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ORIGINAL ARTICLE

Papaverine selectively inhibits human prostate cancer cell (PC-3) growth by inducing mitochondrial mediated apoptosis, cell cycle arrest and downregulation of NF-κB/PI3K/Akt signalling pathway

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Summary

Purpose: *The main objective of the current research work was to investigate the antitumor effects of papaverine in PC-3 human prostate cancer cells along with testing its toxicity in the normal human fibroblast (NHF) cells.*

Methods: *The cytotoxic effects of papaverine were examined by the MTT cell viability assay. Flow cytometry using annexin V-FITC/PI was used to study the effects on apoptosis, including its quantification. Effects on cell cycle progression were analyzed by flow cytometry while as effects on apoptosis-related proteins, NF-kB and PI3K/Akt pathways were estimated by Western blot assay.*

Results: *The results indicated that papaverine could induce significant, highly selective and dose-dependent cytotoxic effects in PC-3 cells without causing too much toxicity in normal cells. Papaverine also led to induction of early and late apoptosis along with inducing sub-G1 cell cycle arrest in a dose-dependent manner. Papaverine induced a dose-dependent reduction in the expression levels of Blc-2 proteins and a dose-dependent increase in the expression levels of Bax protein. The expression levels of NF-kB were decreased markedly in comparison to the untreated control. Papaverine treatment also led to a dose-dependent downregulation of PI3K and phospho-Akt expression.*

Conclusion: *Papaverine showed selective antitumor properties against PC-3 human prostate cancer cells by inducing early and late apoptosis, sub-G1 cell cycle arrest, modulation of apoptosis-related proteins like Bcl-2, Bax, Bid, XIAP and cytochrome C along with downregulation of NF-kB, PI3K/Akt signalling pathway.*

Key words: *antitumor activity, early apoptosis, papaverine, prostate cancer, sub-G1 phase*

Introduction

Prostate cancer is the most common cancer in males and is also the second most common malignancy in men worldwide. Prostate cancer is also the second leading cause of cancer-related deaths in men worldwide. The commencement and advancement of prostate cancer involves a multifaceted series of both exogenous and endogenous factors. Although prostate cancer is potentially curable if it is localized and in early stage, however, metastatic prostate cancer is incurable with a survival range of 3–7 years, and most men die of it [1,2]. Treatment strategies differ for localized and metastatic prostate cancer; the former is usually treated by surgical resection or radiotherapy, while the latter involves surgical castration or hormonal manipulation using gonadotropin-re-

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leasing hormone (GnRH) agonists, antiandrogens, or both. Cytotoxic chemotherapy has also been found to improve patient survival in prostate cancer [3,4]. Its long lack of expression, slow progression, and high occurrence rate make prostate cancer ideal for targeted chemopreventive therapies. The chemotherapeutic drug docetaxel has been reported to improve overall survival in patients with prostate cancer. No new successful anticancer chemotherapeutic agents have entered the market since the approval of docetaxel in 2004 by the Food and Drug Administration (FDA) [5]. Therefore, there is a pressing need for novel, selective and promising anticancer agents which can target prostate cancer cells selectively without causing too much damage to normal cells.

Plant-based natural products have been reported to show chemopreventive effects against prostate cancer. Consumption of these naturally occurring compounds has been linked with reduced risk and /or slow progression of prostate cancer. Most of these natural products have a great potential to be used as chemopreventive agents against prostate cancer because of their low toxicity, specificity to cancer cells, relatively low cost, acceptability and easy availability [6-10]. Therefore, the aim of the present research work was to demonstrate the antitumor effects of papaverine, a naturally occurring alkaloid against PC-3 human prostate cancer cells along with evaluating its effects on the mode of action on several biochemical pathways including apoptosis, cell cycle and NF-κB/PI3K/Akt signalling pathways.

