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Potential of piperine in modulation of voltage-gated K^+ current and its influences on cell cycle arrest and apoptosis in human prostate cancer cells

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Abstract. – OBJECTIVE: Piperine is an attractive therapeutic alkaloid from black pepper that exhibits a broad spectrum of pharmacological properties over various pathological disorders including cancer. Voltage-gated K^+ channels (K_V) play an important role in regulating cancer cell proliferation and are considered as potential targets for the treatment of cancer. However, there is a paucity of information with regard to the implication of piperine in KV associated anticancer activities on human prostate cancer cells LNCaP and PC-3 cells. Therefore, the primary objective of the present study was to elucidate the anticancer action of piperine that might be mediated via voltage-gated K^+ current (I_K) blockade.

PATIENTS AND METHODS: Whole-cell patch clamp was used to record the modulatory effects of piperine on IK expressed in LNCaP and PC-3 cells. Moreover, the anticancer activity of piperine was evaluated by MTT assay, flow cytometry and live/dead assay.

RESULTS: Piperine significantly inhibited I_K in a dose-dependent manner with an effective IC_{50} dose 39.91 μM in LNCaP and 49.45 μM in PC-3 cells. Also, piperine induced a positive shift in the relative activation curve in both cells. Blockade of I_K by piperine exerted G0/G1 phase cell cycle arrest that led to inhibition of cell proliferation and induced apoptosis in a dose-dependent manner.

CONCLUSIONS: We showed that the anticancer effects of piperine are directly correlated with the blockade of IK in LNCaP and PC-3 cells. The study also confirmed that IK inhibition by piperine might be responsible for its anticancer activities in prostate cancer cells.

Key Words
Prostate cancer, Piperine, IK, Proliferation, Cell death.

Abbreviations

K_V, Voltage-gated K^+ channels; I_K, Voltage-gated K^+ current; PARP-1, Poly [ADP-ribose] polymerase 1; PSA, Prostate specific antigen; TRPV1, Transient receptor potential subfamily V member 1; $K_{2p,}$ Two-pore domain potassium channel; Bcl-2, B-cell lymphoma 2.

Introduction

Prostate cancer is the second leading cause of cancer-related deaths amongst men in the United States[1]. However, the mortality rate of advanced and metastatic prostate cancer is even higher despite the androgen deprivation therapy and chemotherapies[2]. Hence, new strategies for the treatment of prostate cancer are desirable. The investigation of the functional properties of ionic channels in cancer progression and metastatic behavior is an emerging strategy as it is a novel approach for the development of effective anticancer treatment. Potassium (K^+) channels in the plasma membrane of tumour cells contribute to a wide range of cellular processes including cell cycle progression, cell proliferation and apoptosis[3]. In particular, K^+ channels play an active role in cell proliferation and their activity is a prime factor for cell cycle progression through the early G1 phase of the cell cycle[4]. So, the regulation of K^+ channel activity contributes significantly to the inhibition of cell proliferation in several cancer cell lines including prostate[5]. In prostate cancer cells, K_V channel is quite prominently expressed and has been reported to be involved in cell proliferation[6]. On the other hand, a recent study reported the inhibition of the K_V channel by K^+ channel blocker 4-aminopyridine (4-AP) leading to growth inhibition in both androgen-sensitive (AT-2) and androgen insensitive (MAT-LyLu) rat prostate cancer cell lines[7]. Furthermore, the channel blockers like dequalinium, amiodarone and glibenclamide have also been observed to induce apoptosis in PC-3 cells[8].

The concept of K_V channels as therapeutic target for prostate cancer treatment is a research hotspot. However, there is a paucity of information with regard to the effective and selective K_V channel modulators. So, this significantly hindered the progression of this form of treatment strategy[9].

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Natural products derived from plants have received widespread attention due to their remarkable anticancer activities. Piperine (1-peperoy piperidine) is the primary pungent alkaloid in black peppercorns derived from the fruit bodies of *Piper nigrum*[10]. Also, numerous studies have described the pharmacological properties of piperine including inhibition of cell proliferation and induction of apoptosis in breast cancer cells[11], cell cycle arrest at the G0/G1 phase, proliferation inhibition, reduced cell migration, apoptosis activation through caspase-3 or PARP-1 protein and suppression of PSA levels in both *in vitro* and *in vivo* models of prostate cancer[12]. Moreover, piperine has also shown its modulatory potential on different ion channel activities in human embryonic kidney cell lines[13], on K_{2p} channels in *Xenopus laevis* oocytes[14], voltage-gated sodium channel activity in human embryonic kidney tsA-201 cells[15] and K_V channel in *Xenopus* embryo spinal neurons[16]. These diverse properties of piperine suggested us to explore the implication of piperine in K_V associated anticancer activities in human prostate cancer cells. Furthermore, the present study is first of its kind; as to the best of our knowledge, Kv channel inhibition and its relevance with the pharmacological mechanisms of piperine in prostate cancer cell lines have not been reported so far. Therefore, it would be worthwhile to examine the modulatory effect of piperine on K_V channel associated anticancer effect on human prostate cancer cells LNCaP and PC-3.

Patients and Methods

Chemicals and Reagents

Live/dead assay kit was obtained from Molecular Probes Invitrogen detection technologies (Thermo Fisher, Waltham, MA, USA). 4',6-Diamadine-2'-phenylindole dihydrochloride (DAPI), tetraethylammonium (TEA) and piperine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Before the experiments, 100 mM TEA was freshly prepared in deionized water and piperine was freshly prepared in dimethyl-sulfoxide (DMSO). However, the desired concentrations were obtained by appropriate dilution in the extracellular fluid (ECF). All the other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

Cell Culture

PC-3 and LNCaP human prostate cancer cell lines were sourced from the American Type Culture Collection (ATCC; Manassas, VA, USA). PC-3 cells were cultured in F12-K medium (Hi-Media Laboratories, West Chester, PA, USA). LNCaP cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA). Both the media were supplemented with 10% fetal bovine serum (FBS) and with 1% antibiotics (penicillin: 100 units/ml, streptomycin: 100 µg/ml) (Thermo Fisher, Waltham, MA, USA). All cells were grown in a humidified incubator at 37°C supplemented with 5% CO_2. The Institutional Ethics Committee of our Institute approved this study and all the experimental procedures.

Electrophysiology

Whole-cell patch clamp recordings in LNCaP and PC-3 cells were performed on single cells at room temperature (20-24° C). Membrane currents were recorded under standard voltage clamp mode using an Axopatch-200B patch clamp amplifier (Axon Instruments, Union City, CA, USA). Borosilicate patch pipettes were pulled with a two-stage vertical puller (PP-830, Narishige, Tokyo, Japan) to a tip resistance of 1.5-3 MΩ. Data for each sample were filtered at 5 kHz, using a low pass filter and sampled at 10 kHz using a 1322-A Digidata converter (Axon Instruments, Union City, CA, USA). Capacitance transient and series resistances were compensated by about 50-60%. The PClamp software (v6.0.3) was used for voltage command protocol and to acquire the data. For whole-cell recordings the external solution (ECF) containing NaCl 140 mM, KCl 5 mM, $CaCl_2$ 2.5 mM, $MgCl_2$ 1 mM, D-glucose 10 mM and HEPES 10 mM (4-(2-hydroxyethyl) piperazine-1-ethane sulfonic acid) adjusted to pH 7.4 with 1M NaOH. Patch pipettes were filled with internal physiological solution containing KCl 140 mM, NaCl 5 mM, $CaCl_2$ 1 mM, $MgCl_2$ 1 mM, HEPES 10 mM and EGTA 11 mM (ethylene glycol-bis (β-aminoethyl ether)-N,N,N,N-tetraacetic acid) adjusted to pH 7.2 with 1M KOH. This solution gave an effective intracellular Ca^{2+} concentration of =15 nM. I_K was recorded by depolarizing the cells from a holding potential of -80 mV; the test potential was stepped from -120 to +70 mV for 200 ms, in 10 mV increments with 30-sec intervals. The holding potential was set to -80 mV for the whole set of experiments. The cell under investigation was continuously focally perfused with an external solution, TEA 10 mM and different concentrations of piperine using Octoflow (ALA Scientific Instruments, Farmingdale, NY, USA) perfusion system.

