Research Article

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Piroxicam induced alteration on membrane depolarization and caspase levels in PC3 prostate cancer cells

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ABSTRACT: The relationship between cancer and inflammation has been known for years, and the interaction between prostate cancer and inflammation and inflammatory agents is still under investigation. Studies have shown that nonsteroidal anti-inflammatory drugs (NSAIDs), which are effective in the treatment of inflammation, can also be effective in some types of cancer. In this study, the efficacy of piroxicam on human prostate cancer cells was evaluated. Piroxicam showed a significant cytotoxic effect in PC-3 cells at a concentration of 50 µM and above at the 48th hour, and induced cell cycle arrest in the S phase. On the other hand, piroxicam was found to increase annexin V binding in PC3 cells, and the early apoptotic cell population was found to be significantly higher at 25 µM and higher concentrations ($p<0.05$). The apoptosis-inducing effect of piroxicam was also confirmed by high total caspase levels ($p<0.0001$). In addition, increased membrane depolarization was observed in PC3 cells treated with piroxicam. In conclusion, this study showed the cytotoxic and apoptotic effect of piroxicam in PC-3 cells, and the obtained findings shed light on further studies on the use of piroxicam as a promising compound in prostate cancer.

KEYWORDS: Piroxicam; PC3; Prostate cancer; Apoptosis; Caspase; Mitopotential.

1. INTRODUCTION

Prostate cancer is defined as cancerous lesions that develop in the prostate gland and may be asymptomatic in their early stages. The most common initial symptoms are urinary problems such as increased frequency and nocturia due to prostatic hyperplasia, and it is very difficult to differentiate these symptoms in early stages from benign prostatic hyperplasia. According to GLOBOCAN 2018 data, prostate cancer is the second most common cancer in men worldwide after lung cancer [1].

The relationship between cancer and inflammation has been known for a long time, and studies have shown that there is a strong correlation between the presence of inflammation and the development of various precancerous conditions [2-3]. It has been reported that the risk of developing prostate cancer is increased by 14% due to prostatitis [4-6] and the risk of developing colorectal cancer is increased by 25% due to ulcerative colitis [7]. In addition, chemokines, cytokines, and growth factors produced in regions of chronic inflammation have also been reported to cause cancer in the host by inducing DNA damage and chromosomal instability [8]. There are two different paradigms for the relationship between inflammation and cancer, namely the intrinsic and extrinsic pathways [9]. In the intrinsic pathway, DNA damage, chromosomal instability, and epigenetic changes lead to inappropriate gene expression. On the other hand, the extrinsic pathway involves inflammatory conditions that promote cancer development, such as inflammatory signals from infections and autoimmune diseases. These two pathways are brought together by transcription factors such as NF-κB and some cytokines and chemokines that recruit leukocytes to the tumor microenvironment [10, 11]. These proinflammatory cascades are signs of cancer such as cell proliferation, inhibition of apoptosis, invasion, metastasis, and angiogenesis [12].

As studies spotlight that many cancers are associated with inflammation, the effectiveness of NSAIDs, which are among the most widely used anti-inflammatory drugs today, on cancer emerges as an important

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research target [13]. It has been reported that there is a tendency to decrease on the risk of prostate cancer in people who use aspirin regularly [14]. A study by Nelson and Harris (2000) also showed that daily administration of ibuprofen or aspirin correlated with 66% reduction in prostate cancer risk [15]. In another study, a lower incidence of prostate cancer was reported in men over 60 years of age who consumed NSAIDs compared to the general population of this age [16].

In vitro inhibitors of cyclooxygenase-2 (COX-2) have been shown to inhibit prostate cancer growth and increase apoptosis in prostate cancer cell lines [17]. Treatment of human PC3 prostate cancer cells with exogenous prostaglandin E2 (PGE2) resulted in increased mitogenesis and upregulation of COX-2, and treatment of PC3 cells with flurbiprofen in the presence of exogenous PGE2 was found to inhibit COX-2 mRNA upregulation and cell growth [18]. Another study provides strong evidence that increased COX-2 / PGE2 expression contributes to the development and progression of prostate cancer through activation of the interleukin-6 pathway [19].

