

Protocatechuic Acid Induces Apoptosis in Human Osteosarcoma Cells by Regulating P13K/AKT/ROS Pathway

(Asid Protokatekuik Mengaruh Apoptosis dalam Sel Osteosarkoma Manusia dengan Mengawal Laluan P13K/AKT/ROS)

QIYUAN MEI, XIAOHU CHEN & WEI LIU*

Department of Orthopedics, Jingzhou Hospital of Traditional Chinese Medicine, Jingzhou, Hubei, China, 434000

† These two authors (Giyuan Mei and Xiaohu Chen) contributed to this work equally

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ABSTRACT

Previous investigations have demonstrated that protocatechuic acid (PCA) provides anti-tumour properties in different tumour cell types. It does, however, have an unknown cause on osteosarcoma cells. In this investigation, the underlying mechanism of the effect of PCA on osteosarcoma cells (MNNG or HOS) was investigated and established. The viability of the cell was assessed with the MTT test. Acridine orange/ethidium bromide staining and Western blot analysis were conducted for assessment of cell apoptosis. Western blot analysis was identified for cell cycle progression. In addition to establishing the above findings, the Western blot analysis demonstrated that PCA mediated osteosarcoma cellular apoptosis by triggering the apoptotic pathway of Caspase-9. Additionally, we found that PCA considerably stimulated osteosarcoma cell apoptosis and arrest of cell cycle proliferation by controlling a pathway involving P13K/Akt/ROS signalling. In short, we observed that PCA prevented the advancement of osteosarcoma through the stimulation of apoptosis in osteosarcoma cells. The mechanism underlying this study also showed that PCA generated effective anti-tumour activity on osteosarcoma cells by controlling the signalling pathways of P13K/Akt/ROS.

Keywords: Caspase-9; MTT; osteosarcoma; protocatechuic acid; western blot

ABSTRAK

Kajian terdahulu telah menunjukkan bahawa asid protokatekuik (PCA) memberikan sifat anti-tumor dalam jenis sel tumor yang berbeza. Walau bagaimanapun, ia mempunyai punca yang tidak diketahui pada sel osteosarkoma. Dalam kajian ini, mekanisme asas kesan PCA pada sel osteosarkoma (MNNG atau HOS) telah dikaji dan ditubuhkan. Daya maju sel telah dinilai dengan ujian MTT. Pewarnaan akridina oren/etidium bromida dan analisis pemblotan Western telah dijalankan untuk penilaian apoptosis sel. Analisis pemblotan Western telah dikenal pasti untuk perkembangan kitaran sel. Di samping memulakan penemuan di atas, analisis pemblotan Western menunjukkan bahawa PCA memediasi apoptosis sel osteosarkoma dengan mencetuskan laluan apoptosis Caspase-9. Di samping itu, kami mendapati bahawa PCA banyak merangsang apoptosis sel osteosarkoma dan penangkapan percambahan kitaran sel dengan mengawal laluan yang melibatkan isyarat P13K/Akt/ROS. Ringkasnya, kami memerhatikan bahawa PCA menghalang kemajuan osteosarkoma melalui rangsangan apoptosis dalam sel osteosarkoma. Mekanisme yang mendasari kajian ini juga menunjukkan bahawa PCA menjana aktiviti anti-tumor yang berkesan pada sel osteosarkoma dengan mengawal laluan isyarat P13K/Akt/ROS.

Kata kunci: Asid protokatekuik; caspase-9; MTT; osteosarkoma; pemblotan Western

INTRODUCTION

Osteosarcoma (OS), one of the world's most prevalent

malignant bone tumours (Endo-Munoz et al. 2010), exists principally in teenagers and young adults. It is associated with major metastasis and high resistance to chemotherapy (Chou et al. 2008; Heymann et al. 2016). It is due to

a variety of matrix components and growth factors in the bone micro-environment which provide a ruthless correlation among normal and tumour bone cells (Chang et al. 2015). Recent studies have proven that pulmonary metastasis is the primary cause of death. (Robl et al. 2015). Another perspective approach for overcoming osteosarcoma chemoresistance is to stimulate apoptosis (Bajpai et al. 2017). Most of the two mechanisms that might cause apoptosis namely caspase-dependent and caspase-independent signaling pathways (Robl et al. 2015). The five-year rate of survival rate enhanced to around 70% (Villegas Rubio et al. 2017), with the advancement of surgeries and adjuvant chemotherapy. Surprisingly, the longer use of chemotherapeutic drugs caused considerable harm to healthy tissues particularly kidneys and liver (Kim et al. 2014, 2012; Srinivasulu et al. 2018). Therefore, it is extremely essential to obtain a safe and reliable osteosarcoma therapeutic medication.

Protocatechuic acid (PCA), a phenolic compound, was shown to have several pharmacological activities namely anti-depression (anti-anxiety), anti-inflammatory, antimicrobial, and anti-coagulant (Srinivasulu et al. 2018). Latest research has shown that PCA seems to have anti-cancer potential in different models of tumours such as Lin et al. (2007), demonstrated that PCA inhibits gastric apoptotic tumour cell and cell cycle arrest by inhibiting the cascade of JNK/p38/MAPK signalling. Tsao et al. (2014) further showed that PCA reduces the proliferation of (human) pulmonary cancer cells and promotes the arrest of the cell cycle by suppressing the Akt pathway. PCA's anti-apoptotic and anti-proliferative stimulating effects were also identified in human squamous oral cells, tumour cells of the pancreas, lung tumour cells, cervical cancer cells, prostate, and breast tumours, respectively (Guttenplan et al. 2016; Semaming et al. 2015; Yin et al. 2009). Together, anti-invasive effects on breast cancer cells were demonstrated by PCA (Yin et al. 2009). Nonetheless, no studies with PCA to assess cytotoxic effects on osteosarcoma cells are available to date. The study, therefore, explored to assess the biological roles of PCA in osteosarcoma cells and the possible mechanism.

MATERIALS AND METHODS

CHEMICALS

Protocatechuic acid (PCA, purity > 98%), LY294002, fetal bovine serum (FBS), Dulbecco's modified eagle medium (DMEM), penicillin/streptomycin and trypsin were purchased from Sigma Alrich (Beijing, China).

NAC (N-Acetyl-L-cysteine) was purchased at Fine Chemicals (Shanghai, China). Caspase-9 specific inhibitor (Z-LEHD-FMK) was obtained from Cell Signaling Technology, China. DCFH-DA (2, 7'-Dichlorodihydrofluorescein diacetate), 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) and DMSO (Dimethyl sulfoxide) were procured from Sigma Alrich (Beijing, China). Antibodies (rabbit) for cdc2; Cyclin B1; p21; Caspase-3; Cytochrome c; p53; Caspase-9; Bcl-2; Caspase-7; Akt; p-Akt; and Bax were procured from Cell Signaling Technology (Beijing, China). Mouse antibody to β -Actin and rabbit antibody to GAPDH and HRP (horseradish peroxidase)-conjugated secondary anti-rabbit antibodies were procured from Cell Signaling Technology (Beijing, China).