Data Analysis

The results are presented as Mean ± SEM (n=number of cells recorded from minimum 6 experiments). The current-voltage curves were analyzed on ClampFit 9.2.1.9 (Molecular Devices, Sunnyvale, CA, USA), Origin Lab 8.6 (OriginLab Corp. Northampton, MA, USA) and Microsoft Excel 2012. All data values were calculated and *p*-value less than 0.05 was considered statistically different. The effects of piperine on I_K peak current density at +70 mV were analyzed at various concentrations (5,10, 20, 50, 100, 200 µM). The half inhibitory concentration (IC_{50}) of I_K current at +70 mV was evaluated and fitted to Hill equation where E is the inhibition of I_K in percentage at concentration C, E_{max} is the maximal inhibition, IC_{50} is the inhibitory concentration required for half-maximal current blockade and b is the Hill coefficient. The current amplitudes were normalized with cell capacitance and expressed as current density (pA/pF). The conductance-voltage relationship (G-V) was determined based on the equation where I_p is the peak current amplitude at the test potential V, V_{rev} is the reversal potential. To determine half-maximal activation voltage of I_K channel, data were fitted with a Boltzmann equation, as follows: G is the conductance, G_{max} is the peak conductance, $V_{1/2}$ is the voltage at which half-maximal conductance occurs, V is the command voltage and k is the slope factor.

Cytotoxicity

The cytotoxic effect of piperine on prostate cancer cell lines LNCaP and PC-3 was evaluated through 3(4,5-Dimethyl-thiazol-2-yl) 2,5-diphenyl-tetrazolium bromide (MTT) assay. Cells ($1x10^6$ cells/ml) were seeded into 96 well plates. Then, the cells were treated with different concentrations of piperine ranging from 5-150 µM obtained by appropriate dilution with dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) and further incubated for 24 h. Following the incubation, 20 µl MTT was added to each microwell and further incubated for another 4 h. Then, the supernatant was removed and replaced with 200 µL of DMSO and finally the optical density of each well was measured with a multimode reader (Tecan, Männedorf, Switzerland).

Cell Cycle Analysis

Control and treated cells were harvested by trypsinization and washed once with cold Phosphate-Buffered Saline (PBS). The pellet was re-suspended in 70% ice-cold ethanol and stored at -20°C. After fixation, cells were centrifuged and re-suspended in cold PBS, and samples were incubated with DAPI staining solution in the dark at room temperature for 10 min before measuring on flow cytometer BD FACSCelesta (Becton Dickinson, San Jose, CA, USA). Data were analyzed using BD FACS Diva 8.0.1.1 software.

Live/Dead Assay

Live/dead assay was performed according to the manufacturer's protocol. In brief, after 24 h treatment, the cells were washed twice with D-PBS and were incubated with freshly prepared working solution containing 1µM calcein Am and 2 µM ethidium homodimer-1 (EthD-1) for 45 min in the dark at room temperature. Following incubation, the cells were washed with D-PBS and the images were captured under fluorescent microscopy Leica-MZ16FA (Leica Microsystems, Heerbrugg, Switzerland). The live and dead cells were quantified using Image J software (National Institute of Health of USA, Bethesda, MD, USA). The percentage of live and dead cells were calculated using the formula.

Results

General Observations

Whole-cell patch clamp recordings were performed on single cells in Petri dishes. No inward currents were noticed in both PC-3 and LNCaP cells. Whole cell capacitances were 45.01 ± 2 pF in PC-3 and 39.23 ± 1.6 pF in LNCaP cells respectively (n=44). The final maximal concentration of DMSO was less than 0.1% and it had no effect on outward currents and anticancer studies.

Electrophysiological Characterization of K_V Channel in LNCaP and PC-3 Cells

First electrophysiological experiments were performed to identify and confirm the outward ion conductance of LNCaP and PC-3 cells. The standard depolarizing protocols were applied, as described in the materials and methods section. Only outward conductance was recorded in both cells at all membrane potentials. I_K was successfully recorded in almost 80% of the cells and the external perfusion of TEA 10 mM reduced the current amplitude in both cells. However, the effect of TEA was reversed in both cells after the ECF perfusion. The current tracings of control, TEA and wash effects are shown in Figures 1A and C. This outward current was characterized by

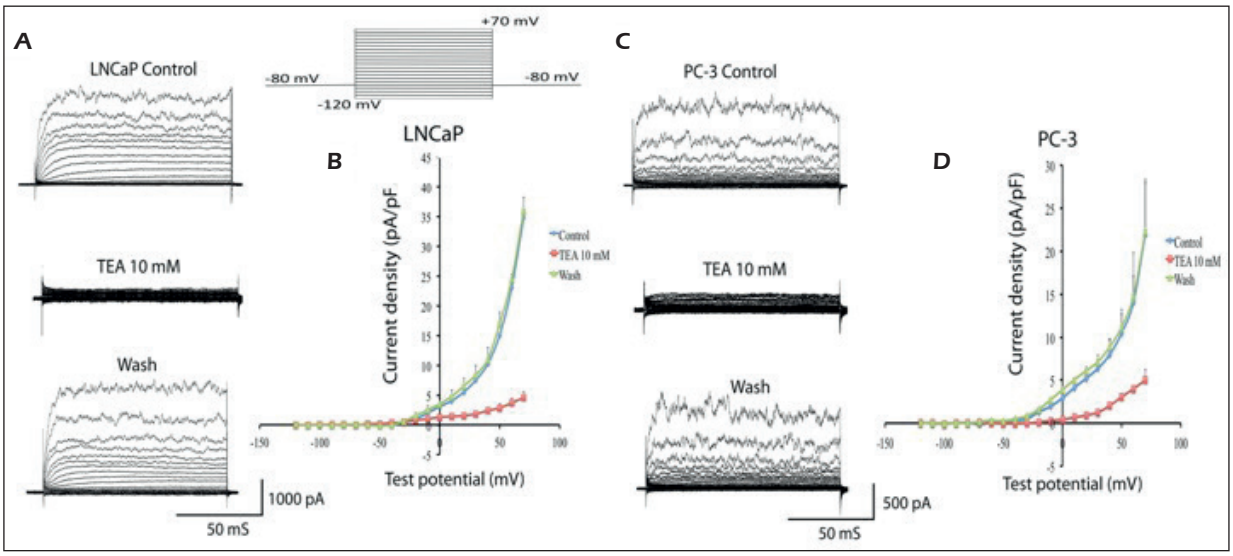

Figure 1. Characterization of I_K current in LNCaP and PC-3 cells. Typical recordings of the whole cell I_K were elicited by voltage command pulses of increasing step pulses from -140 mV to +70 mV in 10 mV increments. **A-C**, Representative traces of I_K current recorded in the presence and absence of 10 mM TEA in LNCaP and PC-3 cells. **B-D**, The I-V relationship in the absence and presence of TEA in LNCaP and PC-3 cells. Data are plotted as mean± SEM (n>7).

the mean current-voltage (I-V) relationship with and without the presence of TEA in both the cells (Figures 1B and D). The average current density of LNCaP cells shows significantly larger current than in PC-3 cells. When compared to control, TEA reduced the peak currents of LNCaP and PC-3 cells by 85 ± 2% (n>8) and 72 ± 3% (n>8) respectively. These characteristics suggested that both LNCaP and PC-3 cells predominantly express I_K current.