Similar to the above data, piroxicam, an NSAID, has been reported to inhibit the synthesis of COX-1 and COX-2, reduce prostaglandin and thromboxane production, prevent polyamine production, and suppress the expression of MMP, the most prominent member of the proteinase alias in tumor development [20, 21]. Considering the above effects of piroxicam, this study aimed to investigate the effect of piroxicam on the induction of apoptosis in human prostate cancer cells by altering caspase levels and mitochondrial membrane potential.

2. RESULTS

2.1. Piroxicam inhibited cell viability through cell cycle arrest at S phase

In order to determine the effect of piroxicam on cell viability, piroxicam solution with a final concentration of 10-1000 µM was applied to PC-3 cells, and inhibition of cell viability was determined by MTT test at 24 and 48 hours. According to the data obtained, cell viability at 24th hour was 78.94±2.22% in the 500 µM group, 76.86±3.73% in the 750 µM group and 66.73±3.10% in the 1000 µM group. ($p<0.05$, Figure 1).

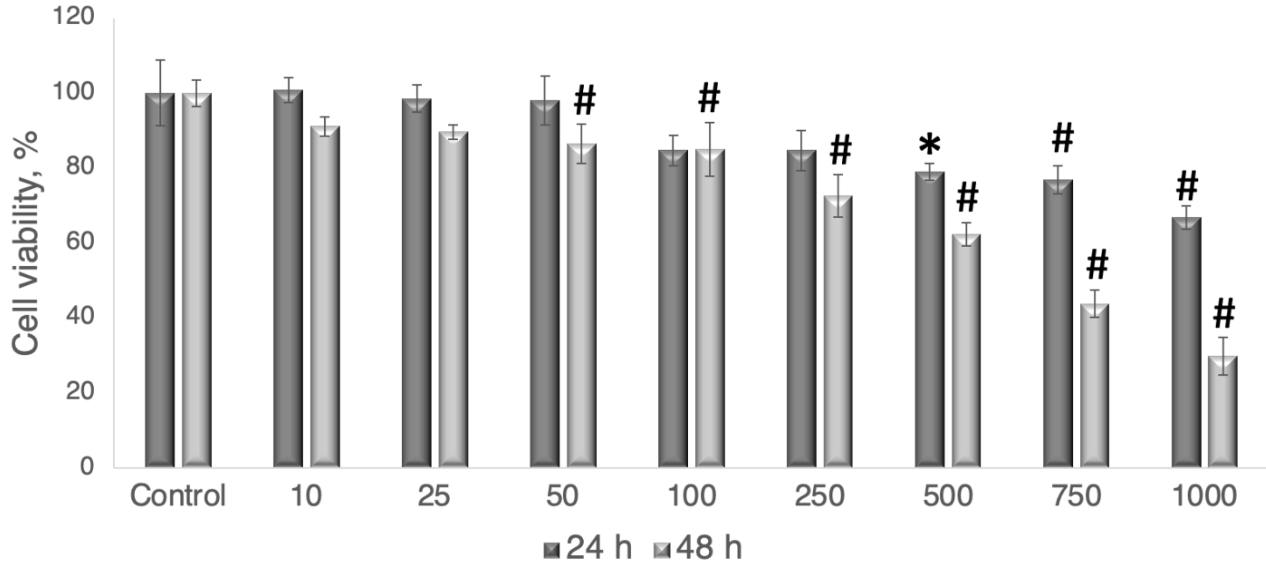

Figure 1. The effect of piroxicam on viability of PC3 human prostate cancer cells. The cells were treated with piroxicam at concentrations ranged between 10 to 1000 µM for 24 and 48 hours and the cytotoxicity was determined by MTT assay. The results are expressed as percentage of the viable cell amount % compared with untreated control. The data represent the mean ± SD in four replicates of two independent experiments. The differences are *$p<0.05$ and # $p<0.0001$ compared with control.

In PC-3 cells that were incubated for 48 hours, cell viability was 86.52±5.21%, 85.03±7.16%, 72.54±5.62%, 62.31±3.19%, 43.73±3.64%, and 29.87±4.99% in the 50, 100, 250, 500, 750 and 1000 µM treated groups, respectively ($p<0.01$, Figure 1) According to the obtained cell viability analysis data, it was

determined that the piroxicam solution showed higher activity at the 48th hour. Therefore, cells were incubated for 48 hours for subsequent analysis.