CELL CULTURE AND TREATMENTS

MNNG or HOS cells (human osteosarcoma cell lines) were purchased from a Research Centre (Shanghai, China). Cell lines were kept in the Dulbecco's modified Eagle medium, enriched with 15% fetal bovine serum and 1.5% penicillin or streptomycin at room temperature in a humid chamber with a supply of 5% CO₂. Different PCA concentrations were used to treat cells placed in a medium that contains 10 percent fetal bovine serum (PCA was dissolved before use in DMSO). Inhibitors like Akt specific inhibitor (30 μ M, LY294002) (Lv et al. 2017), Caspase-9 specific inhibitor (40 μ M, Z-LEHD-FMK) (Chen et al. 2016) and N-Acetyl-L-cysteine (5 mM, antioxidant) (Wang et al. 2016) were pre-treated for one hour before PCA was allowed to treat.

ASSAY ON CELL VIABILITY

The MTT study assessed the survival of osteosarcoma cells. Cells were incubated for 24 h in 96 well plates, and then processed for an additional 24-, 48- and 72-h treatment with various PCA concentrations (0 to 15 μ M), for every PCA concentration in parallel with six wells. 10 μ L MTT solutions were transferred to each well after treatment with PCA. The medium was exchanged after incubating for 4 h with the 200 μ L DMSO solution for dissolving the formazan precipitates. The absorbance was recorded at 470 nm. The percent of cell viability was determined from the treated group absorbance divided by the non-treated group absorbance \times 100. IC₅₀ values were computed using GraphPad Prism 6 (GraphPad, San Diego, CA, USA).

APOPTOTIC CELLULAR MORPHOLOGICAL MODIFICATIONS ASSESSED BY APOPTOTIC ASSAYS

Phenotypic differences in the stimulation of apoptosis in osteosarcoma cells were specifically identified through an inverted contrast microscope. Osteosarcoma cells were exposed for 24 h with various PCA doses. After that, cells were directly viewed through the inverted contrast microscope with a magnification of 100 \times . Then, we studied the apoptotic cells nuclear morphology using acridine orange or ethidium bromide staining. The cells were treated with various PCA (10 μ M PCA in the presence and absence of caspase -9 inhibitor) concentrations over 24 h, double-washed with phosphate buffer saline, followed by staining with 400 μ L of freshly developed mixed staining solution comprising of 100 μ g/mL of ethidium bromide and acridine orange, respectively, in the darkness at 37 for 10 min. A fluorescence microscope with a magnification of 400 \times was used to observe the stained cells. Randomly selected five fields in each group were counted, and the apoptotic cell percentage was calculated from the overall number of counted cells.

ASSESSING THE INTRACELLULAR PRODUCTION OF ROS

The fluorescent probe DCFH-DA was employed to explore intracellular reactive oxygen (ROS) production in osteosarcoma cells. DCFH is oxidized to produce a fluorescent material, DCF, in the presence of ROS. The rate of green fluorescence is closely related to the quantity of ROS intracellular fluorescence can be assessed to detect ROS levels in the cells. The cells were exposed to 10 μ M PCA with or without N-Acetyl-L-cysteine for 24 h, separated by trypsin, centrifuged, twice washed using phosphate buffer saline and incubated with 10 μ M DCFH-DA in the darkness at room temperature for 1 h. The overall concentration of ROS was analyzed using a confocal laser microscope with a magnification of 400 \times and assessed using the Image J program for transitions in fluorescent intensity.

WESTERN BLOT ANALYSIS

The western blot technique was employed to further validate the underlying mechanisms. Cells (after 24 or 48 h of PCA treatment) were harvested by trypsin and the RIPA lysis buffer was used to lyse the cells. The cell lysates were allowed to centrifuge for 12 min at 16,000 g (4 $^{\circ}$ C). The supernatants were acquired and analyzed. SDS-PAGE isolated the protein samples and then shifted them onto the PVDF membranes. Upon transfer, the membranes

were blocked by 5 percent fat-free milk in Tris-Buffered saline containing 0.5% Tween 20 (TBS-T; pH 7.4) for 2 h at 37 $^{\circ}$ C. The membranes were incubated with primary antibodies such as ascdc2, Cyclin B1, p21, Cytochrome c, p53, Caspase-3, -7 -9, Bax, Bcl-2, Akt and p-Akt were diluted in the ratio of 1:1000 in TBS-T (pH 7.4) at 4 $^{\circ}$ C for 12 h, washed with TBS-T (pH 7.4) three times and incubated with secondary antibodies (anti-mouse and anti-rabbit, 1:5000) in Tris-Buffered saline containing 0.5% Tween 20 (pH 7.4) at 37 $^{\circ}$ C for 3 h. Western blot detection with enhanced chemiluminescence was used to detect all proteins. The band density was measured using Image J and normalized to internal protein (β -Actin/GAPDH; 1:5000) references.

STATISTICAL ANALYSIS

All statistical analyzes were accomplished with the software, GraphPad Prism 6 (GraphPad, San Diego, USA) including a one-way ANOVA and student's t-test. The data were expressed as mean \pm SEM and analyzed in three independent tests. Statistically, significance was considered at $P < 0.05$.

RESULTS AND DISCUSSION

PCA DECREASES OSTEOSARCOMA CELLULAR VIABILITY

First, we examined PCA's inhibitory effect on osteosarcoma cells with MTT assay. As seen in Figure 1, PCA effectively reduced the viability of human cell lines of osteosarcoma (MNNG or HOS cells) in a dose and time-dependent approach. The significant ratio of growth inhibition was also noticed at 5 μ M at 24 h, which reasonably increased with 15 μ M at 72 h. The IC_{50} value of PCA for (MNNG or HOS cells) was 15.23, 10.43, and 8.56 μ M ($P < 0.01$) relative to control cells at 24, 48, and 72 h, respectively. Depending on the results obtained, we selected the effective drug concentrations 5, 10, and 15 μ M of PCA for successive studies (IC_{50} for 48 h) (Table 1). The significant marker for tumour growth is uncontrolled cellular proliferation. Consequently, inhibiting tumour cell development or promoting apoptosis is the primary aim of suppressing the growth of tumours (Li et al. 2015). Our study had shown that PCA suppressed MNNG or HOS cellular viability substantially in a dose and time-dependent approach. Analysis of cellular morphological findings (Acridine orange and ethidium bromide staining) also showed that cell apoptosis was associated with a decline of MNNG or HOS cellular viability in a dose and time-related approach triggered by PCA.