Effects of Piperine on I_K in LNCaP Cells

We further investigated whether piperine exerts any modulatory effect over I_K in LNCaP cells. After perfusion of piperine in LNCaP cells, significant inhibition of I_K amplitude was observed in a dose-dependent manner. However, this inhibitory effect was reversible immediately after ECF perfusion. Figure 2A shows the representative current tracings of I_K in the absence (control) and presence of piperine at (0.1, 25, 50 and 100 μM) concentrations. Figure 2B shows the I-V relationship of piperine at different concentrations. At +70 mV, piperine (0.1, 25, 50 and 100 μM) caused significant I_K inhibition by 18.3 ± 1.8%, 28.6 ± 1.3%, 66.8 ± 2.7% and 84 ± 1.2% respectively. The inhibitory concentration-response of piperine was constructed from the peak current (+70 mV) and the Hill equation was used to fit the data points best. The IC_{50} value for I_K inhibition was 39.9 μM and the Hill coefficient and E_{max} was 0.49 and 85.2% respectively (Figure 5A). These results suggest that piperine induced inhibition of I_K in a dose-dependent manner in LNCaP cells.

Effects of Piperine on I_K in PC-3 Cells

Because of high I_K inhibitory efficacy of piperine on LNCaP cells, we further examined whether piperine causes any modulatory effect on I_K in PC-3 cells. Figure 3A shows the representative current traces of I_K recorded in the absence and presence of piperine at 0.1, 25, 50 and 100 μM concentrations. Perfusion of piperine induced a concentration-dependent decrease of I_K in PC-3 cells. However, this inhibitory effect of piperine was recovered immediately after ECF perfusion. Figure 2B represents the I-V relationship of piperine at different concentrations. Extracellular perfusion of 100 μM piperine reduced the peak current by almost 76%, the subsequent dosage 50, 5 and 0.1 μM significantly reduced the peak currents by 51.1 ± 6.3%, 19.5 ± 2.5% and 11.5 ± 1.7% respectively. The IC_{50} value for I_K inhibition was 49.4 μM. The Hill coefficient and E_{max} were 0.38 and 75.1% respectively (Figure 5A). Thus, piperine appears to have pronounced inhibitory effect over I_K in PC-3 cells.

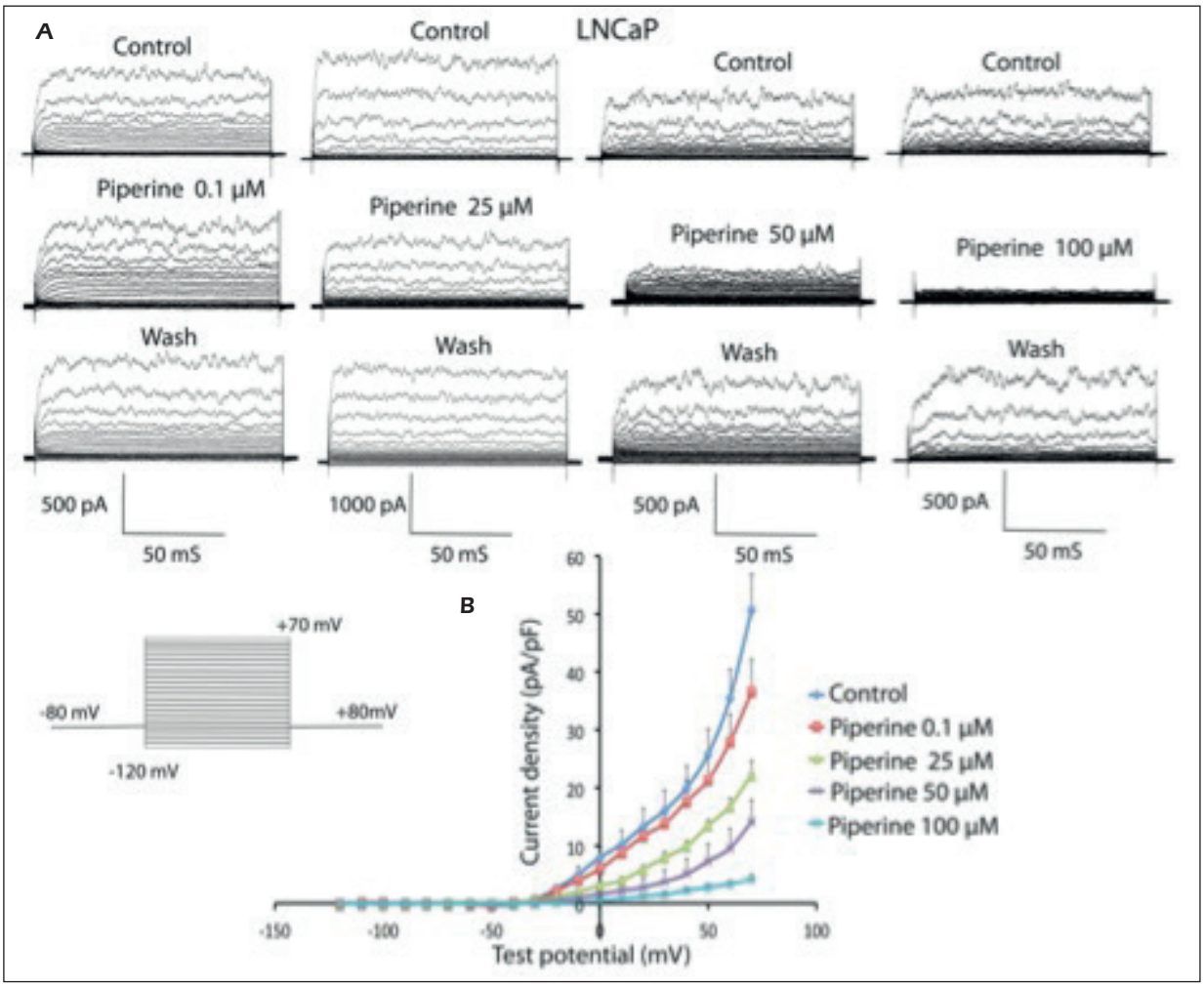

Figure 2. Dose-dependent effect of piperine on I_K current in LNCaP cells. **A**, Representative traces of I_K currents recorded in the absence and presence of piperine at different concentrations (0.1, 25, 50 and 100 µM). **B**, The mean I-V of I_K in the absence and presence of piperine at different concentration. Data are plotted as mean± SEM (n>7).

Effects of Piperine on K_V Channel Activation Kinetics in LNCaP and PC-3 Cells

K_V channel activation kinetics was determined from the relative G-V curve, constructed from I-V under control conditions and in the presence of 100 µM piperine. Mean values were fitted to the Boltzmann distribution to obtain *V1/2* and *k* of the K_V channel. Piperine modulated I_K current activation by shifting the conductance curve towards positive potential in LNCaP cells (Figure 4A). In the presence of piperine the Boltzmann distribution yields V1/2 and k were -5.88 ± 1.4 mV and 10.22 ± 0.5, respectively and in the absence of piperine V1/2 and k were -12.06 ± 1.4 mV and 9.01 ± 0.6, respectively (Figure 4B). Similarly, piperine induced a positive shift in the half-maximal activation potential in PC-3 cells. The Boltzmann distribution bit data points shows V1/2 and k were 50.12 ± 1.3 mV and 9.52 ± 0.4, respectively, in the presence of piperine and 46.27 ± 1.6 mV and 9.49 ± 0.3, respectively, in the absence of piperine (Figures 4C and 4D). These results suggest that piperine significantly modified the K_V channel activation kinetics in LNCaP and PC-3 cells.