Cell Cycle Assay Kit was used to evaluate the effect of piroxicam on the cell cycle, and piroxicam solution was applied at concentrations ranging from 25-250 µM in PC-3 cells. According to the data obtained, the percentage of cell population in the G0/G1 phase was 36.05±0.0% in the control group, while it decreased significantly in the piroxicam-administered groups ($p<0.05$). While the percentage of cell population in S stage was 28.65±0.0% in the control group, it was found to be 37.05±0.5%, 37.8±1.98% and 42.5±4.81% in the 25, 100 and 250 µM piroxicam solution groups, respectively ($p<0.05$). The percentage of cell population in the G2/M phase was determined as 35.05±0.1% in the control group; however, no significant difference was found between the groups treated with piroxicam solution and the control group. According to the obtained data, it was determined that piroxicam solution induced a cell cycle arrest in PC-3 cells at S phase (Figure 2).

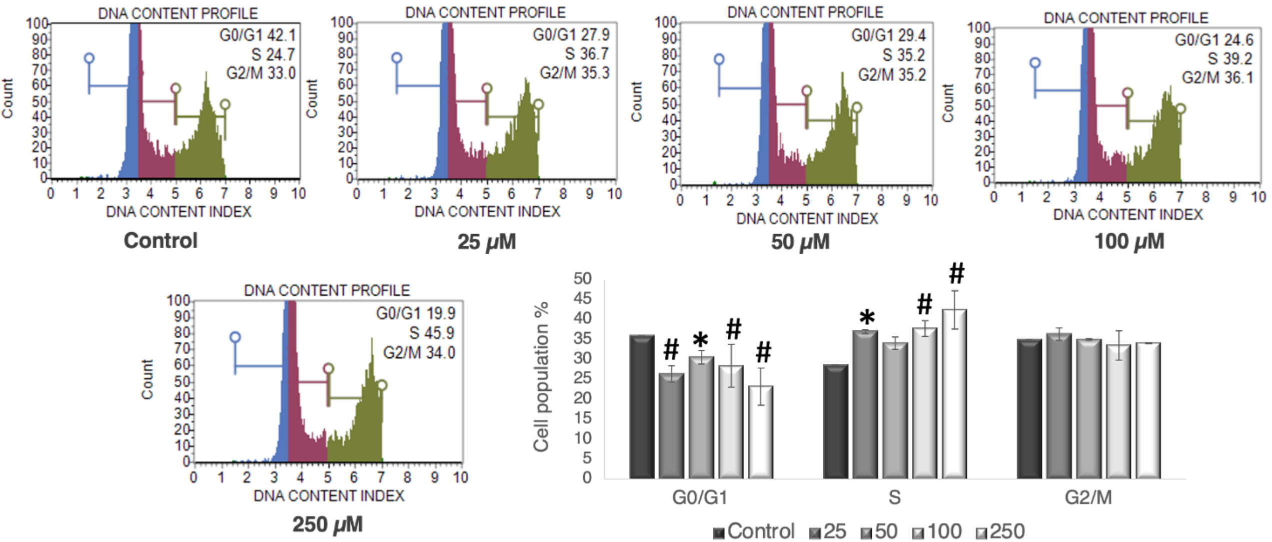

Figure 2. The results of cell cycle analysis. The PC3 cells were treated with 25, 50, 100 and 250 µM of piroxicam, for 48 hr and the cell population percentage at different phases of cell cycle including G0/G1, S, and G2/M were detected by Muse Cell Analyzer (Millipore, Germany). The results were given for three independent experiments.

2.2. Piroxicam induced Annexin V binding to PC3 cells significantly

The apoptotic activity of piroxicam on PC-3 cells was investigated by Annexin V binding assay. According to the findings, the early apoptotic cell population in 25, 100 and 250 µM applied groups were respectively; 25.45±0.99%, 24.83±1.31% and 28.45±0.42%, respectively ($p<0.01$) when compared with the control group. There was no significant difference in the late apoptotic and dead cell population compared to the control. The findings showed that piroxicam increased the apoptotic cell population in PC-3 cells (Figure 3).