Methods

Chemicals and other reagents

Papaverine hydrochloride, and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium-bromide (MTT) were procured from Sigma-Aldrich (St. Louis, MO, USA). Papaverine was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich). To prepare working solutions, the stock solution was further diluted with culture media to yield the desired papaverine concentrations. An equivalent volume of DMSO in complete culture medium was used as the vehicle control. To eliminate the cytotoxicity of DMSO, the final concentration of DMSO for all experiments was kept at less than 0.35%. Minimum Essential Medium (MEM) and RPMI, fetal bovine serum (FBS), penicillin, streptomycin, trypsin, phosphate-buffered saline (PBS) with calcium chloride and magnesium chloride were purchased from Merck Co. (Hangzhou, China). Propidium iodide (PI) and annexin V-FITC-Propidium Iodide Apoptosis Detection Kit were purchased from Boster Biological Technology Co., Ltd. (Wuhan, China).

Cell line and cell culture medium

Human prostate cancer cells (PC-3) and normal human fibroblast (NHF) cells were obtained from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (China) and maintained in MEM medium with 4 g/L Na2CO3, 5 mM l-glutamine, 3 g/L glucose, 4 mM sodium pyruvate supplemented with 5% heat inactivated FBS and penicillin-streptomycin (100 U/ml) at 37°C in a humidified atmosphere containing 5% CO_2.

MTT assay for cell cytotoxicity evaluation

The cytotoxic effects of papaverine on PC-3 cell proliferation as well as on NHF were determined by MTT assay. The cells ($2x10^5$ cells/well) were seeded and cultured with increasing doses of papaverine (0, 2.5, 5, 10, 20, 40, 80, 120 and 200 μM each) for 24 hrs. Subsequent to drug treatment, the medium was changed and MTT 2 mg/ml was added for 3 hrs. The number of viable cells is equal to the formation of formazan crystals which were dissolved in ethanol and the optical density was measured on a microplate reader (ELX 800; Bio-tek Instruments, Inc., Winooski, VT, USA) at a wavelength of 490 nm.

Annexin V-FITC assay for evaluating apoptosis/necrotic cell death

Annexin V-FITC assay (Annexin V-FITC apoptosis detection kit) was used to quantify the extent of apoptosis induced by papaverine in PC-3 human prostate cancer cells. In brief, PC-3 cells at a density of $2x10^6$ cells/ml were seeded and treated with papaverine at different doses (0, 10, 80 and 120 μM). Afterwards, the cells were incubated for 48 hrs, washed with PBS and then stained with propidium iodide and Annexin V-FITC as per manufacturer's instructions. The cells were analyzed by flow cytometry using FACS Calibur instrument (BD Biosciences, San Jose, CA, USA) equipped with Cell Quest 3.3 software.

Cell cycle analysis

PC-3 cells at a density of $2x10^6$ cells/ml were seeded in 60-mm dishes and treated with 0, 10, 80 and 120 μM of papaverine for 48 hrs. Following drug treatment, the cells were trypsinized and washed three times with PBS. Subsequently, the cells were fixed with 70% cold ethanol overnight, then treated with 10 μg/mL RNase A, and stained with 5 μg/mL of propidium iodide. Finally the DNA content and cell cycle distribution was analysed by flow cytometry (FACS Calibur instrument, BD Biosciences, San Jose, CA, USA), equipped with Cell Quest 3.3 software).

Western blot assay

The effect of papaverine on various apoptosis-related proteins was evaluated by Western blot assay.

PC-3 human prostate cancer cells were treated with different doses of papaverine (0, 10, 80 and 120 μM) for 48 hrs. The cells were then harvested and washed twice with PBS and then lysed in RIPA buffer and protease inhibitor for 30 min. Then, after centrifugation, the protein content was estimated by BCA method. The protein lysates (10 μg/lane) were separated by 10% SDS-PAGE and blotted onto nitrocellulose membranes (Millipore, MA, USA). Each membrane was blocked with 5% skim milk, and then incubated with the designated primary antibodies overnight at 4°C. Subsequently, the membrane was incubated with the secondary antibodies (HRP-conjugated goat anti-rabbit or goat anti-mouse IgG) for 1 hr at room temperature. Immunodetection was performed using an enhanced chemiluminescence detection kit. Antibodies against NF-kB, PI3K, p-Akt, Akt, Bcl-2, Bax, cytochrome C, Procaspase 3 and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Statistics

Data are presented as the mean ± SEM of the control. All experiments were repeated at least three times. The differences between groups were analyzed by one-way ANOVA with Tukey's *posthoc* tests. The significance of difference was indicated as *p<0.05, **p<0.01.