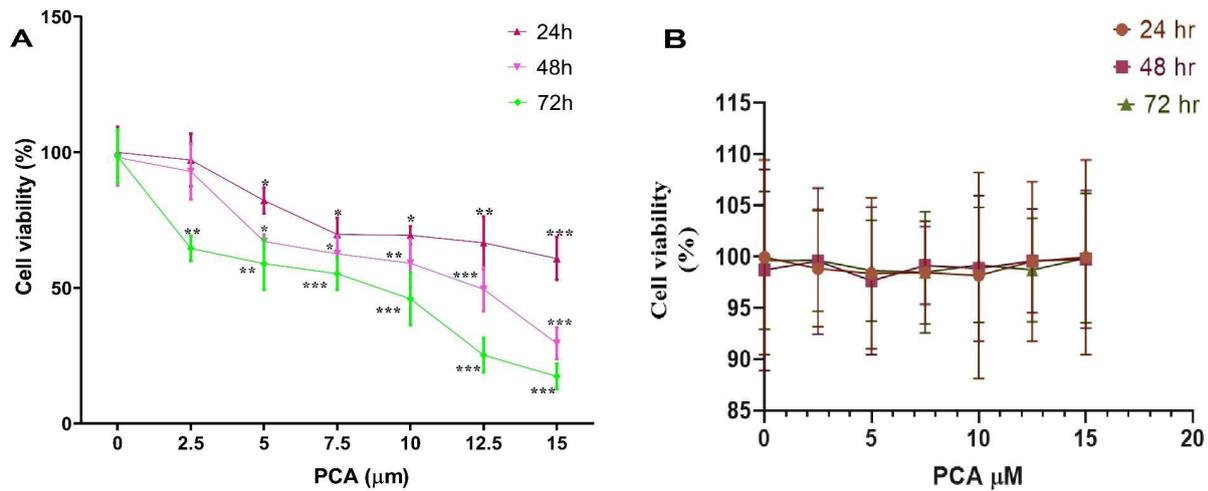


FIGURE 1. Protocatechuic acid (PCA) reduces the viability of cells in osteosarcoma. A) The growth inhibitory effect was assessed by MTT on MNNG cells with PCA concentrations ranging between 0 and 15 µM for - 72 hours. The growth inhibition was dependent on time and concentration. B) The PCA effect was shown on normal human osteoblast cells isolated from human trabecular bone samples indicating non-cytotoxic effect of PCA on non-cancerous cells. All statistical tests were compared to the control group (*P < 0.05, **P < 0.01). Three independent studies were conducted to represent the data as mean±SEM

TABLE 1. IC₅₀ values and selectivity index of PCA at different h on MNNG and normal cell lines (normal human osteoblasts)

Cell line	Drug IC ₅₀ (µM)			Selectivity index		
	24 h	48 h	72 h	24 h	48 h	72 h
MNNG	17.2 ± 1.23	11.67 ± 0.56***	7.87 ± 0.13**	> 8.89	> 8.22	> 7.59
Normal	> 75	> 75	> 75			

Data are the mean ± SD (n = 3). Statistical significance expressed as *** p < 0.001 compared to control (normal human osteoblasts); ** p < 0.01 compared control (normal human osteoblasts)

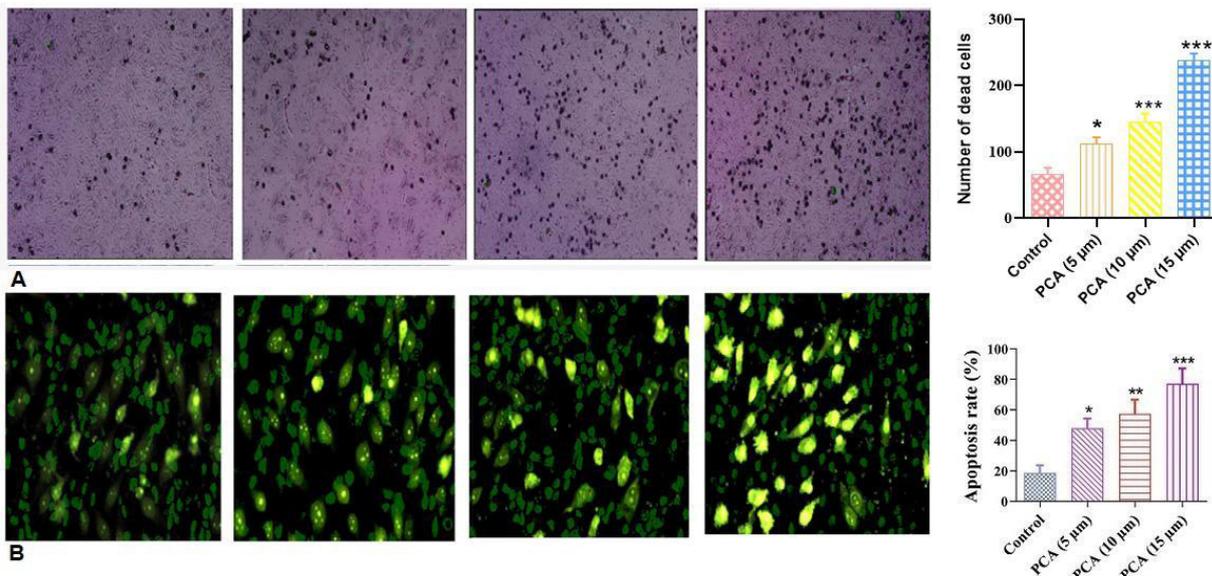


Figure 2: Morphological presentation of effect of protocatechuic acid (PCA) displayed on osteosarcoma cells. A) Morphological appearance of MNNG cells

FIGURE 2. Morphological presentation of effect of protocatechuic acid (PCA) displayed on osteosarcoma cells.

A) Morphological appearance of MNNG cells subjected to 0-15 µM to PCA for 48 hours was examined with 400X magnification using inverted phase-contrast microscope. The apoptotic cell percentage was greatly increased base on the concentration of PCA. B) The nuclear morphology of MNNG cells exposed to 0-15 µM PCA was observed for 48 hours stained with acridine orange and ethidium bromide at 400X magnification of the inverted phase contrast microscope.

The proportion of apoptotic nuclei were increased substantially based on the concentration of PCA

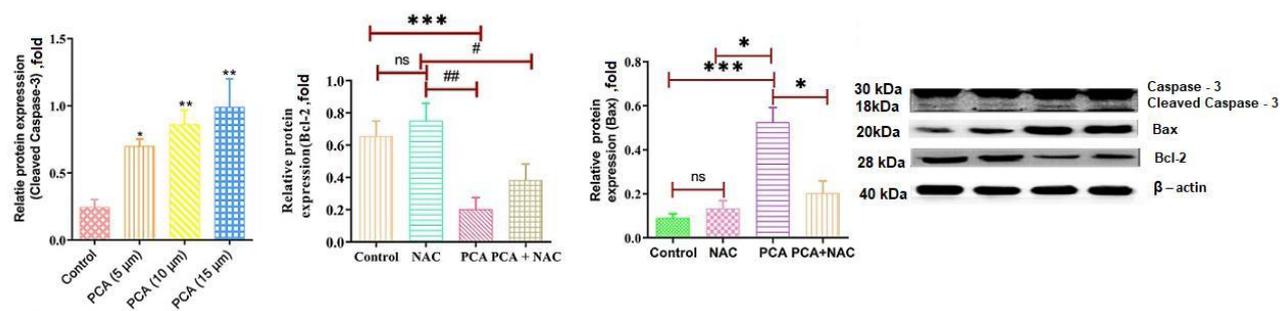


FIGURE 3. Protocatechuic acid (PCA) triggers osteosarcoma cell apoptosis. Western blot test were performed to evaluate the expression of apoptosis-related proteins in the MNNG cells. The internal reference was β -actin. PCA greatly increased Bax and Cleaved Caspase-3 expression and reduced the expression of Bcl-2 in a concentration-dependent manner. All statistical tests were compared to the control group (* $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$) and NAC group (# $P < 0.05$ and ## $P < 0.02$ respectively). Three independent studies were conducted to represent the data as mean \pm SEM