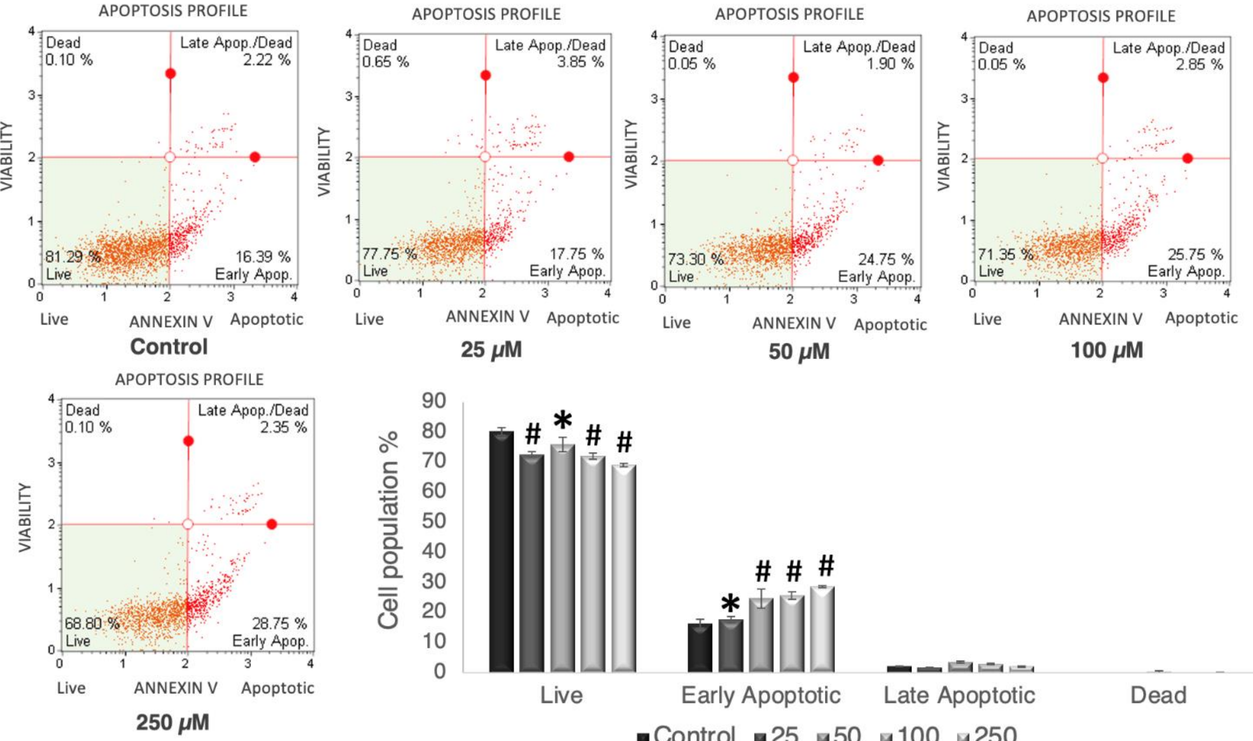

Figure 3. The results of Annexin V binding assay. The cells were treated with 25, 50, 100 and 250 µM of piroxicam for 48 hr, and the apoptosis was detected by Muse cell analyzer (Millipore). Nontreated cells were used as control. The cell population % were determined as live (7-AAD negative, AnxV negative), early-apoptotic (7-AAD negative, AnxV positive), late-apoptotic (7-AAD positive, AnxV negative), and dead (7-AAD positive, AnxV positive) cells. The figure represents cytofluorimetric dot plots of three independent experiments performed for annexin V detection and mean ± SD of late apoptotic population % (bar graph). The differences are * and # from control: $p<0.05$, and $p<0.0001$, respectively.

2.3. Piroxicam increased total caspase levels in PC3 cells

Caspase levels were also measured to evaluate the apoptotic effect of piroxicam on PC 3 cells. According to the data obtained from cells exposed to piroxicam for 48 hours, the caspase+ cell population in the control group was 24.08±3.73%; while it was significantly high in piroxicam treated groups as 40.55±1.70%, 32.88±0.60%, 35.13±3.43% and 34.05±1.13% in the 25, 50, 100, and 250 µM concentrations ($p<0.05$, Figure 4).