Results

Papaverine shows selective antitumor effects in PC-3 human prostate cancer cells

Papaverine (Figure 1) is a naturally occurring plant alkaloid known to have various biological activities. The effects of papaverine on the cell proliferation in PC-3 and NHF cells are shown in Figure 2. The results clearly indicate that papaverine

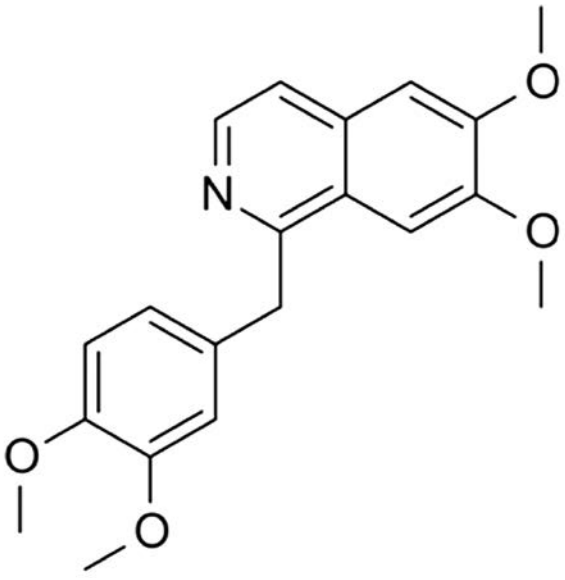

Figure 1. Molecular structure of papaverine.

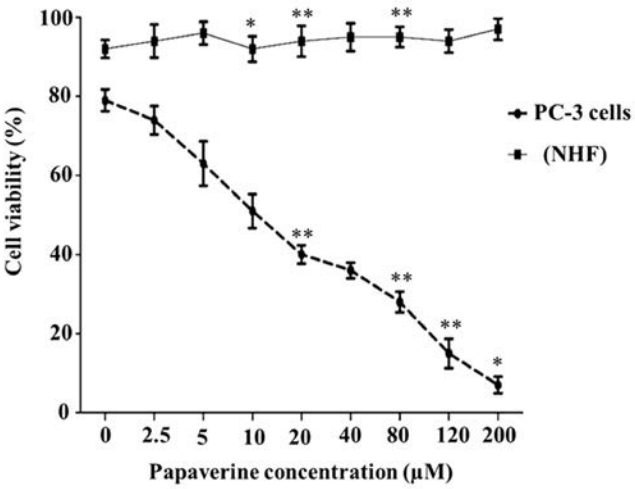

Figure 2. Selective antiproliferative effects of papaverine in human prostate cancer cells (PC-3) and normal human fibroblasts (NHF) cells were evaluated by MTT cell viability assay. Data are shown as mean ± SD of three independent experiments. *p<0.05, **p< 0.01, vs 0 μM (control).

exhibits significant, selective and dose-dependent cytotoxic effects against PC-3 prostate cancer cells without showing too much cytotoxicity against the normal human fibroblasts. Such results are promising since most of the currently used clinical anticancer drugs show significant toxicity not only in cancer cells but also in normal cells.

Papaverine induces early and late apoptosis in PC-3 human prostate cancer cells

Further experiments using annexin V-FITC assay and flow cytometry revealed that papaverine was able to induce early and late apoptosis in PC-3 cells in a dose-dependent manner. The results which are depicted in Figure 3 A-D indicate that untreated control cells showed only 3.4% of the cells in the apoptotic stage (Figure 3 A). However, papaverine with 10, 80 and 120 μM dose-treated cells showed 18.4, 45.7 and 78.5% of the cells in the apoptotic phase (Figure 3 B-D). This indicates that papaverine induces apoptosis and not necrotic cell death in PC-3 human prostate cancer cells. Q1, Q2, Q3 and Q4 quadrants in the figure represent necrotic, early apoptotic, viable and late apoptotic cell populations respectively.