PCA STIMULATES OSTEOSARCOMA CELL APOPTOSIS

We then evaluated the growth inhibition effects with 5, 10, and 15 μ M PCA for 24 h following treatment. Phenotypic variations were noted in both osteosarcoma cells through an inverted contrast microscope. The control cells had a large spindle or hexagonal shape and were homogeneously bound to the culture surface. Nonetheless, in PCA-treated groups, certain cells were tiny and circular and evenly suspended in a medium. In PCA-treated cells, the presence of dead (non-adherent) cells was substantially greater compare to that of the control group ($P < 0.01$), respectively in a dose-dependent approach (Figure 2(A)). We have also noticed morphological variations in acridine orange and ethidium bromide staining under an inverted fluorescence microscope of PCA-treated MNNG or HOS cells. The regular cell nucleus was shown green and the apoptotic cell nuclei displayed a lighter green fluorescence and the cellular structure was compressed. As seen in Figure 2(B), the proportion of apoptotic cell nuclei increased substantially in a concentration-dependent manner in PCA-treated groups, relative to normal control ($P < 0.01$). We identified Caspase and Bcl-2 proteins through western blot analysis to understand the underlying molecular pathways. Cleaved Caspase-3 and Bax expression were enhanced dramatically and Bcl-2 expression declined substantially ($P < 0.05$) in a dose-dependent manner, relative to the control group (Figure 3). All these findings suggested that PCA mediated apoptosis in osteosarcoma (MNNG or HOS) cells in a dose-dependent approach. Next, we studied apoptosis-related proteins expression through western blot analysis. PCA regulated expressions of basic

cellular apoptosis involving caspase and Bcl-2 family. The families of caspase and Bcl-2 are the regulating proteins of the apoptosis-associated with mitochondrial signalling pathways, which are essential apoptosis regulators (Lv et al. 2016). This research, therefore, indicates the mitochondrial apoptotic pathway was often implicated in apoptosis triggered by PCA in MNNG or HOS cells.

PCA STIMULATES OSTEOSARCOMA CELL CYCLE ARREST THROUGH SUPPRESSION OR ACTIVATION OF PROTEINS

We also identified protein expression related to the cell cycle (p21, p53, cyclin B1, and cdc2) employing Western blot analysis to clarify the underlying molecular mechanism. Expressions from cyclin B1 and cdc2 were dramatically reduced and expressions of p21 and p53 were vastly increased through a concentration-dependently relative to the control group in PCA-treated samples ($P < 0.05$, $P < 0.01$), respectively (Figure 4). All these findings showed that PCA triggered cell cycle arrest of MNNG or HOS cells through a concentration-dependently. By literature search, we found that the function of PCA is not precisely the same in various tumour cells. A variety of studies have recorded that PCA induces cell cyclic arrest in cells of pancreatic ductal adenocarcinoma (Tsao et al. 2014), lung cancer cells, (Semaming et al. 2015). Several other studies had shown that PCA contributes to the cell cycle arrest of cholangiocarcinoma (Wang et al. 2012), colorectal cancer cells (Tanaka et al. 1995), melanoma cells (Lin et al. 2011), gastric cancer cells (Lin et al. 2007), leukaemia cells (Tseng et al. 2000) and bladder cancer cells (Hirose et al. 1995). Nevertheless, the PCA function

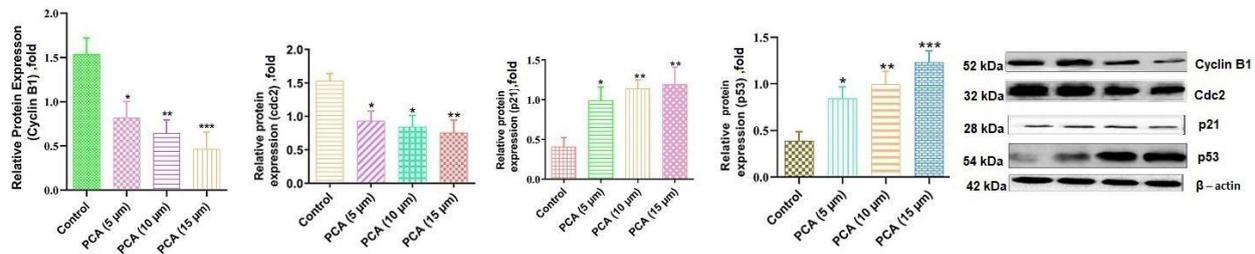


FIGURE 4. Protocatechuic acid (PCA) triggers osteosarcoma cell apoptosis, Western blot tests were performed to evaluate the expression of apoptosis-related proteins in the MNNG and Hos cells. The internal reference was β -actin. PCA greatly increased p21 and p53 expression and reduced remarkably the expression Cyclin B1 and cdc2 in concentration-dependent manner. All statistical tests were compared to the control group (* $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$). Three independent studies were conducted to represent the data as mean \pm SEM

was not documented in osteosarcoma cells. We noticed that PCA triggered a dose-dependent cell cycle arrest in MNNG or HOS cells, which was reliable with earlier findings. Subsequently, we also studied the activation of cell cycle proteins through the western blot technique. The Cyclin B1, cdc2, and p21 expression levels were controlled by PCA. Consequently, we concluded that the mitosis was obstructed progressively following the increase in PCA dose levels that cause apoptotic cell death of MNNG or HOS cells by promoting cell cycle arrest. Furthermore, we proposed that PCA acts as an amicable induction of MNNG or HOS cells for cell apoptosis and arrest of cell cycle, and provide potential remedies for osteosarcoma.

PCA STIMULATES APOPTOSIS OF OSTEOSARCOMA CELLS THROUGH AN APOPTOTIC PATHWAY DEPENDENT ON CASPASE-9

To assess whether the apoptotic pathway dependent on Caspase-9 was involved in PCA-mediated apoptosis of MNNG or HOS cells, a Caspase-9 specific inhibitor (Z-LEHD-FMK) was employed. Furthermore, 10 μ M PCA was chosen for pre-treatment. In the PCA-treated group, the apoptotic cell nuclei were substantially ($P < 0.01$) enhanced relative to the control group (10 μ M). Such pattern was reversed ($P < 0.01$) by the inclusion of a specific inhibitor for caspase-9 in comparison with a single PCA-treated group without inhibitor (10 μ M) (Figure 5). Besides that, with western blot analysis, we explored the underlying molecular mechanisms. As seen in Figure 5, the PCA-treated group considerably enhanced ($P < 0.01$) the expression of Cleaved Caspase-9, Cleaved Caspase-3, and Caspase-7 relative to the control group. Nevertheless, in the presence of Caspase-9 inhibitor, the expressions of