Figure 4. The effect of piroxicam on total caspase levels of PC3 cells. The cells were treated with 25, 50, 100 and 250 µM of piroxicam for 48 hr and total caspase levels were detected with multicaspase assay kit. Values are mean ± SD of three samples of medium from wells containing PC3 cells. The differences are *, and # from control: $p<0.05$, and $p<0.0001$, respectively.

2.4. Piroxicam induced an alteration on mitochondrial membrane potential of PC3 cells

Mitopotential Assay was performed to evaluate the effect of different concentrations of piroxicam on mitochondrial membrane depolarization after 48 hours of incubation with PC-3 cells. According to the data obtained, while the live cell population in the control group was 65.10±0.0%; it significantly decreased to data, it was concluded that piroxicam may induce apoptosis through its effect on mitochondrial membrane potential (Figure 5).

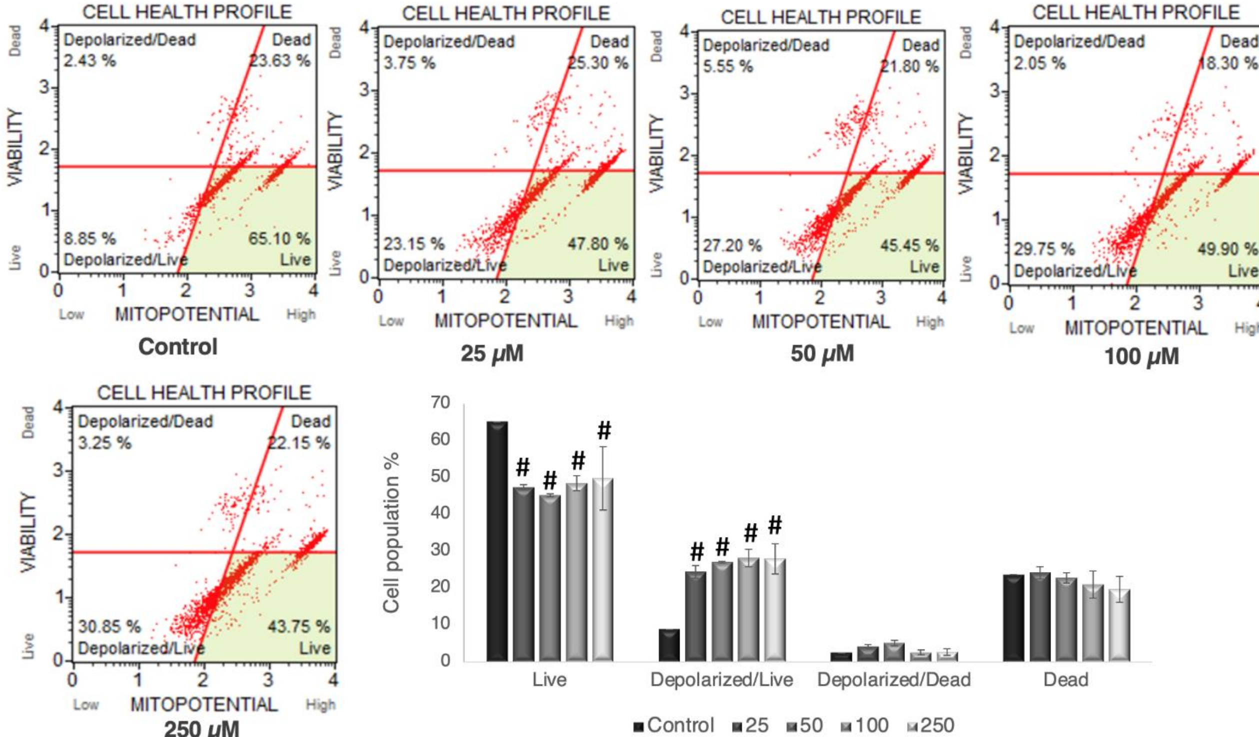

Figure 5. The effect of piroxicam on mitochondrial membrane potential of PC3 cells. The cells were treated with piroxicam at 25, 50, 100 and 250 µM concentrations and stained with Muse Mitopotential assay kit. The histogram obtained from Muse Cell Analyzer showed the percentage of depolarization in PC3 cells following treatment. Data represent of three independent experiments with similar results. The differences are # p<0.0001 compared to control.

