUC3, as well as the underlying mechanism.

EXPERIMENTAL

Materials

Human bladder cancer cell line UMUC3 was provided by Shenzhen Haodi Huatuo Biotechnology Co. Ltd. Berberine hydrochloride was obtained from Shanghai Baoman Biotechnology Co. Ltd.

Cell culture experiments

Cells in logarithmic growth phase were digested and blown into single-cell suspension, and the cells were plated and cultured for 12 h at 37 ^{o}C in a medium of 5 % CO_2 so as to make the cells adhere to the wall. Thereafter, the culture medium was changed, and berberine hydrochloride solutions of concentrations of 10, 15, and 20 µM were added to different wells, followed by a 24-h culture. There was no berberine hydrochloride in control well.

Determination of parameters

Assessment of UMUC3 cell cycle distribution by flow cytometry

The cells were routinely harvested, centrifuged, pre-cooled with PBS, and washed twice. Then, cell suspension in PBS (0.2 mL) was made into a single cell suspension, followed by fixing in 2 mL of 70 % ice-cold ethanol and refrigeration overnight at 4 ^{o}C. The supernatant was removed via centrifugation, after which the cells were rinsed twice with pre-cooled PBS, followed by the addition of 50 µL of RNase and 450 µL of PI staining solution. The mixture was thoroughly shaken and incubated at room temperature for 15 min in the dark, for DNA detection. The cell proportion in each cell cycle phase was determined with Modifit software.

Analysis of apoptosis rate of UMUC3 cells by flow cytometry

After routinely rinsing the cells with PBS and centrifuging at 1000 g for 5 min, half of cell suspension in binding buffer was put in each of 2 tubes, with 200 µL in each tube. One of the tubes served as blank which was used to adjust the instrument, while the cells in the other tube were incubated with FITC-labeled Annexin V reagent in a dark chamber for 15 min at room temperature, followed by incubation with PI for another 10 min in the dark. The BD facsuite software was employed for analysis of UMUC3 cell apoptosis in each group.

UMUC3 cell cycle, apoptosis and PI3k/Akt pathway-related protein expressions

The cells were incubated with graded levels of berberine hydrochloride in a 6-well plate for 24 h, after which the liquid medium in the cell culture plate was discarded. Then, after washing with PBS, the 6-well plates were placed on ice, followed by lysing with RIPA120. After spinning at 12,000 rpm for 20 min at 4 ^{o}C, lysate protein concentration was determined with bicinchoninic acid procedure, after which SDS-PAGE was used for protein resolution. The bands were electro-transferred to PVDF diaphragms which were subsequently incubated with 5 % skimmed milk, after which a 12-h incubation at 4 ^{o}C with 1^{o} immunoglobulins against P21, CDKI, Cyclin BI, bax, bcl-2, caspase-9, caspase-3, PARP, PI3K, Akt, p-Akt and CDV25C, was done. Then, the membranes were incubated with HRP-linked 2^{o} immunoglobulin for 120 min at laboratory temperature. Visualization of bands was done using chemiluminescence image processing software, followed by photographing and analysis using image processing software.

Statistical analysis

Data analysis was done with SPSS version 20.0 software package. All measurement data are expressed as mean ± SD, and *t*-test was used for two-group comparison. Count data are expressed as numbers and percentages, n (%), while comparisons amongst groups were carried

out by chi-squared (χ^2) test. Differences were assumed to be statistically significant at $p < 0.05$.

RESULTS

UMUC3 cell cycle distribution amongst the groups

As shown in Table 1, there was significantly higher population of G2/M phase cells in each berberine hydrochloride dose than in control cells, and it increased with increase in berberine dose. However, there was a significantly lower number of cells at G0/G1 phase in each group than the corresponding number in control cells, and it decreased with increase in berberine dose.

Expression levels of UMUC3 cell cycle-related proteins

Immunoblot assay of G2/M phase-related cyclins showed that with increase in berberine dose, p21 protein level was raised, while protein levels of CDK1, cyclin B1, CDC25c decreased ($p < 0.05$; Table 2).

Apoptosis of UMUC3 cells

The apoptosis rate was significantly higher in each berberine dose group than in control cells and was accentuated with increase in berberine dose ($p < 0.05$; Table 3).

Table 3: Apoptosis rate of UMUC3 cells in each group (mean ± SD)

Group	Apoptosis level
Control	3.46±0.16
Low-dose	8.97±0.82[a]
Medium-dose	27.64±3.48[ab]
High-dose	53.61±10.15[abc]

[a]$P < 0.05$, vs. control; [b]$p < 0.05$, vs. low-dose; [c]$p < 0.05$, vs. medium-dose group

Expression levels of apoptosis-related proteins in UMUC3 cells

Immunoblot analysis of apoptosis-related proteins in UMUC3 cells showed that with increase in berberine dose, there were increases in protein expression levels of Bax, c-caspase-9, and c-caspase-3, while protein levels of Bcl-2, caspases-3/-9, and PARP decreased ($p < 0.05$; Table 4).