the aforementioned proteins were substantially reduced as compared to a single PCA treated group without inhibitor ($P < 0.01$). These findings showed that MG63 cells were induced by PCA through the apoptotic pathway dependent on caspase-9. Results from the present study illustrated that PCA triggers apoptosis of osteosarcoma cells via the apoptotic mechanisms that depend on Caspase-9. The fundamental PCA mechanisms that promote cellular apoptosis and arrest of the cell cycle of the osteosarcoma cells could be correlated with the PI3K/Akt/ROS pathway. During apoptosis, mitochondria are known to act as a significant facilitator in apoptosis. Mitochondrial apoptosis is among the major apoptosis pathways and is extremely complex in its mechanism. Shortly, it relates to pro-apoptotic modulators that limit the rate of apoptosis-associated proteins that are present on the membrane of mitochondria namely the Bcl-2 family. It improves the permeation of the mitochondrial external membranes, liberates apoptosis-dependent proteins, stimulates the cascading of Caspase-3 and -9, contributes to cellular apoptosis and eventually induces apoptosis. Therefore, it was established that PCA induces MNNG or HOS cell apoptosis through the mitochondrial apoptotic mechanism. Using Acridine orange and ethidium bromide staining we found that the level of cell apoptosis in the PCA tested group was considerably higher in comparison to the control group. The rate of cell apoptosis was however inhibited when Z-LEHD-FMK was added. In conjunction with the above findings, we indicated that cell apoptosis triggered by PCA was carried out by a Caspase-9 driven pathway. The main enzyme of mitochondrial apoptosis is Caspase-9, the critical caspase initiator. The alterations of Caspase-7, -3, -9 proteins through western blot analysis were further confirmed. Compared to the control group

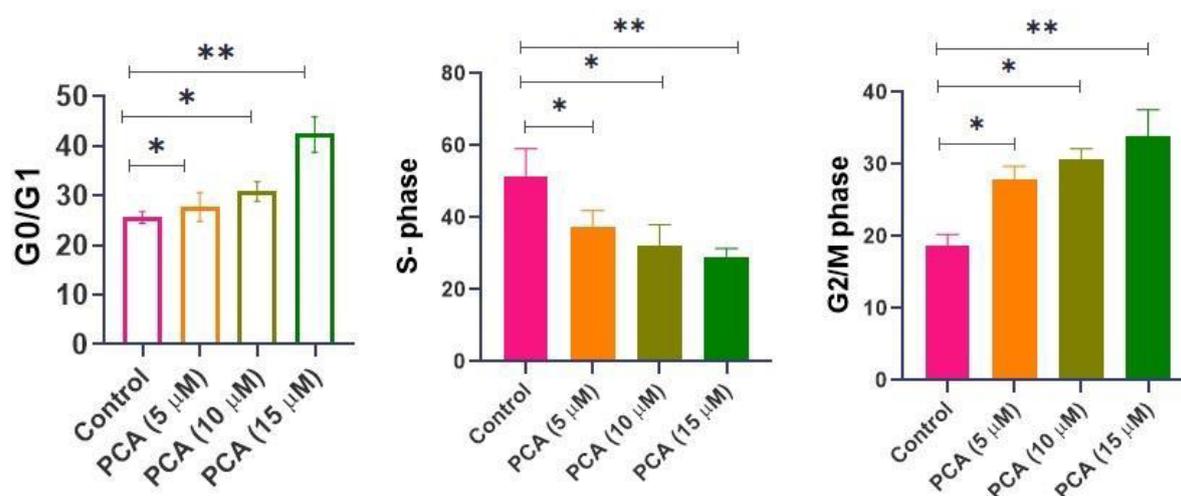


FIGURE 5. Effect of protocatechuic acid (PCA) on cell cycle progression of MNNG cells stained with PI and evaluated using flow cytometry. All statistical testw were compared to the control group (* $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$). Three independent studies were conducted to represent the data as mean \pm SEM

the above caspase protein expressions in PCA tested group were increased significantly but completely suppressed by the presence of Z-LEHD-FMK. In brief, our findings proved that PCA causes apoptosis of MNNG or HOS cells via the apoptotic pathway dependent on Caspase-9.

PCA CAUSES APOPTOSIS AND THE ARREST OF CELLS IN OSTEOSARCOMA CELLS BY ROS PRODUCTION

Next, DCFH-DA fluorescent probe and antioxidant (N-Acetyl-L-cysteine) were employed to determine if PCA causes apoptosis and arrest of cell cycle and by generating ROS. We selected 10 μ M PCA for pre-treatment. The magnitude of green fluorescence in the PCA tested group was substantially increased ($P < 0.01$) by contrast to the control group. The addition of NAC disrupted ($P < 0.01$) this pattern compared with the single PCA-tested group with NAC (Figure 6). After that, with western blot analysis, we explored the underlying molecular mechanism. As seen in Figure 7 pro-apoptotic proteins expression (Cytochrome c, Cleaved Caspase-3 and Bax) was dramatically improved, whilst the anti-apoptotic protein (Bcl-2) in the PCA tested group was remarkably declined ($P < 0.01$) compared to the control group. Nevertheless, the aforementioned protein expressions were greatly ($P < 0.05$; $P < 0.01$) reverted when the NAC was included in contrast to the single PCA group without NAC. From Figure 7, Cell cycle stimulation protein expression (Cyclin B1 and cdc2) declined substantially, and proteins of cell cycle

arrest (p21 and p53) increased dramatically ($P < 0.01$) in the PCA-tested group compared to the control group, while above-compared proteins expression significantly ($P < 0.05$; $P < 0.01$) inhibited with NAC relative to the single PCA tested group. These observations indicate that PCA induces apoptosis in MNNG or HOS cells and causes cell cycle arrest through the generation of ROS. An irregular cell metabolism generates huge quantities of ROS, which overrates the antioxidant reduction capacity and contributes to an oxidative stress state in the cells that induces modifications in the apoptosis dependent gene expressions and stimulates the apoptotic pathways. Recent studies have confirmed that the production of ROS is crucial to the pro-apoptotic activities of different anticancer drugs (Chao et al. 2017; Raj et al. 2011; Tu et al. 2016). A study by Tseng et al. (2000) showed that the PCA contributes to the mortality of active myeloid leukaemia cells apoptotically and autophagically by triggering the stimulation of ROS generation. Another study (Semaming et al. 2015) stated that PCA causes the death of tumour cells by ROS-dependent CHOP stimulation and, also shown that PCA prevents the apoptotic induction and cell growth of pancreatic tumour cells by ROS-dependent downregulation of cMyc. Even though earlier studies have also shown that PCA can influence anti-proliferative activity by triggering ROS production, it is not established if its anti-tumour effect is coherent in various cells. We, therefore, investigated if PCA mediated osteosarcoma cellular apoptosis was linked to ROS production. During our evaluation, we employed the