3. DISCUSSION

The link between inflammation and cancer has been known for a long time. Therefore, the treatment of nascent inflammation and the use of anti-inflammatory agents in the treatment of cancer have been the focus of interest for researchers for many years. In addition to the effects of inflammatory agents on cell proliferation and apoptosis, the demonstration of an increase in these agents in cancer tissues has helped to identify molecules that can be used as targets for treatment [22].

COX-1 and COX-2 enzymes and metabolites, which play an important role in the inflammatory mechanism, are among the targets in cancer treatment [23]. COX-2 is an inducible enzyme and plays an important role in pathophysiological processes in some cancers such as breast, pancreatic, prostate, lung, cervical, colorectal, and skin cancers [24-26]. COX-2 expression has been associated with inflammation, cell survival, proliferation, angiogenesis, invasion, and metastasis [27, 28]. The increase in COX-2 level plays an important role in the production of prostaglandin and VEGF for tumor proliferation [29]. Due to their COX-2 inhibitory effects, various molecules have been investigated for cancer treatment and chemopreventive effects.

In this context, NSAIDs, one of the popular drug groups with anti-inflammatory effects, also attract great attention. Many experimental studies have investigated the mechanisms underlying the anticancer effects of NSAIDs using either cell lines or animal models. Although there are fewer epidemiological studies on cancers other than colorectal cancer, long-term use of NSAIDs is associated with breast, lung, prostate, ovarian, esophagus and stomach tumor incidence has been reported to be inversely correlated [30-31]. Epidemiological studies reporting that NSAIDs reduce the risk of developing cancer have also been supported by experimental animal model studies on breast and other cancer types.

Studies have shown that celecoxib [32] and naproxen sodium [33] have also anti-cancer effects. In an in vivo study using a rat breast model, celecoxib was shown to cause 90% tumor regression and 25% reduction in the number of palpable tumors [32]. In another study, it was reported that mouse and human colorectal cancer cell proliferation was inhibited by ibuprofen. Furthermore, loxoprofen has been shown to inhibit the growth of implanted Lewis lung carcinoma in mice. Mice treated with this drug had a lower

density of intratumoral vesicles and reduced VEGF mRNA expression. In the same study, patients with small cell lung cancer treated with loxoprofen (120 mg/day) for one week had decreased VEGF plasma levels and, in the study, it was suggested that the inhibition of angiogenesis may result from the inhibition of VEGF [34]. In another study in which aspirin was combined with doxorubicin, it was found to have a synergistic anticancer effect on HepG2 human hepatocellular carcinoma cells both in vivo and in vitro. When the two drugs are used in combination, they show strong synergism in cell cycle arrest, growth inhibition and apoptosis in vitro and synergistic antitumor activity was observed in HepG2 cell xenography in nude mice [35].

In a study using piroxicam in actinic keratosis patients in 2019, a decrease and disappearance of dermoscopic features of the skin were observed in patients treated with piroxicam and sunscreen and it has also been reported to reduce the expression of matrix metalloproteinases, which have proven to play an important role in skin cancer [36]. It has been found that lornoxicam, which is very similar in chemical structure to piroxicam, reduces TLR 2 and TLR-4 expression and the production of proinflammatory cytokines after intravenous use [37]. In another study, it was shown that lornoxicam decreased the expression of chemotactic proteins associated with chronic inflammation and it was suggested that the compound in question could be used to stabilize immune functions [38]. When the effects of piroxicam, diclofenac and dexamethasone on apoptosis and MMP-2 activity on the fibrocarcinoma cell line were examined, it was concluded that piroxicam stopped MMP-2 activity and induced apoptosis. It has also been shown that piroxicam is better tolerated compared to diclofenac and dexamethasone [39].

Polard and Luckert (1980) demonstrated suppression of tumor growth, metastasis and bone degeneration in transplantable rat prostate adenocarcinoma III cells treated with piroxicam [40]. In another study, a rat model of prostate carcinoma F344 chemically induced and supplemented with indomethacin reported a tumor suppressive effect and a significant decrease in PGE2 level. A study by Rai et al. in 2015 examined the efficacy of piroxicam on MCF-7 human breast cancer cells. Researchers reported that piroxicam decreased cell viability and induced apoptosis by increasing intracellular reactive oxygen radicals in cells [41].