Expression levels of PI3k/Akt pathway-related proteins in UMUC3 cells

Western blot assay of Akt/PI3K signal route-associated proteins in UMUC3 cells showed that with increase in dose, the protein expression

Table 1: UMUC3 cell cycle distribution in each group

Group	G2/M	S	G0/G1
Control	7.58±0.58	24.93±2.46	67.49±5.85
Low-dose	14.52±1.02a	45.33±4.61	40.15±4.16a
Medium-dose	23.76±2.74ab	47.77±3.91	28.47±3.58ab
High-dose	35.21±3.46abc	46.15±3.42	18.64±1.28abc

[a]$P < 0.05$, vs. control; [b]$p < 0.05$, vs. low-dose group; [c]$p < 0.05$, vs. medium-dose group

Table 2: Levels of UMUC3 cell cycle-related proteins in each group (mean ± SD)

Group	P21	CDK1	Cyclin B1	Cdc25c
Control	0.46±0.11	1.21±0.34	1.18±0.23	1.17±0.27
Low-dose	0.65±0.23[a]	0.86±0.25[a]	0.77±0.19[a]	0.81±0.23[a]
Medium-dose	0.87±0.26[ab]	0.63±0.16[ab]	0.59±0.20[ab]	0.63±0.21[ab]
High-dose	1.13±0.32[abc]	0.47±0.13[abc]	0.35±0.14[abc]	0.46±0.18[abc]

[a]$P < 0.05$, vs. control; [b]$p < 0.05$, compared with the low-dose group; [c]$p < 0.05$, compared with the medium-dose group

Table 4: Apoptosis-related protein levels in UMUC3 cells in each group (mean ± SD)

Cell type	Control	Low-dose	Medium-dose	High-dose
Bcl-2	0.89±0.26	0.73±0.24[a]	0.62±0.23[ab]	0.53±0.18[abc]
Bax	1.21±0.34	1.42±0.36[a]	1.66±0.39[ab]	1.79±0.42[abc]
Caspase-9	1.23±0.28	0.89±0.18[a]	0.74±0.21[ab]	0.62±0.18[abc]
C-caspase-9	0.27±0.11	0.46±0.18[a]	0.77±0.28[ab]	1.19±0.32[abc]
Caspase-3	1.24±0.32	0.89±0.28[ab]	0.76±0.24[ab]	0.65±0.21[abc]
C-caspase-3	0.28±0.09	0.49±0.11[ab]	0.76±0.26[ab]	0.98±0.32[abc]
PARP	0.87±0.24	0.68±0.21[a]	0.58±0.19[ab]	0.35±0.23[abc]

[a]$P < 0.05$, vs. control; [b]$p < 0.05$, vs. low-dose; [c]$p < 0.05$, vs. medium-dose group

Table 5: Expression levels of PI3k/Akt pathway-related proteins in UMUC3 cells in each group

Group	PI3K p85	PTEN	P-Akt	Akt
Control	0.82±0.34	0.32±0.11	1.06±0.34	1.06±0.28
Low-dose	0.75±0.27[a]	0.56±0.21[a]	0.84±0.28[a]	1.13±0.21
Medium-dose	0.66±0.25[ab]	0.89±0.37[ab]	0.76±0.21[abc]	1.09±0.18
High-dose	0.49±0.19[abc]	1.19±0.43[abc]	0.43±0.23[abc]	1.10±0.17

[a]$P < 0.05$, vs. control; [b]$p < 0.05$, vs. low-dose; [c]$p < 0.05$, vs. medium-dose group

levels of PI3K p85 and p-Akt decreased, while the expression level of PTEN protein increased ($p < 0.05$). However, Akt protein level was comparable in all groups. These data are depicted in Table 5.

DISCUSSION

Bladder cancer has high recurrence frequency, high progression rate, and high death rate. The pathogenesis of bladder cancer is poorly understood, and the clinical treatment effect is usually unsatisfactory. Therefore, bladder cancer not only brings a heavy economic burden to society and families, but it also severely impacts the quality of life of the patients [8]. Recent studies have demonstrated that Chinese herbal extracts suppress tumors by blocking the cell cycle, inducing apoptosis, reversing multidrug resistance of tumor cells, exerting anti-tumor angiogenesis effect, and improving immunity.