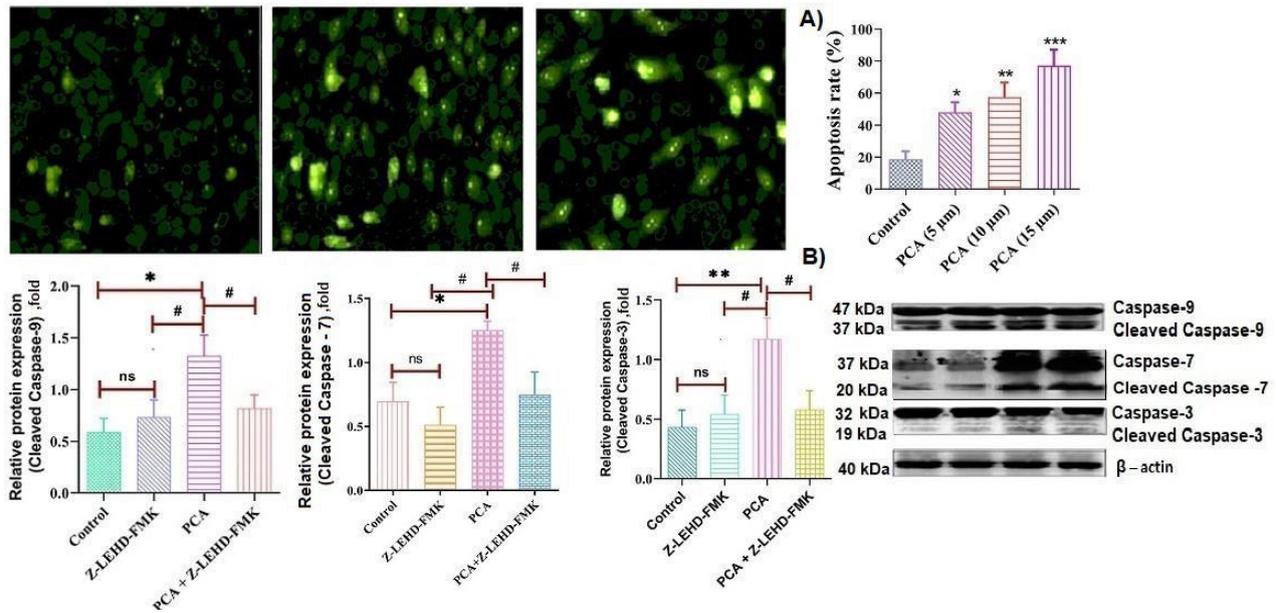


FIGURE 6. Protocatechuic acid triggers MNNG cell apoptosis via the pathway of dependent on Caspase-9. A) The nuclear morphological appearance of PCA-exposed MNNG cells with or without Z-LEHD-FMK was observed for 48 h under acridine orange and ethidium bromide staining and phase-contrast inverted microscope at magnification of 400X. The percentage of apoptotic nuclei in PCA+Z-LEHD-FMK was reduced dramatically in comparison with PCA group ($^{\#}P < 0.01$). B) Western blot analysis was used to further validate whether the independent Caspase-9 pathway is implicated in PCA-induced apoptosis of MNNG cells. The internal reference was β -actin. Cleaved Caspase-3, Cleaved Caspase-7 and Cleaved Caspase-9 expression in the PCA group was significantly increased in comparison to control group ($*P < 0.05$; $**P < 0.01$). In comparison to the PCA group ($^{\#}P < 0.01$), Z-LEHD-FMK substantially reversed expression of the related proteins in PCA+Z-LEHD-FMK. Three independent studies were conducted to represent the data as mean \pm SEM

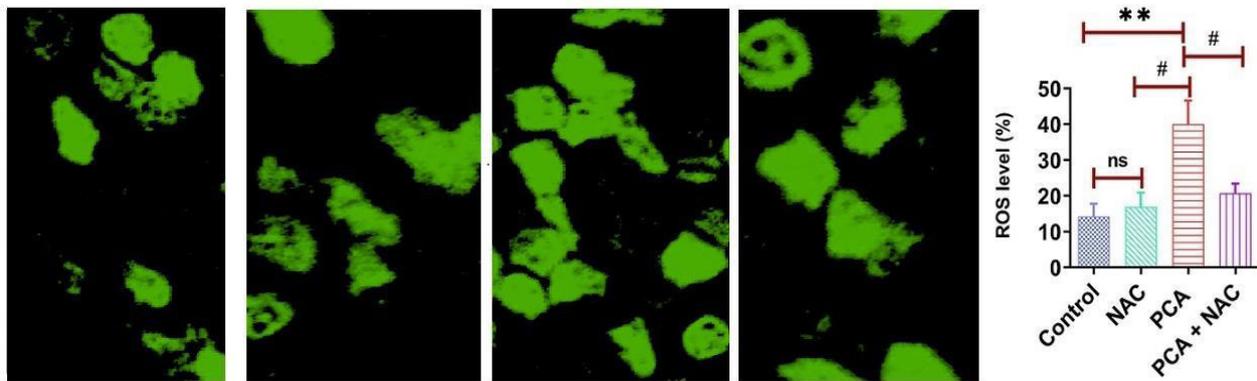


FIGURE 7. Protocatechuic acid (PCA) induces MNNG cell apoptosis through the production of ROS. After 24-hour treatment with PCA in the presence of absence of NAC, ROS generation was observed by the DCFH-DA fluorescent probe and laser scanning confocal microscope at 400X magnification. In comparison to the control group, the intensity of fluorescence (ROS) of the MNNG cells was vastly enhanced in the PCA group ($^{\#}P < 0.05$, $**P < 0.01$; $***P < 0.001$). Besides that, the increase in PCA induced ROS generation in the PCA+NAC group has greatly decreased relative to the PCA group ($^{\#}P < 0.05$). Three independent studies were conducted to represent the data as mean \pm SEM

DCFH-DA fluorescent probe to identify the production of ROS and noticed that ROS levels were considerably higher in the PCA-tested MNNG or HOS cells and the NAC (ROS inhibitor) greatly inhibited the above findings. In the meantime, reliable findings were found from western blot analysis that showed that ROS production was strongly correlated to PCA mediated apoptosis and MNNG or HOS cell cycle arrest.

PCA PREVENTS PI3K/AKT SIGNALING PATHWAYS IN OSTEOSARCOMA CELLS

PI3K/Akt signalling pathways were further investigated to explore the role of PCA to cause cellular apoptosis and arrest of the cell cycle in MNNG or HOS cells. The findings of the Western blot analysis demonstrated that p-Akt was declined remarkably ($P < 0.01$) through a concentration-dependently in PCA tested groups compared to the control group. Until then, in each group, there was no noticeable variation in the Akt expression (Figure 8). Besides that, we examined the ROS effects on the signalling pathways of PI3K/Akt. In the presence of NAC, the expression p-Akt was substantially reversed, comparing with the single PCA tested group without NAC ($P < 0.01$). In each group, there were no significant variations in Akt expression (Figure 8). The above hypothesis was also supported by 20 μM of a

specific inhibitor of Akt phosphorylation (LY294002). As seen in Figure 9, in a PCA + LY294002 group, p-Akt was greatly declined ($P < 0.01$) relative to the PCA group without inhibitor. The Akt expression in each group was not changed significantly. Similarly, in the group PCA + LY294002, the cellular apoptosis and cell cycle-associated proteins were disturbed in contrast to the PCA group ($P < 0.05$, $P < 0.01$) (Figure 10). The above findings showed that the pathway of PI3K/Akt signalling was implicated in PCA caused cellular apoptosis and arrest of the cell cycle in MNNG or HOS cells. We noticed that PCA inhibition in distinct tumour cells was therefore related to various signal transduction pathways through reviewing the literature. Lin et al. (2007) study have found PCA diminish proliferation, progression, and invasion of gastric tumour cells via the suppression of JAK1, 2/STAT3 signalling pathways. A study showed that PCA prevents the development of growth of pulmonary cancer cells through suppressing NF- κ B signalling pathways (Yin et al. 2009). Another study also stated that PCA contributes to apoptosis and autophagy by blocking receptors for leukemic cells by suppressing the mTOR/PI3K/Akt signalling pathway and stimulating the p38 signalling pathway (Tseng et al. 2000). A report indicated that PCA-induced apoptosis of colorectal human tumour cells by triggering the JNK signal pathways (Tanaka et al. 1995). In our investigation, we explored the possible