Effects of Piperine on Cell Proliferation in LNCaP and PC-3 Cells

Furthermore, we examined the concentration-dependent effect of piperine on cell proliferation in LNCaP and PC-3 cells, assuming that this result would provide the evidence for the association of I_K with cell proliferation. The treatment with piperine exhibited dose-dependent inhibition of cell

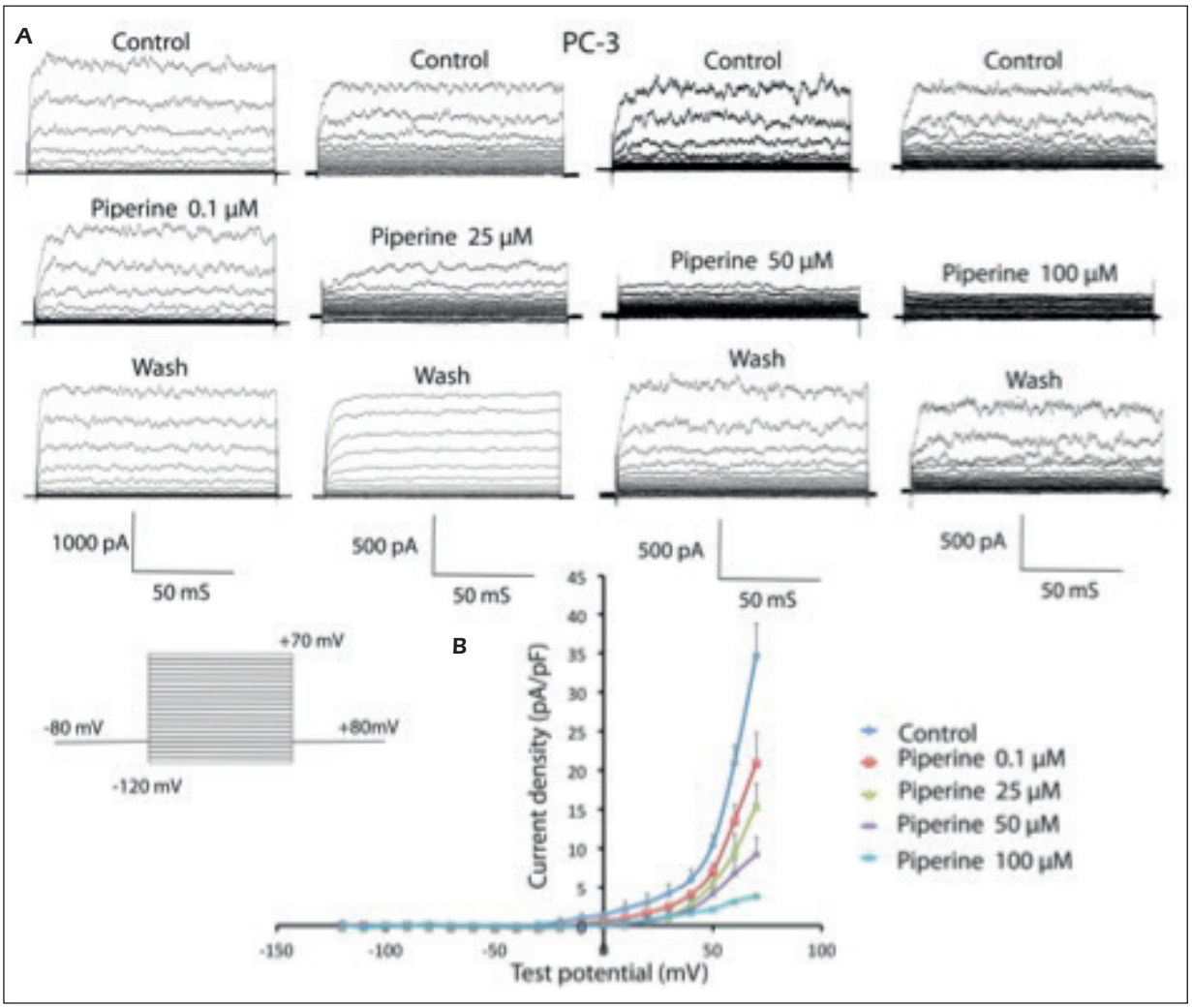

Figure 3. Dose-dependent effect of piperine on I_K current in PC-3 cells. **A**, Representative traces of I_K currents recorded in the absence and presence of piperine at different concentrations (0.1, 25, 50 and 100 μM). **B**, The mean I-V of I_K in the absence and presence of piperine at different concentration. Data are plotted as mean± SEM (n>7).

viability in both cell lines. The IC_{50} value of piperine in LNCaP and PC-3 cells were 55.34 μM and 72.21 μM, respectively (Figure 5B). It was noticed that, compared with PC-3 cells, LNCaP cells were more sensitive to piperine treatment. In addition, the bright field microscopic images also show morphological changes during the treatment with various concentrations of piperine (Figures 6A and B). As compared to control cells, a concentration 100 μM of piperine inhibited 82% cell proliferation in LNCaP cells whereas only 73% was inhibited in PC-3 cells. (Figure 6C). These results suggest that piperine inhibits the proliferation of both LNCaP and PC-3 cells in a concentration-dependent manner.

Piperine Induces Cell Cycle Arrest at G0/G1 Phase in LNCaP and PC-3 Cells

To gain more insight about the possible mechanism of current outward agonist involved in cell cycle progression, we performed flow cytometric cell cycle analysis. Subconfluent cultures of LNCaP and PC-3 cells were treated with different concentrations of piperine 0.1, 25, 50 and 100 μM for 24 h. The proportion of cell population was dose-dependently increased at G0/G1 phase, accompanied by a significant decrease of the cell population in S and G2/M phases in both the cells (Figures 7A and B). However, treatment of LNCaP cells with the highest dose of piperine (100