4. CONCLUSION

In this study, the apoptotic activity of piroxicam on human prostate cancer cell line was investigated for the first time. The results showed that piroxicam increased cell death in PC3 cells by arresting the cell cycle in S phase at high concentrations and also triggered apoptosis by increasing mitochondrial membrane depolarization and total caspase level in the cells. Although further studies are needed to investigate the mechanism of action of the anti-cancer activity of the compound in question, the results of this study are promising and suggest that piroxicam may be effective in the treatment of prostate cancer.

5. MATERIALS AND METHODS

5.1. Cell culture

Human PC3 prostate epithelial adenocarcinoma cell line was purchased from American Type Culture Collection. Cells were cultured in DMEM media supplemented with 10% FBS, 1% penicillin/streptomycin and 1% L-glutamine and incubated in a 5% CO2 humidified atmosphere at 37°C.

5.2. Cell viability

The effects of piroxicam on viability of PC3 cells were evaluated by MTT assay. The cells were treated with piroxicam at 10-1000 μM concentrations and the cell viability were evaluated at 24 and 48 h. The reduced formazan crystals formed by MTT reagent were dissolved in DMSO and the absorbance at 540 nm was measured by a spectrophotometer (Thermo, Germany). The results were given as mean ± SD and untreated cells were used as control.

5.3. Cell cycle assay

The PC3 cells were treated with piroxicam at 25-250 μM concentrations for 48 h and the effects of compounds on cell cycle were analyzed using Cell Cycle assay kit [42]. The cells were fixed with ethanol for 2 h, the cell cycle reagent was applied to cell lysates and the cell population % at G0/G1, S and G2/M stages of the cell cycle were determined by Muse Cell Analyzer (Millipore).

5.4. Annexin V binding assay

The effect of piroxicam on Annexin V binding in PC3 cells were performed by Annexin V binding assay using Annexin V/Dead Cell assay kit [43]. The cells were treated with piroxicam for 48 h. The cells were than harvested, Annexin V and 7 aminoactinomycin (7-AAD) dye were applied for 20 min at RT. Based on Annexin V and/or 7-AAD positivity, four different cell population were monitored as follows: non-apoptotic live (7-AAD negative, Annexin V negative), early apoptotic (7-AAD negative, Annexin V positive), late apoptotic (7-AAD positive, Annexin V positive) and non-apoptotic dead (7-AAD positive, Annexin V negative).

5.5. Multicaspase assay

Total caspase levels were measured by Multicaspase Assay Kit according to instructions. The PC3 cells treated with piroxicam for 48 h and the cell suspensions were diluted and incubated in multicaspase working solution for 30 min at 37°C. Following incubation, 7-AAD solution were added and caspase levels were analyzed on Muse Cell Analyzer (Millipore). The detected cell population % were as follows; Live cells (caspase -, 7-AAD -), Pan Caspase activity (caspase +, 7 AAD -), Late stage of caspase activity (caspase +, 7-AAD +) and necrotic cells (caspase -, 7 AAD +).

5.6. Mitopotential assay

The effect of piroxicam on alteration of membrane potential was analyzed by Mitopotential Assay Kit [44]. The cells were treated with piroxicam for 48 h, and mitopotential working solution were applied to the cell suspension. The cells were incubated at 37°C for 20 min, then 7-AAD dye was added and re-incubated at room temperature for 5 min. The data were obtained for live, depolarized-live, depolarized-dead and dead cell population % on Muse Cell Analyzer (Millipore).

5.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism 6.0 version (GraphPad Software Inc.). Data obtained from the cell culture experiments were expressed as mean ± SD, and one-way ANOVA test were applied for multiple comparisons.

Author contributions: Concept –F.B.A.; Design – C.S.K, F.B.A.; Supervision – F.B.A.; Resources – F.B.A..; Materials – F.B.A.; Data Collection and/or Processing – C.S.K, F.B.A.; Analysis and/or Interpretation – C.S.K, F.B.A.; Literature Search - C.S.K, F.B.A.; Writing – C.S.K, F.B.A.; Critical Reviews – F.B.A.

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