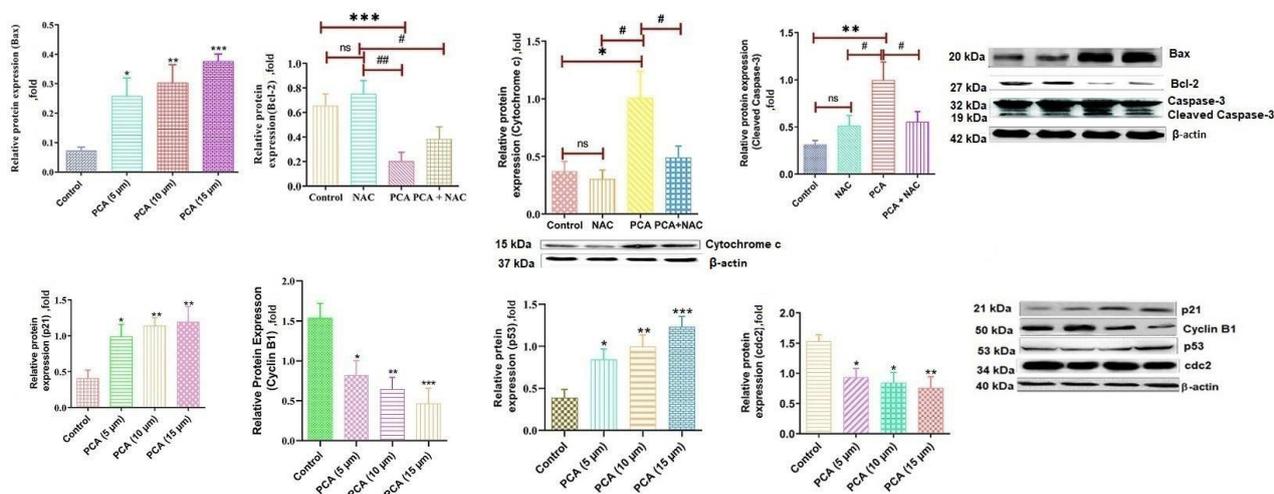


FIGURE 8. Improvements in apoptosis and proteins related to cell cycle following NAC treatment. (A) Western bolt analysis for MNNG cells was performed to evaluate apoptosis-related protein expression. The internal reference was β -actin and GAPDH. NAC considerably diminished Bax, Cytochrome c and Cleaved Caspase-3 expression and enhanced Bcl-2 expression in PCA+NAC relative to the control (* $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$) and PCA groups (# $P < 0.05$ and # $P < 0.01$) respectively. (B) Western blot analysis was also used to identify the presence of protein related to the cell cycle in MNNG cells. Similarly, β -actin was used as an internal reference. Cyclin B1/cdc2 expression was greatly enhanced by NAC, and p21 and p53 in the PCA=NAC group were decreased relative to PCA group (* $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$). Three independent studies were conducted to represent the data as mean \pm SEM

signalling mechanism by western blot analysis for PCA induced apoptosis of osteosarcoma cells. Ironically, we noticed that the PI3K/Akt signalling pathway to PCA-triggered MNNG or HOS cell apoptosis, following other previous studies on tumour cells (Lin et al. 2007; Yin et al. 2009). Phosphorylation of the Akt was reduced

according to the dose of PCA used in the study. The PI3K/Akt signalling pathway was believed to be associated with the activation of a variety of biological processes, such as cell survival, apoptosis, development, and metastasis (Maddika et al. 2007; Zhou et al. 2015). Triggering Akt phosphorylation is known to be a key

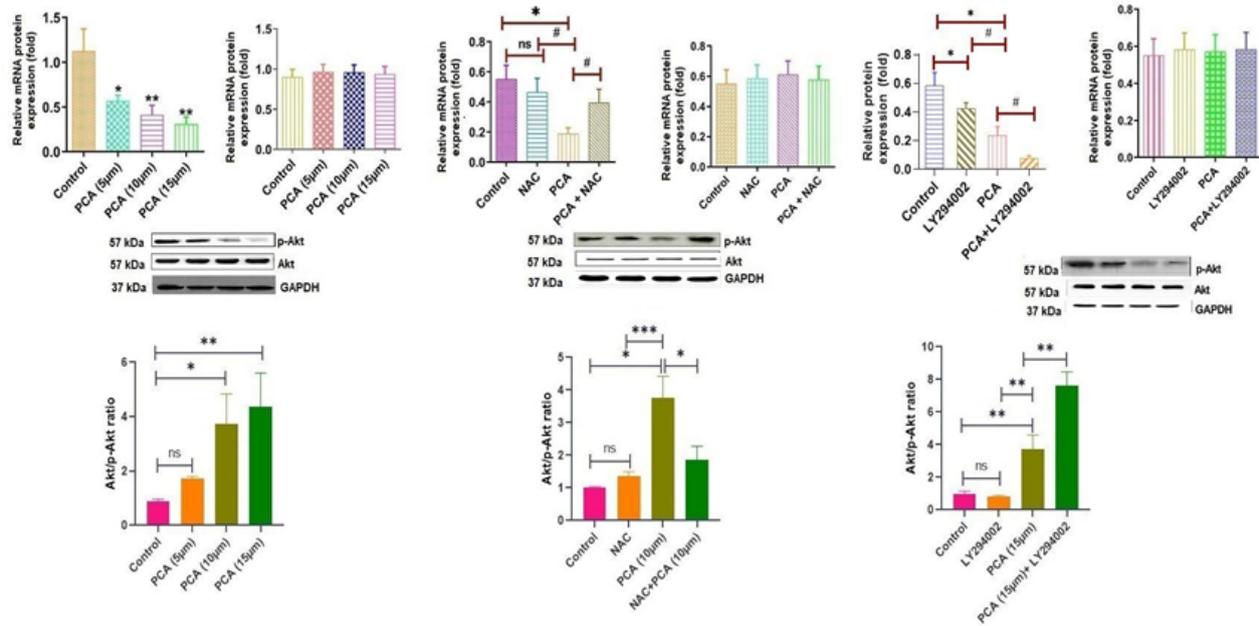


FIGURE 9. Protocatechuic acid causes apoptosis and the cell cycle arrest by inhibiting PI3K/Akt pathway on the MNNG cells. A) Western blot analysis was used to focus on the Akt phosphorylation effect for PCA. As an internal reference, GAPDH was used. Akt phosphorylation was substantially reduced by PCA in concentration-dependent manner relative to the control group (* $P < 0.05$, ** $P < 0.01$). NAC was used to test whether ROS was involved in Akt phosphorylation regulation. Akt phosphorylation significantly increased for the PCA+NAC group relative to the PCA (# $P < 0.01$) and NAC (# $P < 0.01$) group respectively. LY294002 was used to validate the Akt phosphorylation effect of PCA. The PCA-mediated Akt phosphorylation reduction relative to PCA group (# $P < 0.05$) was greatly increased by LY294002 groups (* $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$) respectively. Three independent studies were conducted to represent the data as mean±SEM