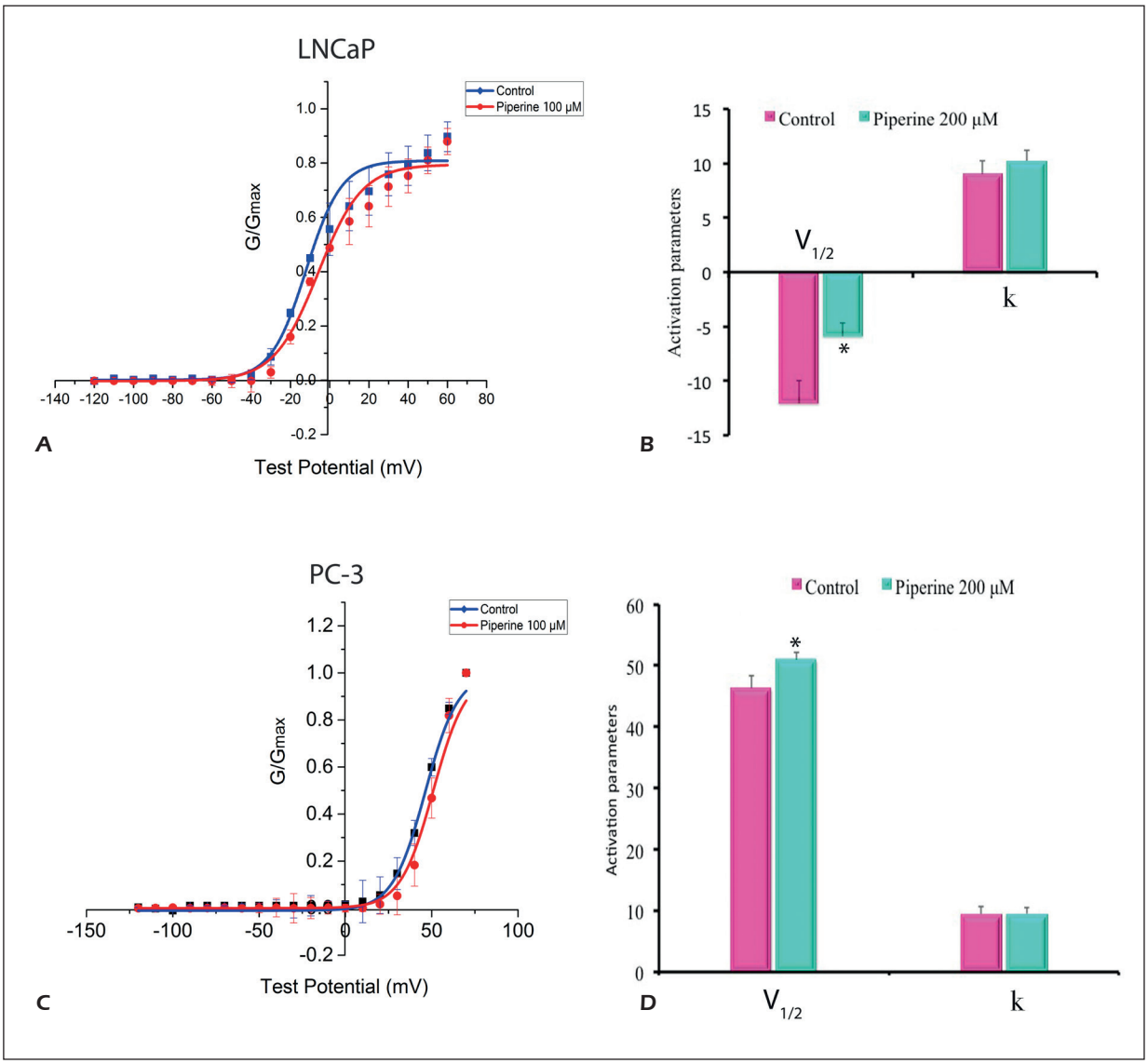

Figure 4. Activation kinetics of piperine on I_K current in LNCaP and PC-3 cells. **A-B**, Activation curves obtained in the absence and presence of piperine (100 µM) in LNCaP and PC-3 cells. Data were fitted with Boltzmann functions, represented as a smooth line. **C-D**, Histogram shows the parameters of activation kinetics of K_V channel ($V_{1/2}$ and k) for control and piperine 100 µM in LNCaP and PC-3 cells. Data are plotted as mean±SEM (n>7 for each concentration). *p<0.05 piperine *vs.* control.

µM) showed increased cell population in the G0/G1 phase (71.8 ± 1.1%) as compared to control cells (49.7 ± 2.1%). Likewise, piperine treated PC-3 cells exhibited G0/G1 cell population (70.7 ± 0.9%) compared to control cells (48.5 ± 2.3%). The effect of piperine on cell cycle arrest in PC-3 cells was slightly lower than that in LNCaP cells (Figures 7C and D). In short, these results suggest that I_K current blocking activity of piperine may be involved in the regulation of cell cycle arrest in LNCaP and PC-3 cells.

Piperine Induces Cell Death in LNCaP and PC-3 Cells

Due to the involvement of piperine in the regulation of cell cycle arrest, we postulated that piperine may also exert cell death. Therefore, the proportion of cell death was determined in both LNCaP and PC-3 cells using Live/dead assay. Ethidium homodimer-1 enters the cells with damaged membrane and positively binds with nucleic acid and emits bright red fluorescence signal. The polyanionic dye calcein AM binds with live cells

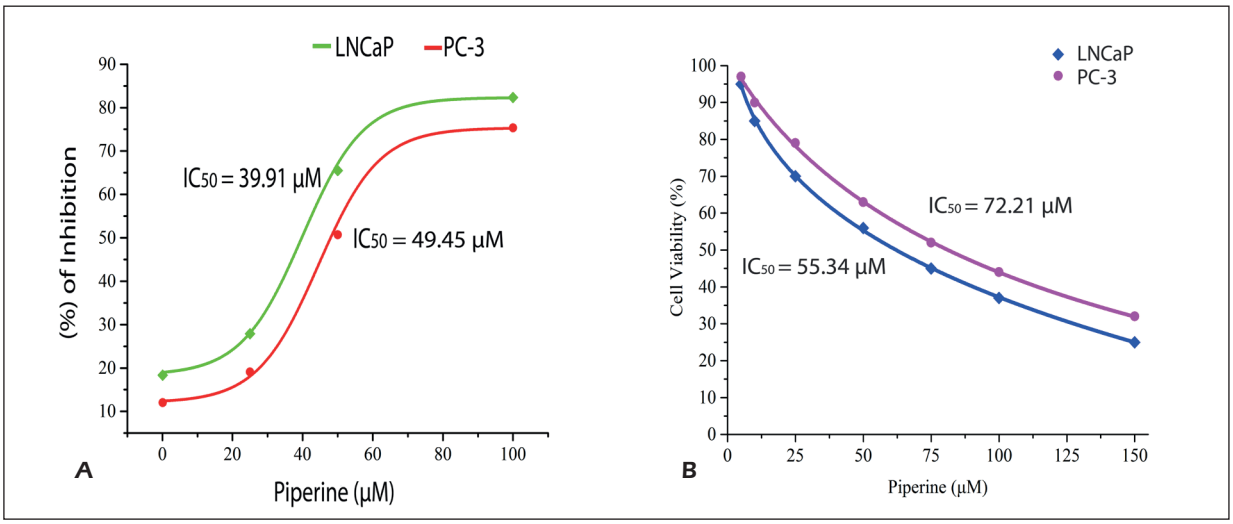

Figure 5. The concentration-response relationship of piperine on LNCaP and PC-3 cells. **A**, Dose-response effect of piperine on inhibition of the peak I_K current in LNCaP and PC-3 cells. Data were fitted with Hill equation, represented as smooth line. **B**, Cell viability curve of piperine in LNCaP and PC- 3 cells determined by MTT assay. Data were fitted with logistic function. Data are plotted as mean±SEM (n>7 for each concentration).