Papaverine induces sub-G1 cell cycle arrest in PC-3 human prostate cancer cells

The effects of papaverine on the cell cycle progression were evaluated by flow cytometry technique using propidium iodide as probe. The results depicted in Figure 4 A-D clearly show that papaverine is a potent agent inducing cell cycle

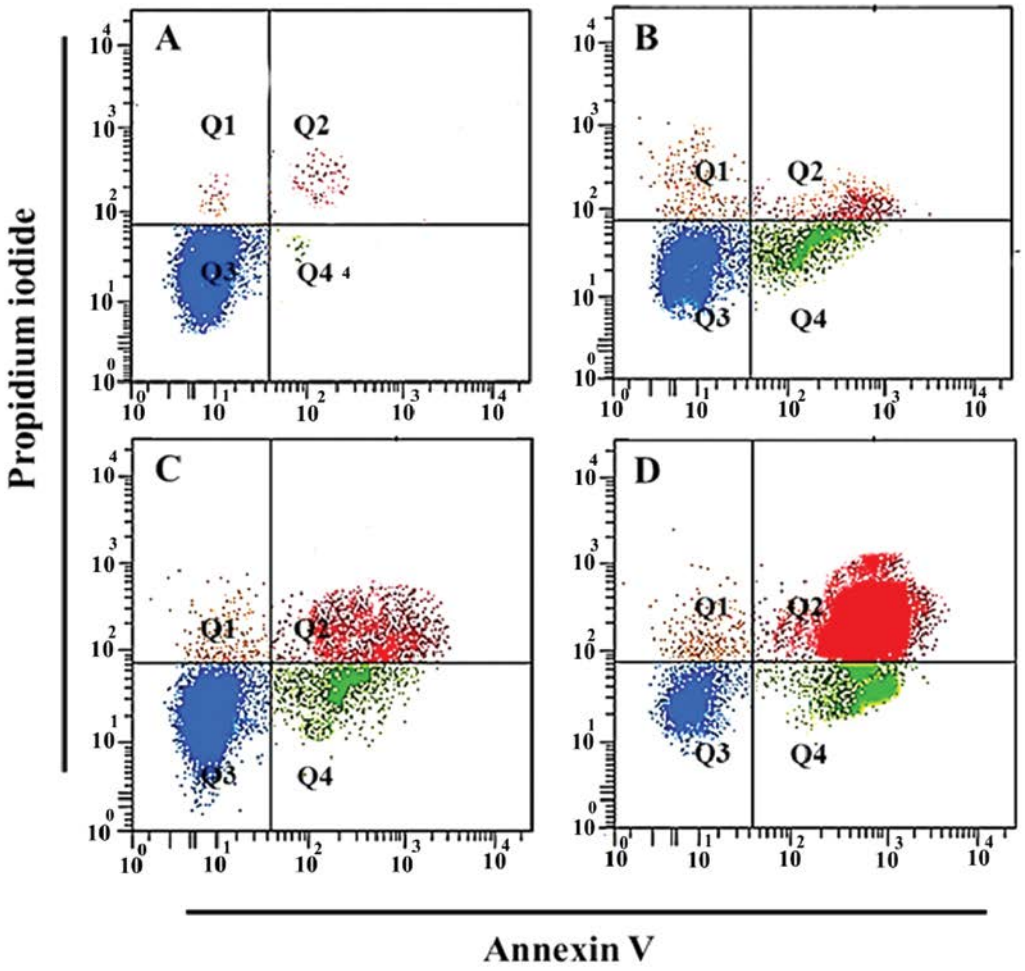

Figure 3. Annexin V-FITC assay for apoptosis quantification induced by papaverine in human prostate cancer cells (PC-3). The cells were treated with 0 **(A)**, 10 **(B)**, 80 **(C)** and 120 **(D)** μM dose of papaverine for 48 hrs, then stained with annexin V-FITC/PI and analyzed by flow cytometry. Q1, Q2, Q3 and Q4 quadrants represent necrotic, early apoptotic, viable and late apoptotic cell populations respectively.

arrest in the sub-G1 phase which is also termed as the apoptotic phase. As compared to the untreated cells which showed only 2.3% of the cells in the sub-G1 phase, the papaverine-treated cells with 10, 80 and 120 μM doses resulted in 12.5, 28.7 and 52.4% respectively of the cells to be in the sub-G1 phase. Cell cycle and tumor progression are closely related biochemical processes and any disturbances in the former will greatly affect the latter.