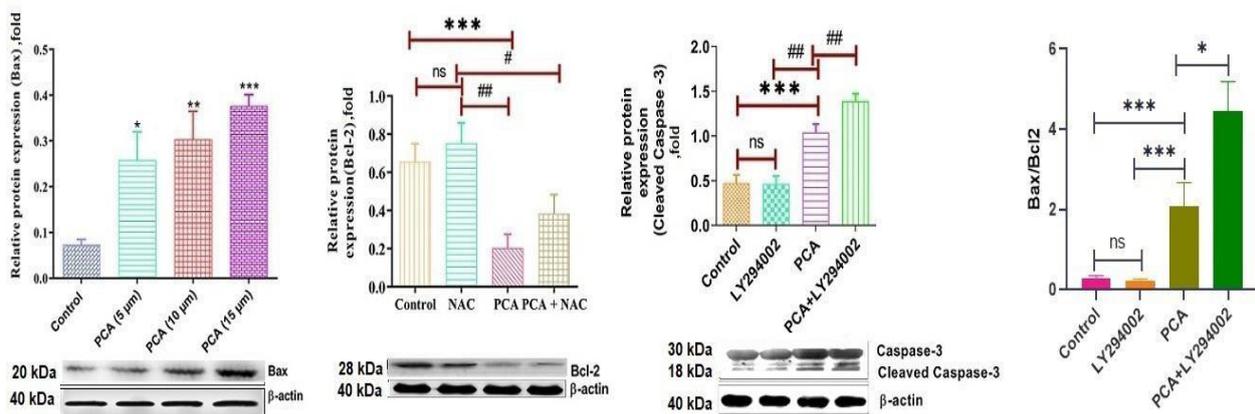


FIGURE 10. LY294002 was used to determine if the pathway PI3/Akt was implicated in PCA-induced MNNG cells apoptosis. A) Western blot analysis was used to determine MNNG cells expression of apoptosis-related proteins. The internal reference was beta-actin. The increase in Bax and Cleaved Caspase-3 and declining Bcl-2 expression in PCA+LY294002 group was noticeably increased by LY294002 as compared with the PCA and LY294002 groups (# $P < 0.05$; ## $P < 0.01$) respectively. B) Effect of PCA on Bax/Bcl-2 ratio in MNNG cells. All statistical tests were compared to the control, NAC and LY294002 groups (* $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$) respectively. Three independent studies were conducted to represent the data as mean±SEM

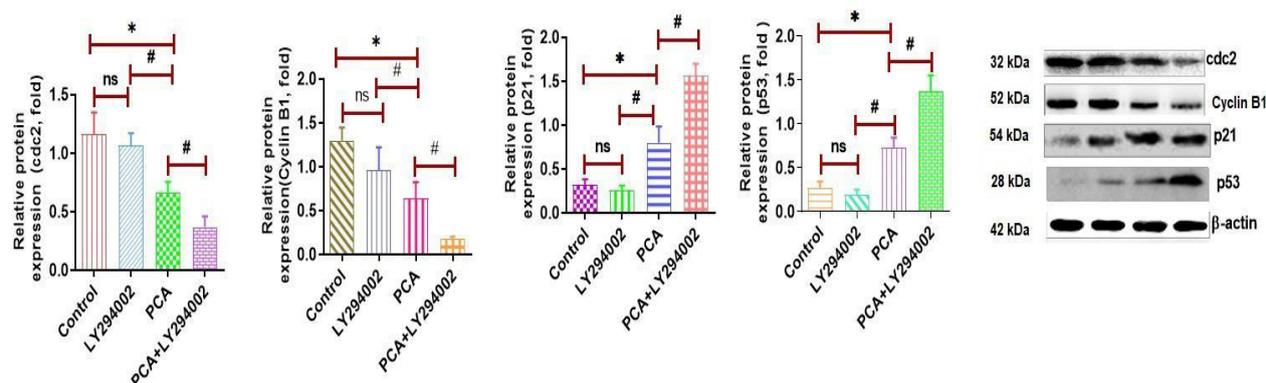


FIGURE 11. LY294002 and Western blot analysis were used to test whether PI3K/Akt pathway was involved in PCA mediated cell cycle arrest of MNNG cells. The increase in expression in p53 and p21 the decrease expression in cdc-2 and Cyclin B1 in the PCA+LY294002 group was drastically enhanced by LY294002 compared with the PCA and LY294002 groups ($^{\#}P < 0.01$) respectively. Three independent studies were conducted to represent the data as mean \pm SEM

contributor to tumour growth (Han et al. 2016). The PI3K/Akt signalling pathway over-stimulation in a variety of tumours is majorly attributable to over-stimulation of Akt (Bai et al. 2014). Earlier studies recorded that ROS promotes apoptosis by inhibiting the pathway PI3K/Akt (Wang et al. 2018; Xie et al. 2018). Our study findings showed that Akt phosphorylation in MNNG or HOS cells was substantially hindered by PCA while N-Acetyl-L-cysteine inhibited the modulation of Akt phosphorylation. Besides that, the specific PI3K-inhibitor LY294002 was used to additionally validate if the PI3K/Akt signalling pathway was shown in MNNG or HOS cell apoptosis mediated by PCA and cell cycle arrest. Evidence from western blot findings reported that not only did LY294002 increase PCA's influence on phosphorylation of Akt, but also increased PCA's effect to promote (stop) pro-apoptotic (anti-apoptotic) expression and arrest of cell cycle promoting proteins, which shows PCA promotes cell apoptosis and the arrest of cell cycle in MNNG or HOS cells through ROS production and suppression of PI3K/Akt signaling pathways (Figure 11).

CONCLUSION

In this research, PCA was shown to be involved in the inhibition of cellular apoptosis and arrest of the cell cycle as the crucial inhibitor of osteosarcoma cellular proliferation. Additional mechanistic studies indicated that the key mechanisms may be triggering the mitochondrial apoptosis pathway, producing ROS, and inhibiting the PI3K/Akt pathways. Our results established a basis for PCA's molecular mechanism for osteosarcoma treatment. The use of PCA can provide

successful osteosarcoma therapeutic approaches. We would later investigate more osteosarcoma cell lines to validate our results and make the assessment more convincing by adding positive controls (p-Akt agonist). In the meantime, we would further justify in an animal model the therapeutic effect, mechanism for action and safety of PCA to offer new and effective medications for the therapeutic intervention of osteosarcoma.

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*Corresponding author; email: 33557355@qq.com