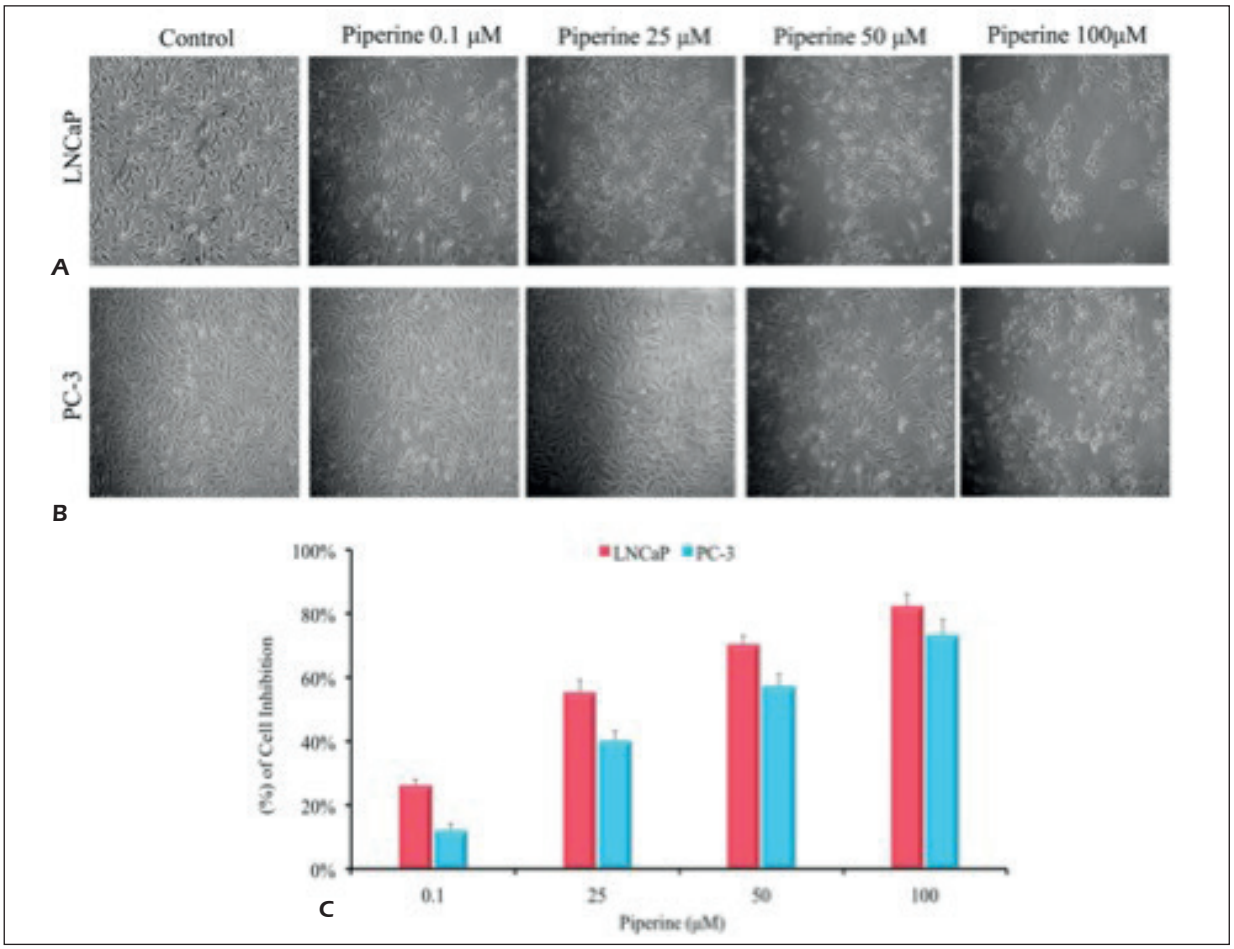

Figure 6. Assessment of cell proliferation in LNCaP and PC-3 cells. **A-B**, Bright field microscopic images of LNCaP and PC-3 cells treated with 0.1, 25, 50 and 100 μM. **C**, Histogram represents the percentage of cell inhibition.

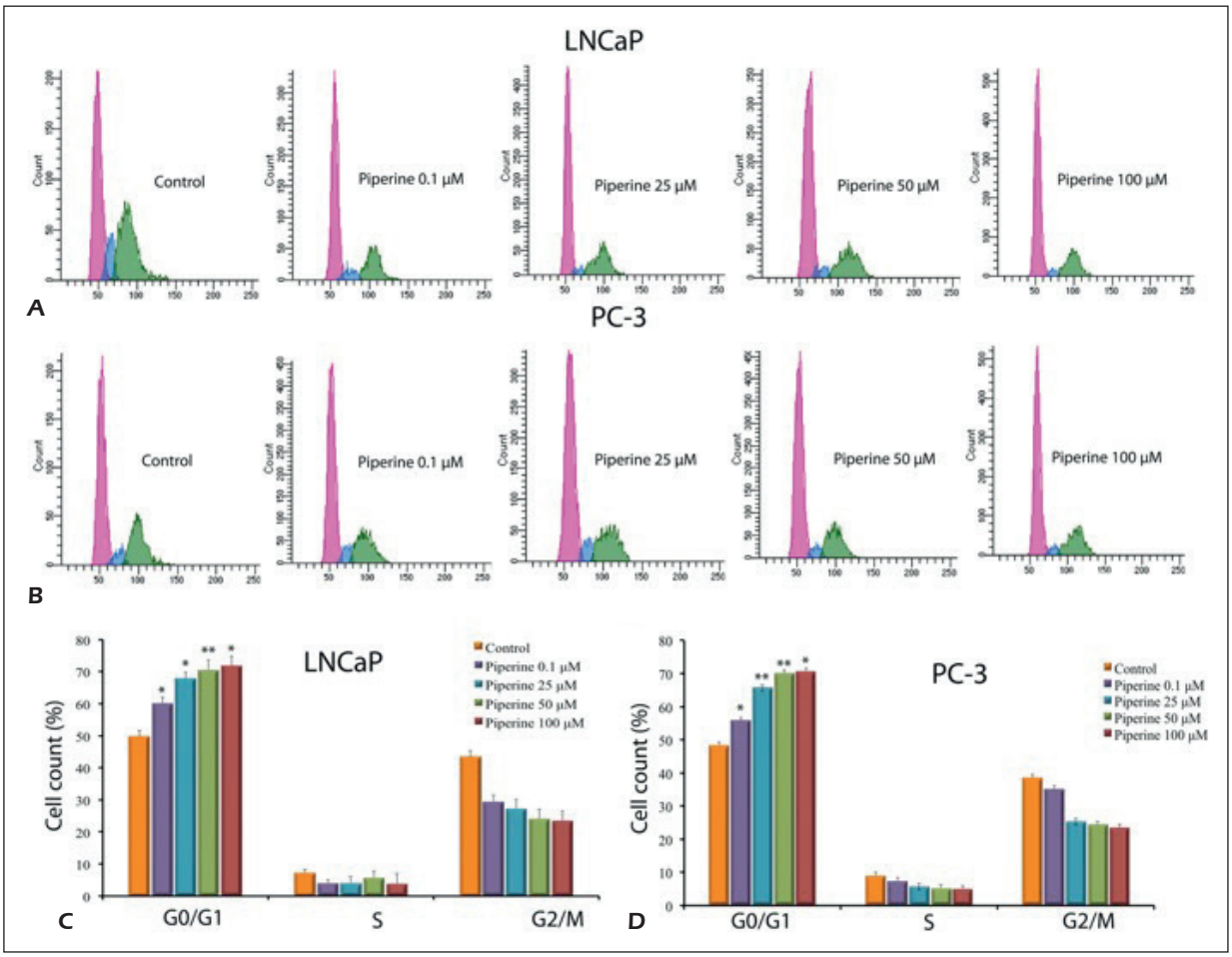

Figure 7. Flow cytometric analyses of LNCaP and PC-3 cells. **A-B**, Cell cycle distribution of LNCaP and PC-3 cells control and treated with piperine 0.1, 25, 50 and 100 µM. **C-D**, Each histogram indicates the percentage of cells at various phases (G0/G1, S and G2/M). All results were obtained from 3 independent experiments. *Indicates a statistically significant difference from control (p<0.05). **Indicates a statistically significant from control (p<0.02).

and produces a uniform green fluorescence signal. Fluorescence microscopic images are shown in Figure 8A and Figure 9A. The treatment with 100 µM of piperine on LNCaP cells caused 55.37 ± 1.2% cell death, following treatment with 50, 25 and 0.1 µM piperine caused 41.76 ± 2.1%, 23.71 ± 1.3%, and 12.24 ± 1.2% of cell death, which significantly differed from the control cells that exhibited 1±0.3% of cell death (Figure 8B). Similarly, treatment with 100, 50, 25 and 0.1 µM of piperine in PC-3 cells exerted 50.37 ± 2.1%, 76 ± 2.1%, 15.71 ± 1.3% and 8.24 ± 1.4% cell death, compared to control cells which exhibited 2.1 ± 0.4% of cell death. These results suggest that piperine induces moderate cell death in LNCaP and PC-3 cells.