Effect of papaverine on the various apoptosis-related proteins

The effects of papaverine on various apoptosis-related proteins, like Bcl-2, Bax, cytochrome C, Bid, XIAP etc were assessed by Western blot assay. It was observed (Figure 5) that papaverine induced a dose-dependent reduction in the expression levels of Blc-2 proteins, while it also led to a dose-dependent increase in the expression levels of Bax protein. Papaverine also led to a dose-dependent release of cytochrome C into the cytoplasm as is shown in Figure 5. Furthermore, the expression levels of XIAP, which is an anti-apoptotic protein, also decreased after papaverine treatment in a dose-dependent manner.

Papaverine led to downregulation of NF-kB, PI3K/Akt signalling pathway

The effects of papaverine on the expression levels of NF-kB, PI3K/Akt in the PC-3 cells were demonstrated by Western blot of the nuclear extracts prepared from the papaverine-treated cells. The results (Figure 6) indicate that the expression levels of NF-kB were markedly decreased in comparison to the untreated control. It is possible therefore, that the antitumor effects of papaverine in PC-3 cells were mediated via NF-kB inactivation. Furthermore, papaverine treatment also led to a dose-dependent downregulation of PI3K and phospho-Akt expression.

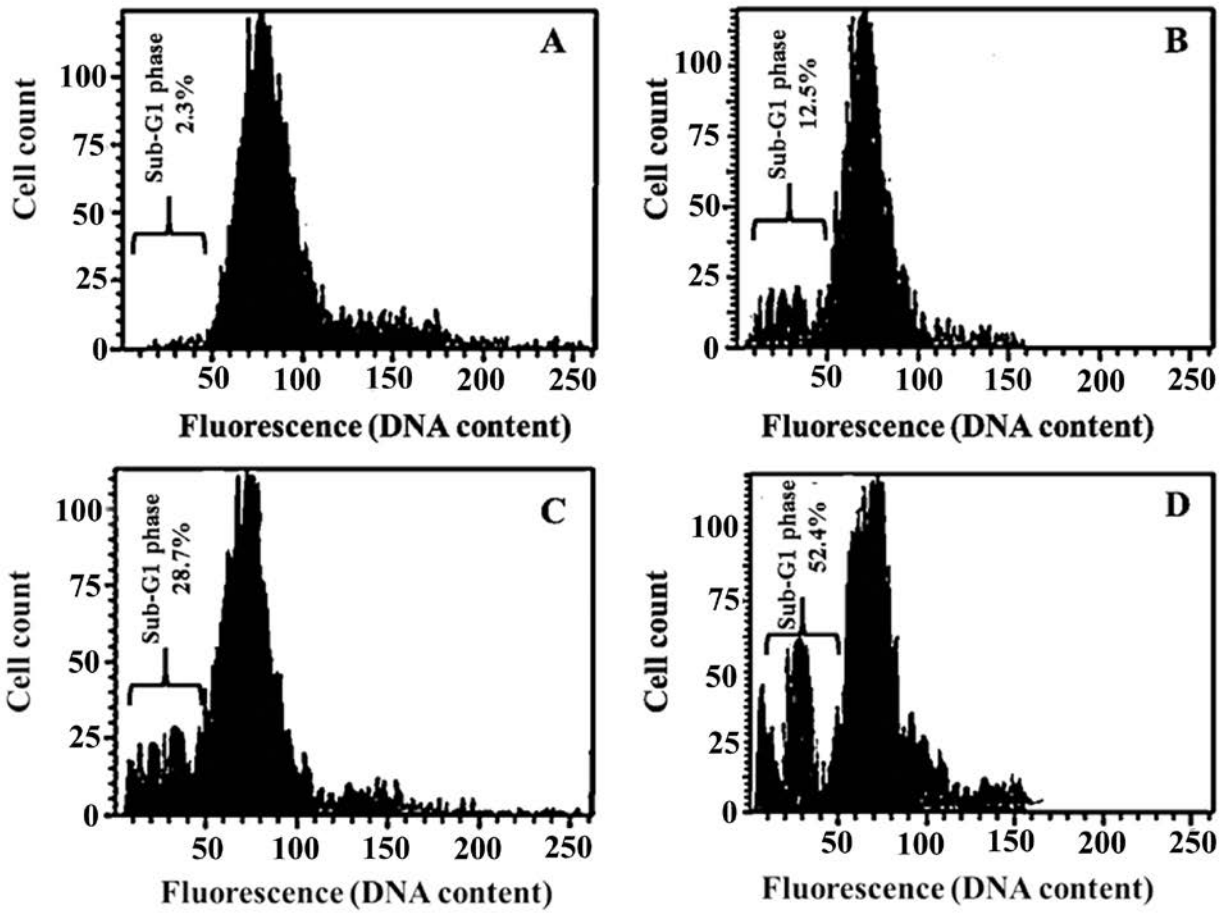

Figure 4. Effect of papaverine on the cell cycle phase distribution in human prostate cancer cells (PC-3) evaluated by flow cytometry using propidium iodide as DNA staining agent. The cells were treated with 0 **(A)**, 10 **(B),** 80 **(C)** and 120 **(D)** μM dose of papaverine for 48 hrs, then stained with PI and analyzed by flow cytometry. Papaverine induced sub-G1 cell cycle arrest as it led to the increase in the population of sub-G1 (apoptotic cell) cells.

Discussion