It has been reported that berberine suppressed mammary cancer cell multiplication and reduced the viability of the cells [9]. Moreover, at low concentrations, it triggered cell cycle arrest in TNBC but did not damage normal human breast cells [10]. A study showed that berberine suppressed multiplication of MDA-MB-468 cells and induced cell cycle arrest in G1/S stage through inhibition of CDK4, while the cell growth suppressor p38 was activated. Another study showed that berberine blocked the proliferation of MDA-MB-231 cells and induced cell cycle arrest in G2/M phase by inhibiting cyclin A/CDK1 and cell growth-associated Erk/Akt/ pathway [11]. In addition, berberine down-regulated top2β levels and induced DNA damage in NSCLC cells, thereby inducing apoptosis in these cells [12]. It has been reported that berberine induced apoptotic changes in NSCLC cells via Mir19a/MAPK/TF signal route [13], and induced apoptosis in these cells through activation of ROS-linked, apoptotic signal-controlled kinase 1/JNK and apoptosis pathway of mitochondria [14]. In the present study, there was a significantly higher population of cells in G2/M phase in each dose group than in control cells, and it increased with increase in berberine dose.

The population of cells in G0/G1 stage was significantly lower in each berberine dose group than in control cells, and it decreased with increase in berberine dose. These data suggest that berberine hydrochloride induced G2/M arrest of UMUC3 cells, thereby affecting cell proliferation. The regulation of cell cycle involves cyclins, CDKs, and CDK1s. The CDKs combine with cyclins to form heterodimer complexes. Different cyclin CDK complexes catalyze the phosphorylation of various substrates, thereby enhancing the transformation of different phases of the cell cycle. Abnormal expressions of CDKs and cyclins lead to abnormalities in regulation of the cell cycle, uncontrolled cell proliferation, and ultimately, tumorigenesis. It is only when CDK1 binds to cyclins that it exerts kinase activity. In particular, p21 is a more common class of CDK1 in clinical research [15]. Western blot assay of the expressions of G2/M phase-related cyclins showed that, with increase in berberine dose, the protein expression of p21 increased, while the protein expression levels of CDK1, cyclin B1, and CDC25c decreased. This suggests that berberine hydrochloride induced the expressions of cell cycle-related proteins, affected cell cycle transition, and eventually led to cell death.

Cancer cell apoptosis is one of the mechanisms of action of cytotoxic anti-tumor drugs [16]. In this study, the apoptosis rate was significantly higher at each berberine hydrochloride dose than in the control cells and was raised as berberine dose increased. This result implies that berberine hydrochloride effectively and dose-reliantly induced apoptosis of cancer cells. Apoptosis is strictly controlled by multiple genes. It is a process in which bcl-2 family and caspase family play important roles. It is known that bcl-2 is an anti-apoptotic protein of bcl-2 family, while bax is a pro-apoptotic protein. An increase in Bax/bcl-2 ratio induces the cleavage of pro-caspase-9 into activated caspase-9, leading to the activation of caspase-3. The activation of caspase-3 inactivates PARP, thereby causing DNA breakage [17-19]. In this study, the expressions of apoptosis-related proteins in UMUC3 cells were determined with immunoblotting. With increase in berberine dose, protein amounts of bax, and c-caspases-9/3 increased, while protein expressions of bcl-2, caspase-9, caspase-3, and PARP decreased, suggesting that berberine accelerated apoptosis of bladder cancer cells

through the bcl-2 family and caspase family. Recent investigations have shown that PI3k/Akt route regulates cell growth, multiplication, differentiation, and metabolism, and an imbalance in this pathway affects the occurrence, development, and invasiveness of tumors, as well as tumor immune escape [20]. The PI3K family members are proto-oncogenes, and they are important kinases that phosphorylate inositol and phosphatidylinositol. The activation of PI3K produces the second messenger PIP3 on the plasma membrane. In turn, PIP3 binds to Akt and PDK1 in the cell. When Akt is translocated to the cell membrane, it becomes catalytically active. Activated Akt phosphorylates p21 and translocates it to the cytoplasm. This results in neutralization of its inhibitory effect on CDK, and enhancement of progression of the cell cycle. In the present study, results from Western blotting showed that with increase in dose, there were decreases in protein expressions of PI3K, p85, and p-Akt, while the expression level of PTEN protein increased. Thus, berberine hydrochloride induced G2/M arrest and apoptosis of bladder cancer cells through inhibition of the PI3k/Akt pathway.

CONCLUSION

The findings of this study demonstrate that berberine hydrochloride arrests cell cycle of bladder cancer cells at G2M stage and accelerates cell apoptosis via a mechanism related to inhibition of PI3/Akt signal pathway. Therefore, berberine hydrochloride is a potential drug for the management of bladder cancer but *in vivo* studies are required to ascertain this.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the author(s) named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors read and approved the manuscript for publication. Tingyong Wu conceived and designed the study. Yougen Hu, Jianming Peng, and Landi Su collected and analyzed the data, while Xiuling Jiang wrote the manuscript.

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