Discussion

It is a well-known fact that the Kv channels play a major role in prostate cancer cell proliferation. Furthermore, differential expression of Kv channel in androgen-sensitive LNCaP and androgen-insensitive PC-3 cell lines has shown to influence their metastatic potential[6]. So, we investigated and verified whether Kv channel expression differs between LNCaP and PC-3 cell lines. The present study confirmed that the whole-cell patch clamp recordings of current amplitude in LNCaP cells were significantly different from PC-3 cells. The current densities were significantly higher in LNCaP cells in comparison to PC-3 cells. This result suggested that higher currents might be associated

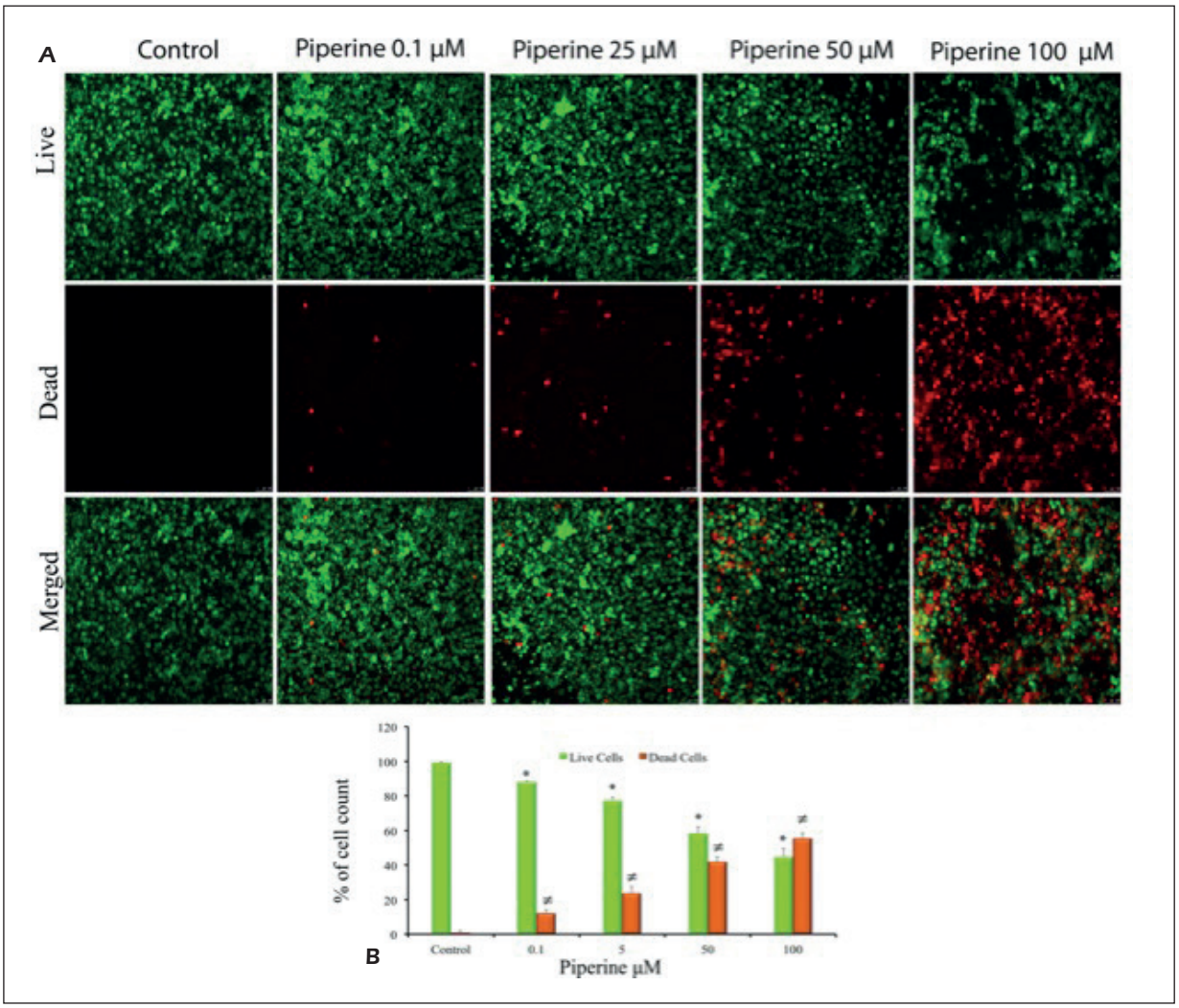

Figure 8. Detection of live and dead in LNCaP cells. ***A***, Fluorescent microscopic image of live and dead cells treated with piperine (0.1, 5, 50, 100 µM) and with out piperine. **B**, The histogram shows quantified live and dead cell percentage from total cell number using Image J. Representative images of one of three independent evaluations. *p< 0.05 live cells *vs.* control. ≠p<0.05 dead cells *vs.* control.

with higher negative resting membrane potential and lesser invasive character. PC-3 cells produced small hyperpolarizing currents, which could lead to more invasive character[17]. In addition, the results showed that both the cells were highly sensitive to TEA (10 mM), exhibiting almost 85% inhibition of peak current in LNCaP and 72% inhibition in PC-3 cells. Altogether, our results confirmed that the outward current recorded in both LNCaP and PC-3 cells predominantly expressed I_K and these results were inconsistent with earlier reports[17,18].

K_V channels are considered as therapeutic targets in variable cancer types[19-21]. Therefore, identifying new I_K modulators is of great interest in oncology. Interestingly, electrophysiological studies on LNCaP and PC-3 cells revealed that piperine potently blocked I_K in a dose-dependent manner in both cells. In addition, study findings confirmed that piperine modulated the channel activation kinetics in both the cells. The $V_{1/2}$ of K_V channel was significantly shifted to a more positive potential of about + 6.18 mV in LNCaP and +3.85 mV in PC-3 cells. So, the above observations suggested that piperine might exert high affinity for the activation state of K_V channels or exerted an open-state blocking action. Although piperine showed pronounced I_K inhibitory effect on both the cells, piperine was more sensitive to I_K in LNCaP cells in comparison to PC-3 cells. This might be associated with the malignant nature of PC-3 cells[6].