The main aim of the current study was to investigate the antitumor effects of papaverine in PC-3 cells and in NHF cells. MTT assay using various doses of papaverine revealed that papaverine can induce selective and dose-dependent cytotoxicity in PC-3 cancer cells without causing too much toxicity to the NHF. These initial cytotoxicity results of papaverine are promising because most of the clinically used anticancer drugs are non-selective, targeting both cancer cells as well as normal cells. In addition, using flow cytometry along with annexin V-FITC/PI staining, the effects of papaverine on the apoptosis induction as well as its quantification were demonstrated. The results showed that papaverine could induce both early and late apoptosis in PC-3 cells in a dose-dependent manner. In comparison to the untreated group, the percentage of early and late apoptotic cells was much higher in papaverine-treated cells. Cell cycle experiment using flow cytometry in combination with propidium iodide indicated that papaverine induced sub-G1 cell cycle arrest in PC-3 cells. There is strong evidence that manipulation of the cell cycle may prevent or induce an apoptotic event. The cell cycle is a conserved mechanism by which eukaryotic cells replicate themselves. Any disturbance in cell cycle will ultimately affect the cell multiplication process and hence the cancer formation [11]. Additionally, the effects of papaverine on the expression levels of various anti-apoptotic and pro-apoptotic proteins were investigated and the results revealed that papaverine induced a dose-dependent reduction in the expression levels of Blc-2 proteins (anti-apoptotic) while it also led to a dose-dependent increase in the expression levels of Bax (pro-apoptotic) protein. Papaverine also led to a dose-dependent release of cytochrome C into the cytoplasm. The expression levels of XIAP, which is an anti-apoptotic protein, also decreased after papaverine treatment in a dose-dependent manner. Apoptosis is regulated by pro-apoptotic and anti-apoptotic proteins