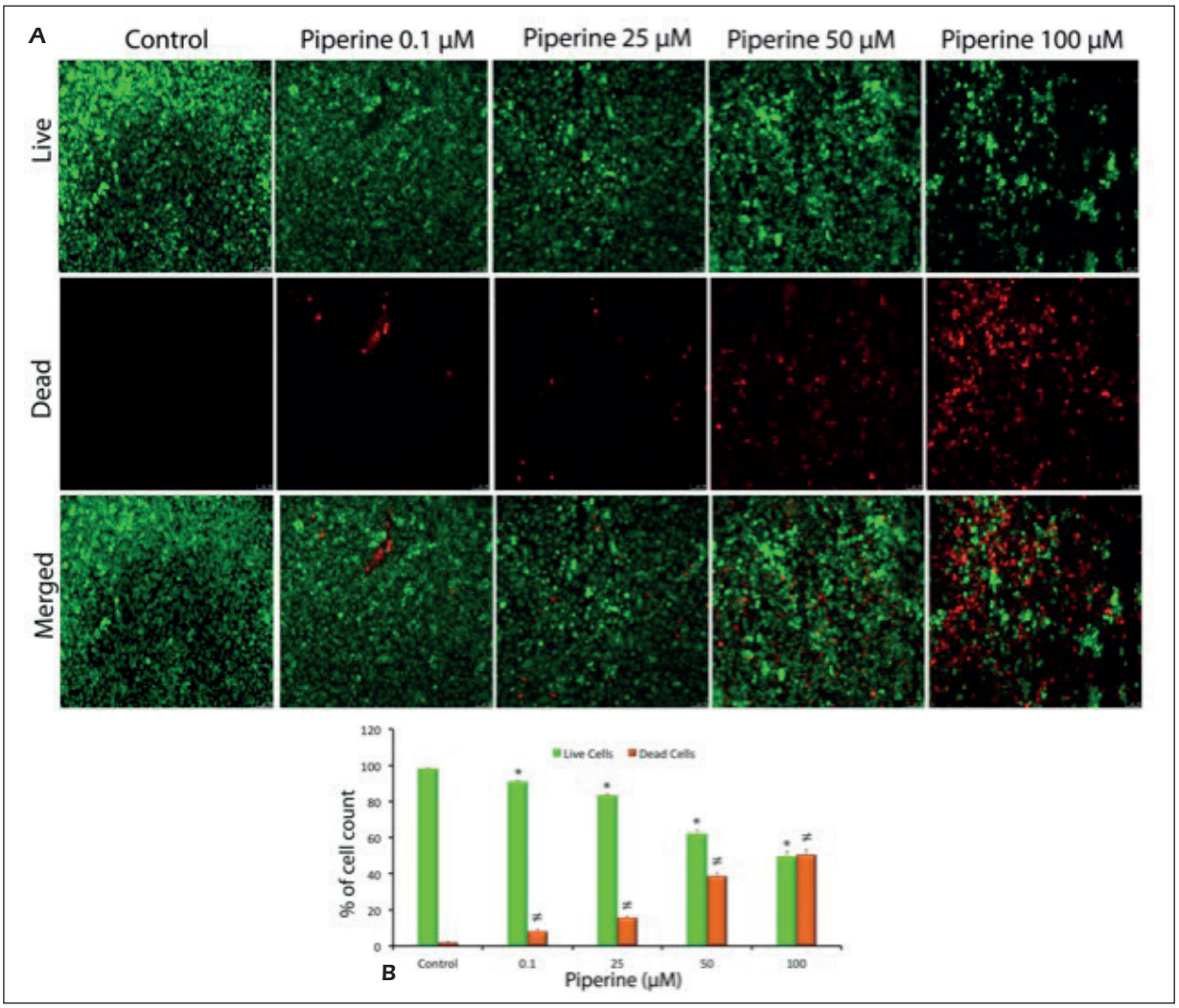

Figure 9. Detection of live and dead in PC-3 cells. ***A***, Fluorescent microscopic image of live and dead cells treated with piperine (0.1, 5, 50, 100 µM) and with out piperine. **B**, The histogram shows quantified live and dead cell percentage from total cell number using image J. Representative images of one of three independent evaluations. *$p<0.05$ live cells *vs.* control. ≠$p<0.05$ dead cells *vs.* control.

There is substantial evidence that proved the active involvement of K_V channels in the cell proliferation and differentiation of various cell types[22]. Thus, the regulation of K_V channel activities contributed to cell proliferation inhibition[23]. Therefore, the concentration-dependent growth inhibitory effect of piperine was determined in LNCaP and PC-3 cells, which revealed that piperine inhibited cell proliferation with IC_{50} 55.34 µM in LNCaP cells and IC_{50} 72.21 µM in PC-3 cells. However, when compared to the dose-response curves, the IC_{50} values for cell proliferation were slightly higher than the IC_{50} for I_K. The reason for this discrepancy is that I_K recordings were performed in serum-free ECF solution whereas the culture medium used for cytotoxic assay contained serum that promotes cell growth. In a fashion similar to that in peripheral blood mononuclear cells (PBCMs) serum reduced the potency of proliferation inhibition by 4-AP, charybdotoxin and TEA, thus suggesting the binding affinity of these drugs to the serum[23]. However, the possible contribution of K_V channel activities in cell progression through the G1 phase of the cell cycle cannot be excluded. As reported in a few studies, blockade of I_K by K_V channel antagonists has shown to induce the G0/G1 cell cycle arrest in various cells[24-25]. In accordance with the previous studies, we postulated that the blockade of I_K by piperine may be contributing to cell cycle arrest.

Of note, our data revealed that piperine treatment induced a significant cell cycle arrest at the G0/G1 phase of the cell cycle in both LNCaP and PC-3 cells. These results were consistent with previous observation of piperine activities on prostate cancer cells[10].

K_V channel in the plasma membrane promotes apoptotic cell death, though this is still conflicting. In a wide variety of cells, the activation of K_V channel expression promotes apoptotic cell death[26]. On the contrary, recent studies showed that K^+ channel blocker clofilium induced apoptosis via mediating Bcl-2 insensitive activation of caspase-3 in human promyelocytic leukemia (HL-60) cell lines[27]. Similarly, K^+ channel antagonists induced apoptosis in porcine granulosa cells[28] and dequalinium, amiodarone and glibenclamide caused apoptosis in human prostate cancer cell lines. To ascertain if blockade of I_K by piperine induces apoptosis, we investigated the apoptotic effect of various doses of piperine in LNCaP and PC-3 cells. The obtained results show that piperine induced a moderate level of cell death in both LNCaP and PC-3 cells. However, 100 μM of piperine caused significant cell death in LNCaP cells than in PC-3 cells. In agreement with the previous findings, piperine possesses a low level of apoptotic cell death but induced autophagic flux in LNCaP and PC-3 cells[10]. To validate our results, there are very few studies dissecting the contribution of K^+ channel blocker in apoptosis. In glioma cells (U87 and A172), 4-AP blocked I_K, inhibited cell proliferation and induced apoptosis[29]. Even more scarce is the information regarding the link between blockade of K^+ current and apoptosis, although some clues are provided by the synthetic N-terminus peptides of Reaper and Grim proapoptotic protein, which inactivate K^+ current by blocking the pores of K^+ channels. This effect was irreversible and suggested that Reaper interacts with K^+ channel protein with multiple domains and initiates apoptosis[30]. Thus, these findings provide evidence that the apoptotic effect of piperine seems to be due to the blockade of I_K current in prostate cancer cells.

Conclusions

We found that piperine inhibits multiple aspects of metastatic behavior of prostate cancer cells LNCaP and PC-3. These concentration-dependent effects on cell proliferation and cell death have provided the evidence that the inhibitory effect of piperine on I_K was responsible for this anticancer effect in prostate cancer cells. Piperine is therefore considered as a novel inhibitor of I_K and could be a promising therapeutic agent against prostate cancer cells.

Conflict of Interests

The authors declare that they have no conflict of interest.

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