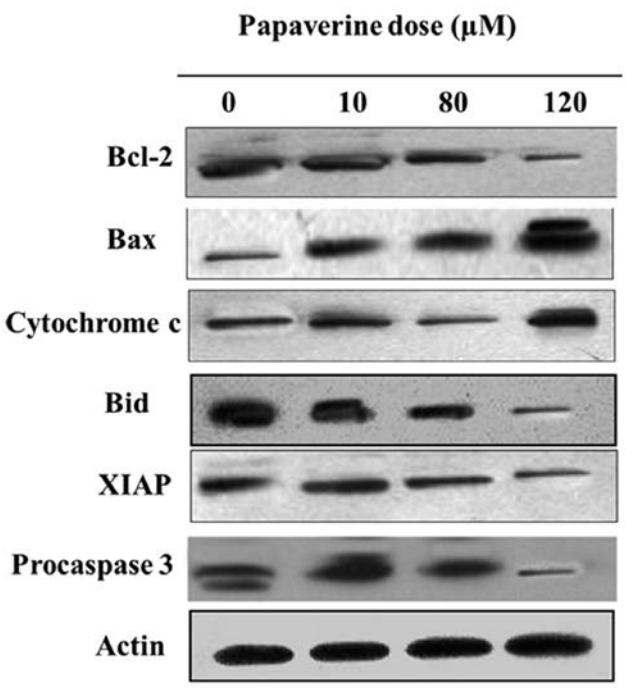

Figure 5. Effect of papaverine on the expression levels of various apoptosis-related proteins in PC-3 human prostate cancer cells. The cells were treated with 0, 10, 80 and 120 μM dose of papaverine for 48 hrs and then the cells were lysed, proteins were separated and analyzed by Western blot using specific antibodies. Actin was used as an internal control. Papaverine induced a dose-dependent reduction in the expression levels of Bcl-2 protein, while it also led to a dose-dependent increase in the expression levels of Bax protein.

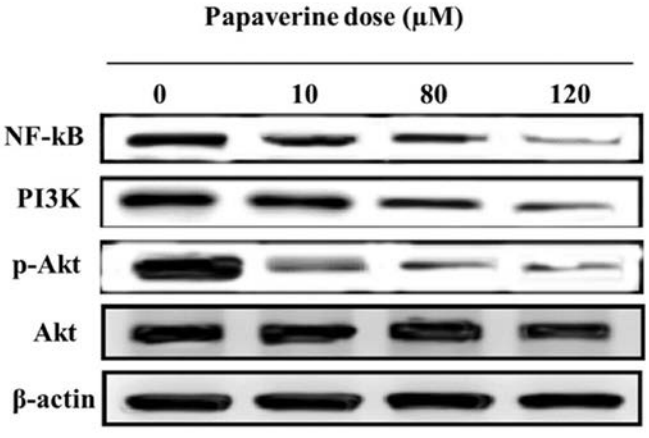

Figure 6. Effects of papaverine on the expression levels of NF-kB, PI3K/Akt in human prostate cancer cells (PC-3). The cells were treated with 0, 10, 80 and 120 μM dose of papaverine for 48 hrs. The cellular levels of NF-kB, PI3K/Akt proteins were analyzed by Western blotting assay. β-actin was used as an internal control. The expression levels of NF-kB, PI3K and p-Akt were markedly decreased in comparison to the untreated control.

that are recruited as a result of apoptotic stimuli such as DNA damage, chemo- and radiotherapy. Bcl-2, an anti-apoptotic factor, is an attractive molecular target in the treatment of prostate cancer [12]. In addition, the effects of papaverine on the NF-kB, PI3K/Akt pathway were also studied. The expression levels of NF-kB were decreased markedly in comparison to the untreated control. Papaverine treatment also led to a dose-dependent downregulation of PI3K and phospho-Akt expression. Phosphatidylinositol 3-kinase (PI3K)/Akt survival pathway plays a key role in carcinogenesis as it has been reported that advanced prostate cancers frequently have elevated levels of phosphorylated (activated) Akt. The Akt pathway also regulates cell growth, proliferation, and angiogenesis through the mTOR (mammalian target of rapamycin) pathway [13]. It is already known that while Bcl-2 is an anti-apoptotic agent, Bax is a pro-apoptotic agent ultimately leading to lowering of the Bcl-2/Bax (anti-apoptotic/pro-apoptotic) ratio, which results in release of cytochrome C to start caspase activation and the ultimate disruption of mitochondrial membrane leading to loss of mitochondrial membrane potential. Both NF-kB and PI3K/Akt pathways have been reported to play crucial roles in the carcinogenesis of various kinds of tumors including prostate cancer [14-16]. Many natural products including alkaloids have been reported to induce apoptosis in a variety of cancer cells targeting other diverse biochemical pathways [17,18].

Conclusion

In brief, papaverine shows selective anti-tumor properties towards PC-3 human prostate cancer cells by inducing early and late apoptosis, sub-G1 cell cycle arrest, modulation of apoptosis-related proteins like Bcl-2, Bax, Bid. XIAP, and cytochrome C along with downregulating the NF-kB, PI3K/Akt signalling pathway.

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Conflict of interests

The authors declare no confict of interests.

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